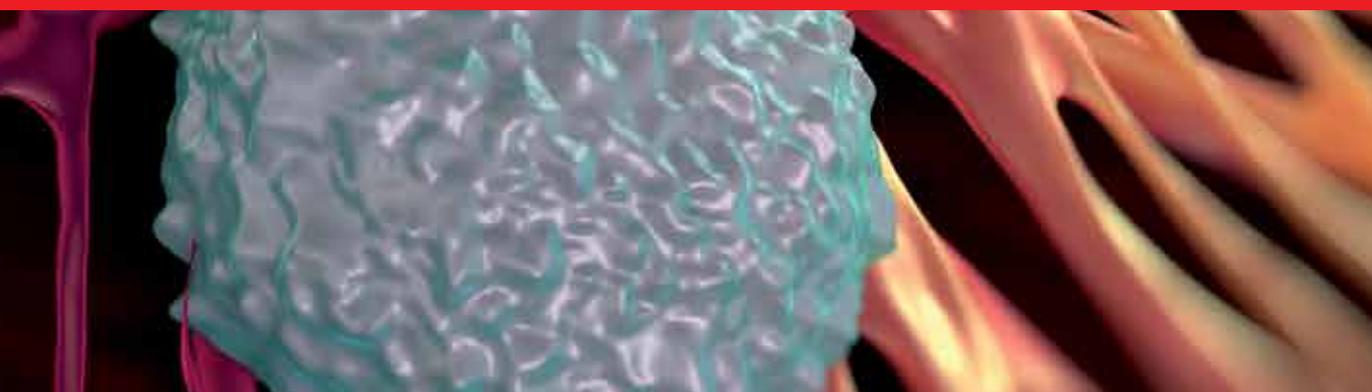


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Current Cancer Treatment
Novel Beyond Conventional Approaches

Edited by Öner Özdemir



CURRENT CANCER TREATMENT – NOVEL BEYOND CONVENTIONAL APPROACHES

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Meet the editor



Assoc. Prof. Dr. Öner Özdemir was born in Alaplı, Zonguldak, Turkey on September 18, 1965. He graduated from İstanbul University İstanbul Medical School and became a medical doctor in 1989. His pediatric residency was completed at the Department of Pediatrics at the Children's Hospital of İstanbul Medical School, İstanbul, Turkey. His clinical fellowship training was completed at the Pediatric Allergy/Immunology program at Louisiana State University Health Sciences Ctr., New Orleans, LA. A part of his clinical fellowship training was done at the pediatric allergy/immunology program in Cincinnati Children's Hospital Medical Center, Cincinnati, OH. Prof. Özdemir's research areas are as follows: LAK-cell generation and cell-mediated cytotoxicity; human mast cell development and mast cell cytotoxicity; and apoptosis -related research. He was 1st place winner of Clemens Von Pirquet Award from ACAAI at ACAAI meeting in 2005 for the best research on allergy/asthma/immunology by a fellow in training. He has 45 international and 20 national publications, as well as 85 international and 25 national presentations. Currently, he is working as Chief of Pediatrics at the third clinic associated with the division of pediatric allergy/immunology at İstanbul Medeniyet University, Göztepe Teaching and Training Hospital, Kadıköy, İstanbul, Turkey.

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Preface

It is a great pleasure for me to be editor of the book titled “Current Cancer Treatment - Novel Beyond Conventional Approaches”. Currently there have been many armamentaria to be used in cancer treatment. This indeed indicates that the final treatment has not yet been found. It seems this will take a long period of time to achieve. As expected, it is really hard to find a definitive treatment for each type of cancer due to various types of tumors and their different behaviors. Thus, cancer treatment in general still seems to need new and more effective approaches.

The book “Current Cancer Treatment - Novel Beyond Conventional Approaches”, consisting of 33 chapters, will help get us physicians as well as patients enlightened with new research and developments in this area. This book is a valuable contribution to this area mentioning various modalities in cancer treatment such as some rare classic treatment approaches: treatment of metastatic liver disease of colorectal origin, radiation treatment of skull and spine chordoma, changing the face of adjuvant therapy for early breast cancer; new therapeutic approaches of old techniques: laser-driven radiation therapy, laser photo-chemotherapy, new approaches targeting androgen receptor signal, V-ATPase inhibitors, Farnesyltransferase inhibitors, cancer treatment with hyperthermia; targeted therapy in hematological malignancies, current and emerging therapies for breast, ovarian and prostate cancer; novel therapeutic modalities: gene and epigenetic therapy; anti-angiogenic treatment in gynecologic cancers, photodynamic therapy, electrotherapy, cancer vaccine, NKG2D-based cancer immunotherapy, xenovaccinotherapy; multimodal therapies in upper gastrointestinal cancers as well as alternative therapies showing the role of Iranian cytotoxic plants in cancer treatment.

In the battle against cancer disease, different treatment options are available which are somewhat efficient; it is thus essential to improve the efficiency of current treatments and to build up newer strategies. Here, I want to mention briefly some new and remarkable approaches in current cancer treatment as pointed out in the book:

One of the most promising among these new therapeutic strategies is cancer gene therapy consisting of the replacement of non-functional gene or suicide gene therapy. Cancer gene therapy is explained by Touati et al in another chapter. In their laboratory they developed a ‘Gene Directed Enzyme Prodrug Therapy’ approach using

cytochrome P450 2B6 and NADPH cytochrome P450 reductase to activate an anticancer prodrug: the cyclophosphamide. The main benefit of this method is the *in situ* metabolism of the prodrug, which allows the production of a large concentration of toxic metabolites in tumors. And the role of epigenetic therapies in the cancer treatment is discussed by Bojang et al. Cancer is known to result from the uncontrolled activation of cancer-promoting genes (oncogenes) or the inactivation of tumor suppressor genes. In the cancer cell microenvironment, epigenetic mechanisms may regulate gene expression by DNA methylation, histone acetylation, histone methylation, etc. It is known that disequilibrium in the level of gene methylation and demethylation, or acetylation and deacetylation, has been linked to the onset and progression of cancer. This active regulation at the epigenetic level has opened a window for the development of novel treatments of cancer, as well-versed in this chapter.

Vaccine therapies in the treatment of cancer are pointed out by Akiyama et al. and Seledtsov et al., respectively. Cancer vaccine therapy is elucidated by Akiyama et al. In the last few decades, identification of tumor-associated antigens has incited the development of different strategies for anti-tumor vaccination, aimed at inducing specific recognition of tumor-associated antigens in order to obtain a persistent immune memory eliminating residual tumor cells and protecting recipients from relapses. As stated in the chapter, recent data from cancer vaccine trials for patients with advanced cancer are not consistent due to the variability of protocols in the preparation of vaccine, the vaccination itself. This chapter reviews data, examined by a private clinic immune cell therapy center, supportive of the clinical responsiveness of advanced cancer. Xenovaccinotherapy for cancer is being discussed by Seledtsov et al. Xenovaccinotherapy is the xenovaccine-based approach to breaking immune tolerance to self differentiation antigens. The authors' own data indicates that a vaccine consisting of murine tumor-associated antigens (B16 +LLC) might be effective in prolonging the survival of patients with advanced melanoma, astrocytoma, colorectal or renal cancer.

NKG2D-based cancer immunotherapy is being depicted in detail by Wu et al. In the eradication of tumors, promising clinical evidence has indicated that NKG2D-mediated cellular immunity can be very effective by activating NK cells, and CD8+T cells. The stimulatory immunoreceptor NKG2D is expressed by all human NK cells, CD8+T cells, and subsets of $\gamma\delta$ T-cells. The NKG2D ligand is generally absent in normal tissues. In precancerous tissues, typically when DNA damage occurs, NKG2D ligand is induced and thus stimulates immune response via NKG2D expression of NK / T-cells and prevents tumorigenesis. In this chapter the authors review the basic understandings of NKG2D function in anti-tumor immunity and the challenges and advances in NKG2D-based cancer treatment. In addition, prospective use of recombinant Bacillus Calmette- Guérin secreting Th1 cytokines in bladder cancer immunotherapy is being described by Luo et al.

Mathematical modeling on electrotherapy for treatment of tumors is described by Bergues Pupo et al. As told, 'the aim of this chapter is to propose a mathematical formalism that allows the 3D visualization of the potential, electric field strength and electric current density on the tumor and its surrounding healthy tissue generated by a point electrodes array and a wires array of a length given'. What an interesting chapter especially for the oncologist! Furthermore, management of small renal tumors by ablative therapies is discussed by Sriprasad et al. It is a fact that targeted destruction of small renal tumors has been performed by a number of different techniques both by laparoscopic and percutaneous routes. The common ablative techniques discussed in the chapter include the following: cryotherapy, radio-frequency/ microwave ablation, interstitial laser coagulation and high- intensity focused ultrasound. This chapter also consists of a section on radio-surgery. The researchers state that the interstitial photon radiation and the more attractive strictly extracorporeal approach using a frameless image guided radio-surgical device (cyberknife) are promising.

As mentioned in the chapter, numerous laser technologies have been used in surgical oncology, including the CO₂ laser for cutting and coagulating, laser-induced thermal therapy for thermal ablation of cancer, and photodynamic therapy for the past five decades. Photodynamic therapy is described as an oxygen-mediated minimally invasive therapeutic modality. As also defined, it involves the administration of a tumor-localizing photosensitizer that is subsequently activated with light of a specific wavelength, thus causing highly selective destruction of tumor cells. The mentioned advantages of photodynamic therapy over other conventional treatments are its minimal invasiveness, target selection and reduced toxicity. Currently, photodynamic therapy is being successfully utilized especially in early oral cavity and larynx carcinomas to save normal tissue and increase cure rates. Moreover, photodynamic therapy in combination with anti-angiogenic approaches to treat bladder cancer is discussed by Bhuvaneshwari et al as well.

Eichbaum et al reveals recent developments in anti-angiogenic treatment in a different chapter. There is increasing evidence that angiogenesis plays a major role especially in the development of gynecologic tumors such as ovarian, cervical and endometrial cancers. This chapter summarizes the current therapeutic experiences and anti-angiogenic treatment models in gynecologic oncology. A hot and controversial topic is the use of antioxidants during cancer treatment discussed by Nepomuceno et al. Considering that the use of antioxidants during treatment is a very sensitive and controversial issue, the purpose of this chapter seems to review studies in animals and humans and to evaluate the use of these antioxidants as a therapeutic intervention in cancer, and their interactions with radiotherapy and chemotherapy. And potential uses of cytotoxic plants from Iran in prevention and cancer treatment were told by Emami et al.

The role of farnesyltransferase (FTase) inhibitors (FTI) in cancer treatment is discussed by Agrawal et al. Ras protein is well-known to play key roles in the control of several

signal transduction pathways, which include cell growth, differentiation, proliferation and survival. Ras mutation is one of the most frequent abnormalities in cancer and plays an essential role in tumorigenesis. As explained in the chapter, Ras requires attachment to the inner surface of the plasma membrane for functioning. The first and most critical modification is the addition of a farnesyl isoprenoid moiety in a reaction catalyzed by the FTase. FTI comprise a novel class of antineoplastic agents recently developed to inhibit FTase with the downstream effect of preventing the proper functioning of the Ras protein. FTIs are currently used in the treatment of leukemias, gastrointestinal/genitourinary systems, head/neck cancers and current combination studies including solid tumors e.g. glioblastoma and breast cancer.

Resistance to chemotherapeutic agents is one of the most common reasons for treatment failure in cancer patients. This book reviews chemoresistance through the chapters on vacuolar ATPase (V-ATPase) and ion channels. Briefly, **V-ATPase** and its role in multidrug resistance is being defined by García et al. V-ATPase is largely responsible for regulating acidity in the microenvironment of solid tumors (and hence interfering with the absorption of chemotherapeutic drugs), seems to be the most important molecule involved in multi-drug resistance in such tumors. The application of specific inhibitors of V-ATPases can decrease the acidity of tumor and may allow the reduction of tumor metastasis, acting on the survival of tumor cells and prevent the phenomena of chemoresistance. Specific V-ATPase inhibitors, thus, may be useful, not only as co-adjuvants in anti-tumor treatments but also as a mechanism for controlling resistance to anti-tumor drugs. On the other hand, ion channels are nowadays emerging as relevant functional hubs in acute leukemias, mentioned by Annarosa et al. Ion channels where they control several processes, related to the progression of the disease. Hence, ion channel modulators are particularly attractive tool for the treatment of acute leukemias, and in particular to overcome chemoresistance.

In conclusion, with these 33 chapters this book enlightens us on fascinating cancer treatment modalities including gene, vaccine, anti-angiogenic therapies as well as immunotherapeutic approaches further than conventional ones. A couple of chapters touch on multi-drug and chemoresistance, which still signifies one of the most important obstacles for cancer treatment success.

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Part 1

Conventional Cancer Therapy Modalities

Breast and Ovarian Cancer Treatment: Facing Forward Women's Health Care

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1. Introduction

In the last decades, the Oncology field has faced an era where the pace of biotechnological advances has promised improvements in cancer diagnosis and treatment in a truly impressive manner. In this context, the enlightenment of cancer biology and carcinogenesis mechanisms have enabled not only more accurate diagnosis of the disease, therefore guiding more specific and efficient therapeutic approaches, but also allowing the discovery of novel biomarkers to fight cancer with molecular targets. Regardless the referred progress in medicine, there is still low tumor responsiveness index to classic chemotherapy regimens, and an epidemiology scenario that shows an increase in cancer-related mortality rate over the years. According to American Cancer Society, in January of 2006, about 11.6 million living Americans had already developed cancer during lifetime. For the year of 2010, there are expected over 1.5 million new cancer cases diagnosis and about 1,500 cancer-related deaths daily in the USA. In the present chapter, the focus will be given to two major types of cancer affecting women's health care: breast cancer(BC) and ovarian cancer(OVCA). Whereas BC accounts for near 23% of all cancers diagnosed and 13.7% of cancer-related deaths in women, OVCA, although not very incident (approximately 3.7% of cancer cases among women), correlates to an extremely high mortality rate of affected women (approximately 4.2% of cancer related deaths among women).

2. Standard regimes for BC and OVCA treatment

Usually, BC diagnosis is based not exclusively on anatomic and pathological aspects of the tumor cells, but also on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) by the referred cells. Actually, tumor staging relies upon the TNM system, which considers the extent of the tumor (T), the extent of lymph nodes invasion (N), and the presence of distant metastasis (M). Besides, women's age and menopausal status at the disease diagnosis, and the nuclear grade of the primary tumor cells are taken into consideration (NCI, 2011; Simpson et al., 2000). Altogether, these

* These authors equally contributed to the elaboration of this chapter

parameters guide tumor treatment decisions, as well as the disease prognosis evaluation (Arnone et al., 2010). It is likely that BC classification may require revision, as a recent cDNA microarray gene expression profiling study has classified BC into 5 distinct subtypes based on variations in gene expression patterns (Haupt, 2010). These 5 subtypes are luminal A and luminal B, normal breastlike, HER2 overexpressing, and basal-like subtypes (Nielsen et al., 2004; Haupt et al., 2010).

BC treatment commonly follows combinatory schemes comprising surgery, radiotherapy, chemotherapy, and/or hormone therapy. Surgical strategies of breast tumors vary according to the extent of the disease, which is evaluated as *in situ* carcinoma or invasive cancer. Lobular and ductal *in situ* carcinomas are treated, respectively, with excisional biopsy then prophylactic use of tamoxifen, or another hormone therapy approach, for five years (depending on the tumor ER status), and mastectomy followed by radiotherapy. Partial mastectomy may also be considered. Although the discussion of BC surgery is beyond the scope of the present work, it is worthwhile to point that the decision to pursue with mastectomy, partial or radical, must consider that its curative benefit overpasses its mutilation impact on women's psychological health (Barros et al., 2009). On the other hand, invasive BC carcinomas are differentially treated depending on the tumor size, free surgical margins and residual post-surgical disease, skin damage, and the existence of metastasis. When BC is diagnosed as a tumor of 3 cm or less with free surgical margins, the recommendation is conservatory surgery, characterized by segmentar resection, followed by radiotherapy (Veronesi et al., 1995). Axillary lymphadenectomy dissection is also advised whenever sentinel lymph node (SLN) tests positive for malignancy, as it indicates lymphoid drainage of the primary tumor micrometastasis (Fisher et al., 1997a). Nonetheless, invasive BC carcinomas larger than 3 cm at the disease diagnosis, both mastectomy and lymphadenectomy are indicated. Breast reconstitution may be considered for patients presenting good clinical conditions (Barros et al., 2009).

Regarding the conventional pharmacologic control of BC, there are several multidrug regimens preconized for neoadjuvant, adjuvant, or palliative chemotherapy approaches. As for any other antineoplastic approach, BC chemotherapy combines drugs with distinct cytotoxic mechanisms of action aiming the avoidance of drug resistant phenotype development by cancer cells. In this context, current BC chemotherapy schemes include drugs classified as anthracyclines, alkaloid taxanes, nitrogen mustard alkylating agents, antimetabolic drugs, and hormone therapeutic agents. Of interest, the relevant pharmacodynamic aspects of the cited drugs will be briefly addressed [Drugs mechanisms of action have been reviewed by Brunton (2010)].

Anthracyclines, which include doxorubicin and epirubicin, are considered cytotoxic antibiotics that comprise a tetracycline ring coupled to a quinone or a hydroquinone ring by a daunosamine sugar. They form stable complexes with DNA and the enzyme topoisomerase II, therefore preventing the DNA double strand to be rebuilt, and subsequently inducing cellular apoptosis. Moreover, anthracyclines react with CYP 450 reductase in the presence of NADPH to form semiquinone radicals that, in turn, react with oxygen species. These free radicals can oxidate DNA nitrogen-bases, additionally resulting in cellular death. It is relevant to point that the reactive free radicals are also the cause of major cardiotoxicity, which can be cumulative and irreversible. Another important antineoplastic drug that inhibits topoisomerase II is etoposide.

The alkaloid taxane paclitaxel interact with β -tubulin within the cytoskeleton microtubules structure, stabilizing the polymer and preventing cellular division, thus inducing cellular

death. It has been also demonstrated that paclitaxel induces apoptosis through the interaction and further inhibition of the anti-apoptotic molecule Bcl-2. Vinorelbine is a synthetic alkaloid that also disrupts microtubule dynamics; therefore, inhibiting cellular division. The ultimate effect is cell cycle arrest; however, major peripheral neurotoxicity is also observed. Although not an alkaloid, a new drug that interferes with microtubule dynamics is the semi-synthetic epothilone B analog, ixabepilone: a 16-membered polyketide macrolide with a chemically modified lactam substitution for the naturally existing lactone that inhibits microtubule.

Nitrogen mustards, as cyclophosphamide, are DNA-alkylating drugs. Specifically in the case of cyclophosphamide, the pro-drug is metabolized by CYP2B in the liver to acrolein and phosphoramidate mustard. Whereas the former can cause hemorrhagic cystitis, the latter undergoes a series of reactions to ultimately alkylate DNA and disrupt its double strand structure, causing cellular apoptosis. In addition, the compound can cause cardiotoxicity, and hepatic vein occlusive disease. In brief, the bis-chloroethyl-amine undergoes an intramolecular cyclization process to form the unstable ethylene-immonium structure that further transforms the tertiary amine into an unstable quaternary amine. Then, the ring opens to form the reactive carbonium ion that reacts majorly with the N7 of guanine within the DNA structure. It has been documented that the 7-alkyl-guanine confers lability to the imidazole ring that opens, inducing DNA depurination through the excision of guanine residues, and cellular death. Moreover, cellular apoptosis seems to be coupled to the tumor suppressor gene p53. Similar mechanism of action has been associated to the platinum-based compounds, as cisplatin and carboplatin.

The rationale to develop antimetabolic drugs to control cancer progress relied on the idea that cancer cells have higher metabolic rates than the normal counterparts. The first antimetabolic drug to become available was methotrexate, which interacts with the catalytic site of the enzyme dihydrofolate reductase, thus decreasing the amount of tetrahydrofolate, and inhibiting the synthesis of thymidylate, purines, serine, and threonine. The critical event is the interruption of DNA, RNA, and protein synthesis, leading to cellular apoptosis. Similarly to the observation with the DNA-alkylating agents, methotrexate mechanism of action seems to be mediated by p53. Of clinical interest, methotrexate can cause dermatitis, pneumonia, nephrotoxicity, and neurotoxicity. Another antimetabolic drug used to control BC is 5-fluorouracil. This is metabolized to 5-fluoro-2'-desoxyuridine 5'-phosphate that forms a stable ternary complex with the enzyme thymidylate synthetase; so, preventing RNA synthesis, and leading to cellular death. In addition, the drug can be converted in 5-fluorouridine, which incorporates into the RNA molecule, altering its processing and function, hence resulting in cellular apoptosis. Other important antimetabolic drugs are capecitabine, which is metabolized to 5-fluorouracil, and gemcitabine. The latter is metabolized to difluorodeoxycytidine diphosphate that inhibits ribonucleotide reductase, then preventing DNA synthesis, and difluorodeoxycytidine triphosphate, which is incorporated into DNA leading to precocious termination of the nascent molecule and cellular death.

Lastly, the well-established role of estrogen in tumorigenesis has corroborated with the use of modulators of the hormone interaction with its specific receptors or of its biosynthesis. The so called hormone therapy consists on the use of ER antagonists, as tamoxifen, raloxifen, and lasofoxifen or aromatase inhibitors. Whereas the former class of drugs inhibits the estrogen-induced transcription of growth-regulating factors, as IGF-1, the latter blocking the conversion of adrenal androgens to estrogens by the enzyme CYP19 aromatase (Sikora et al.,

2009). In addition to the anti-estrogen action, hormonotherapy may also cause hot flashes, nausea, vomiting, menstrual irregularities, vaginal bleeding, hepatic and endometrial cancer (related to tamoxifen), thromboembolism, visual impairment, and osteoporosis. Despite the consequences, hormonotherapy is consensually prescribed to ER-positive and/or PR-positive BC carriers.

Neoadjuvant chemotherapy is a preoperative tumor-debulking strategy, usually recommended in the control of inoperable tumors, as well as in the treatment of operable ones, in which case it might enable more conservative surgery methods (Fisher et al., 1997b). Current neoadjuvant regimens include the anthracyclines doxorubicin or epirubicin, associated with taxanes or cyclophosphamide, and fluorouracil administered for 3 to 4 cycles, depending on the patients' responsiveness. It has been established that tumor resection by neoadjuvant chemotherapy serves as a predictor of patients' disease-free and overall survival rates (Bonadonna et al., 1998). Also of relevance, the success of breast conservation after preoperative chemotherapy depends on careful patient selection to receive neoadjuvant chemotherapy, and the achievement of negative surgical margins during the surgical process (Buchholz et al., 2008). In any event, the benefit of enrolling a BC patient in neoadjuvant chemotherapy schemes has been increasingly considered in routine clinical decisions.

On the other hand, adjuvant chemotherapy is prescribed to BC post-surgical patients, aiming the prevention of disease recurrence or the elimination of residual tumor. Tumor size is considered a major parameter in the guideline of BC adjuvant chemotherapy. With this regard, if the tumor is smaller than 1cm, there is an implicit low risk of axillary lymph node metastases occurrence; hence, leading some clinicians and investigators to argue against the routine axillary dissection in these women. In agreement, because the disease prognosis of these patients is generally favorable, regular prescription of adjuvant systemic therapy is considered unjustifiable by the same group of health professionals. Nonetheless, others have documented that some patients with apparent small tumors at BC diagnosis, thus considered to carry disease with low invasive potential, may actually present small invasive cancers that, indeed, may progress to axillary nodal involvement and/or metastatic disease (Chen & Schnitt, 1998). Despite the complexity of the matter, careful and methodical evaluation of each case should substantiate the patients eligibility to receive adjuvant chemotherapy, which might follow the specifications discussed thereafter. Conversely, BC patients with tumor size larger than 1cm at diagnosis might receive adjuvant polychemotherapy. In this context, different polychemotherapy regimens are recommended according to the status of axillary lymph node commitment, as: i) negative lymph node status: cyclophosphamide, methotrexate, 5-fluorouracil or 5-fluorouracil, doxorubicin, cyclophosphamide, or doxorubicin and cyclophosphamide, for 6 months; ii) positive lymph node status: docetaxel, doxorubicin, and cyclophosphamide (because the anthracyclines-based regimens work better than cyclophosphamide and should be preferential indicated) (Barros et al., 2009). One important consideration should be made regarding the clinical value of taxanes in the treatment of lymph node-negative BC: whereas the actual risk/benefit of the drug remains unproven for these cases, some authors have found effectiveness in treating carriers with the docetaxel, doxorubicin, and cyclophosphamide scheme compared to the 5-fluorouracil, doxorubicin, cyclophosphamide regimen (Brunton, 2010).

As previously mentioned, adjuvant hormonotherapy is prescribed to ER-positive and/or PR-positive BC carriers and relies on two strategies: either selective modulators of ER (SERMs), as tamoxifen, raloxifen, or lasofoxifen, or aromatase inhibitors (AIs), including the third-generation nonsteroidal compounds anastrozole and letrozole, and the steroidal

compound exemestane (Sikora et al., 2009). Due to its peripheral action, aromatase inhibitors are not used in premenopausal women; rather is indicated exclusively to post-menopausal patients. In this context, AIs are becoming the hormonal treatment of choice for postmenopausal women with early BC, while tamoxifen can be used by pre- or postmenopausal women with or without the use of chemotherapy (Hortobagyi, 2002). Recent large, well-controlled clinical studies have established the efficacy and safety of initial adjuvant therapy with letrozole or anastrozole versus the previous standard of 5 years of adjuvant tamoxifen (20mg/day), and have supported the use of AIs following tamoxifen for 2-3 years (early 'switch' treatment) or 5 years (extended adjuvant treatment) (Bria et al., 2010). Therefore, these studies have indicated that initial therapy with AIs, which reduced early distant recurrence events, can be expected to improve long-term survival outcomes in eligible hormonotherapy BC patients (Bria et al., 2010).

BC clinics have been dramatically impacted by newly classified triple-negative tumors, which are ER-negative; PR-negative, and HER2-negative. It is worthwhile to emphasize that triple-negative BC cases account for approximately 15% of all BC diagnosis, but rather correspond to a disproportionate share of mortality. Indeed, triple-negative BC is mostly characterized by an aggressive behavior with a poor prognosis course (De Laurentiis et al., 2010). Thus, to date, chemotherapy remains the only possible therapeutic option to control these tumors subtypes (Gluz et al., 2009). Given the lack of standard molecular targets, patients with triple-negative BC are unlikely to benefit from currently viable targeted therapy, such as endocrine or anti-HER2 agents. Therefore, the only systemic treatment option available for these patients is chemotherapy with standard cytotoxic agents. Fortunately, several studies have shown a marked chemosensitivity among these tumors, especially with regard to the neoadjuvant regimens. In fact, reports derived from diverse clinical trials, these subtypes of BC have demonstrated high response rates to neoadjuvant chemotherapy, including the schemes with anthracyclines and taxanes (Rouzier et al., 2005; Carey et al., 2007; Liedtke et al., 2008; Esserman et al., 2009). In these trials, the response of triple-negative BC is usually higher than those of other BC subtypes, but despite initial responsiveness, they show a poorer overall survival rate: such apparently surprising behavior is often referred to as "triple-negative BC paradox" (De Laurentiis et al., 2010). Last but not least, it seems that the triple-negative BC cases are extremely heterogeneous; thus, opening an intriguing avenue to investigate the distinct genomic signatures of the BC subtype carriers in an attempt to enlighten this challenge in the oncology field.

Notwithstanding the improvements in the early detection of BC, and the development of more effective adjuvant therapies to control the disease, the actual scenario remains fearsome. Indeed, epidemiology studies have revealed that about 30% of BC patients with early disease detection will relapse with distant metastases (Early Breast Cancer Trialists' Collaborative Group, 2005). In the meanwhile, metastasis BC is a heterogeneous disease presenting a variety of different clinical scenarios, ranging from a solitary metastatic lesion to diffuse and multiple organ involvement. Although the survival rate of patients with metastatic BC is gradually improving, many physicians believe that the disease remains largely incurable. In this context, another important aspect of cancer treatment has been explored: the palliative chemotherapy. The aims of palliative chemotherapy for metastatic BC are to prolong patients' survival rate while maintaining a good quality of life. However, clinical observations have clearly demonstrated that only in a minority of cases it is possible to obtain long-term survival (>5 years) using standard treatments (Iwata, 2010). The choice of drug, or drug combination in palliative therapy is determined by the subjective

symptoms of the patient, as well as by more objective parameters, such as patients age at diagnosis and general health status, localization of metastases, and aggressiveness of the disease, which is described by the necessity to achieve remission (Paepke & Kiechle, 2003).

The treatment options to prolong patients' survival rate, providing the best quality of life possible, in metastatic BC includes hormonotherapy, chemotherapy, radiotherapy, trastuzumab (a monoclonal antibody anti-HER2; to be discussed latter in this chapter) and bisphosphonates (Barrett, 2010). ER-positive and/or PR-positive, HER2-negative BC cases must always be treated with hormonotherapy, except in critical situations where there is an urge for rapid tumor response (Barros et al., 2009). In case of disease progression, the option is to move to a subsequent endocrine therapy with different class of medication, such AIs. If aggressive visceral metastasis is detected, the option is to interrupt hormonotherapy, and to promptly introduce chemotherapy. In some cases, however, initial treatment is already based on polytherapy, aiming a more rapid response, even though the most frequent recommendation is the use of drugs, alone or sequentially to each disease progression. The monotherapy treatment for metastatic BC is based on the following drugs: epirubicina, doxorubicina, paclitaxel, docetaxel, capecitabine, vinorelbine, ixabepilone or etoposide. On the other hand, current recommendations for patients with progressive or resistant disease include the combinations: capecitabine/docetaxel, gemcitabine/paclitaxel, gemcitabine/docetaxel, cyclophosphamide/methotrexate, docetaxel/ doxorubicin, gemcitabine/cisplatin, cisplatin/vinorelbine, paclitaxel/ bevacizumab (to be further discussed in this chapter) (Rivera & Gomez, 2010).

The combination of anti-angiogenic drugs with standard chemotherapy has also increased both patients' objective response rate and median progression-free survival rate when compared with chemotherapy alone (Milano et al., 2007). At the time, blockade of growth factor receptors is the landmark of targeted therapy in metastatic BC. Monoclonal antibodies such as trastuzumab and bevacizumab represent the first generation of molecular-based therapies (Miller et al., 2007). HER2 inhibitors and the vascular endothelial growth factor antagonists have shown synergism with a broad spectrum of cytotoxins, thus being approved as the first-line treatment of metastatic BC in combination with taxanes (Bischoff & Ignatov, 2010).

More recently, tyrosine kinase inhibitors have been incorporated in the routine of BC clinics as an alternative approach for targeting HER2. This concurrent inhibition in ErbB1-expressing and ErbB2-overexpressing tumors blocks the activating signaling cascades in the MAPK and PI3K pathways, resulting in cellular growth arrest and/or apoptosis (Xia et al., 2001; Rusnak et al., 2001). A representative of tyrosine kinase inhibitors is lapatinib, an orally active small molecule (Xia et al., 2001; Rusnak et al., 2001), which reversibly inhibits both ErbB1 and ErbB2. The lapatinib effectiveness depends on the inherent biological profile of a tumor, since dependence on the EGFR and/or HER2 for cell proliferation and survival is the ideal target for lapatinib. Tumors with innate or evolved survival mechanisms which are not EGFR- and/or HER2- dependent are resistant or have reduced sensitivity to this therapy. Although lapatinib targets both EGFR and HER2, its effects on HER2 appear to be critical to its efficacy (Oakman et al., 2010). Lapatinib has demonstrated activity in trastuzumab-pretreated metastatic BC patients in combination with capecitabine (Walko & Lindley, 2005). Furthermore, chemotherapy-free regimens (trastuzumab or lapatinib plus AIs) have been identified as additional options for hormone receptor- negative and HER2-positive patients (Bischoff & Ignatov, 2010). An alternative is to substitute capecitabine by gemcitabine that is also an excellent agent in combination therapy with paclitaxel and trastuzumab (Suzuki et al., 2009)

Multitarget tyrosine kinase inhibitors have the potential to inhibit several signaling pathways involved in BC-related angiogenesis. Until now, they have failed to show a clear benefit in metastatic BC. On the other hand, poly (ADP-ribose) polymerase (PARP) inhibitors, represent an exciting new therapeutic direction in oncology. The rationale behind PARP inhibitors design is targeting tumor-cell vulnerability during DNA repair (Care & Sharpless, 2011). These new strategies are being rapidly developed and approved by the health vigilance agencies, due to the exciting preliminary clinical studies, in which their activity as single agents in the control of BRCA mutation-associated BC, and in combination with chemotherapy in triple-negative BC has been demonstrated (Telli & Ford, 2010). There is an enormous expectation that these treatments might offer hope for patients with refractory BC (Ellisen, 2011).

Future directions of research, particularly in HER2-positive BC, focus on the evaluation of novel antibodies (pertuzumab, T-DM1), and irreversible tyrosine kinase inhibitors (neratinib, BIBW 2992), as well as inhibitors of HER2-related downstream signaling molecules (mTOR, TORC 1/2, PI3K/Akt), and of receptor cross-talks (IGFR) (Bischoff & Ignatov, 2010).

Regarding OVCA treatment, cytoreductive surgery and chemotherapy platinum-based (an alkylating agent) are the basis of conventional treatment. Surgical evaluation is indicated for most women with known or suspected OVCA. Surgery is generally recommended, provided there are no medical contraindications, and the distribution of disease is deemed resectable on preoperative evaluation. The goals of the initial surgery are to obtain pathologic diagnosis, accurately determine the extent of the disease and, when feasible, optimally cytoreduce the OVCA. According the Gynecologic Oncology Group (GOG), optimal surgical cytoreduction is defined as residual tumor less than 1cm. Treatment is often driven by the surgical stage, as expressed by the International Federation of Gynecology and Obstetrics (FIGO) staging system. Precise surgical staging is critical for the patient in terms of both therapy and prognosis guidance (Ramirez et al., 2011).

In patients with early disease (FIGO stage I-II), apparently confined to the pelvis without extra-abdominal metastatic disease, the recommendation is total abdominal hysterectomy and bilateral salpingo-oophorectomy with omentectomy, peritoneal washing, peritoneal biopsies, evaluation of the entire abdominal cavity and retroperitoneal assessment that involves both the pelvic and para-aortic area. In selected patients who desire to preserve their childbearing potential, unilateral salpingo-oophorectomy with adequate staging may be performed after proper counseling (Colombo et al., 2010).

Adjuvant chemotherapy for early stage OVCA remains a controversial topic. Meta-analysis of three trials with adequate data, assessing 1,008 women, indicated that women who have received adjuvant platinum-based chemotherapy had better overall survival (OS) rates than those who did not received the treatment (hazard ratio (HR) 0.71; 95% CI 0.53 to 0.93). Likewise, meta-analysis of four trials with adequate data, assessing 1,170 women, has indicated that women who have received adjuvant chemotherapy had better progression-free survival (PFS) rate than those who did not get the treatment (HR 0.67; 95% CI 0.53 to 0.84) (Winter-Roach et al., 2009). To evaluate the clinical benefit and toxicity of two regimens used to treat early epithelial ovarian cancer, single agent carboplatin and a carboplatin/paclitaxel combination, a retrospective review including women treated with adjuvant chemotherapy between 2002 and 2005 has been performed. Five year OS rate was 62% (95% CI 44-81%) for carboplatin, and 73% (95% CI 61-85%) for carboplatin plus paclitaxel (P = 0.316). For the subgroup with stage I disease and good performance status 5-

year OS rate was 80% (59-100%) for carboplatin, and 79% (63-95%) for carboplatin plus paclitaxel ($P = 1.0$). For those patients with stage 2 disease, 5-year OS rate was 29% (95% CI 0-62%) for carboplatin, and 63% (95% CI 44-82%) for carboplatin plus paclitaxel ($P = 0.025$). Recurrences of the disease and patients death rates have been proven similar in both cohorts. It is clear that well-designed trials are needed to identify the optimum chemotherapy regimen to control OVCA (Adams et al., 2010). With this regard, the European Society for Medical Oncology, based on a meta-analysis data evaluation, has recommend six cycles of single-agent carboplatin as the adjuvant treatment in patients with intermediate and high-risk early stage OVCA. Low risk of recurrence includes stage IA–IB grade 1 tumor; medium risk includes stage IA and IB grade 2; high risk includes stage IC all grades, IB or IC grades 2 and 3, clear cell histology (Colombo et al., 2011).

For advanced OVCA (FIGO-Stage III and IV), on the other hand, the standard initial treatment consists of cytoreductive surgery followed by a combination platinum-based chemotherapy. Since 1996, the combination of platinum plus paclitaxel has become the standard treatment for the disease (Colombo et al., 2010). A study conducted by GOG (GOG 111) has demonstrated a survival benefit of the combined regimen with cisplatin and paclitaxel in comparison to cisplatin and cyclophosphamide. The authors have observed that the PSF was significantly longer in the cisplatin-paclitaxel group than in the cisplatin-cyclophosphamide group (median, 18 versus 13 months). OS has been also significantly longer in the cisplatin-paclitaxel group when compared to the cisplatin-cyclophosphamide group (median, 38 versus 24 months) (McGuire et al., 1996). In the study denominated GOG 158, the investigators have demonstrated that patients with advanced ovarian cancer would benefit from the chemotherapy regimen consisting of carboplatin plus paclitaxel, due to lower systemic toxicity when compared to the scheme with cisplatin plus paclitaxel. Besides, the former is at least as effective as cisplatin plus paclitaxel (Ozols et al., 2003).

The phase III Gynecologic Cancer Intergroup (GCIG) trial (GOG 0182-ICON 5) has been established to determine if additional cytotoxic agents in the front-line setting against OVCA would further extend patients PFS or OS. Each arm of the trial have included 8 cycles of triplet (carboplatin–paclitaxel–gemcitabine and carboplatin–paclitaxel–pegylated liposomal doxorubicin, or sequential-doublet chemotherapy, which provided a minimum of 4 cycles with the experimental treatments (carboplatin–topotecan and carboplatin–gemcitabine) while maintaining at least 4 cycles with carboplatin and paclitaxel, or 8 cycles of standard treatment (carboplatin–paclitaxel). Compared with standard paclitaxel and carboplatin, addition of a third cytotoxic agent provided no benefit in patients PFS or OS after optimal or suboptimal cytoreduction (Bookman et al., 2009). Paclitaxel plus carboplatin remains the standard front-line intravenous therapy in the fight against OVCA.

Concurrent with the development of intravenous treatment approaches, intraperitoneal treatment has also been shown to be a valuable strategy against OVCA, as it offers the possibility of targeting the therapy to the site of disease while minimizing systemic toxicities. In this context, the GOG 172 study has shown that the intraperitoneal therapy is associated with longer survival rate in surgically treated patients added to intravenous therapy as compared with intravenous therapy alone (65.6 versus 49.7 months of median survival with a 21.6% reduction in death). However, only 42% of patients were able to complete 6 cycles of intraperitoneal treatment, because toxicities were higher in the intraperitoneal treated group than in the intravenous-treated group (Armstrong et al., 2006). Then, the following drug combination has been recommended as initial chemotherapy for epithelial OVCA in advanced stages, intravenously: i) paclitaxel is given over a 3-hour

intravenous infusion followed by carboplatin over a 1-hour intravenous infusion on day 1. This combination is given every 3 weeks for a total of 6 cycles; ii) paclitaxel is given over a 1-hour intravenous infusion on days 1, 8 and 15 plus carboplatin over a 1 hour intravenous infusion on day 1 only. This combination is given every 3 weeks for a total of 6 cycles. Intraperitoneal chemotherapy may be used in combination with intravenous drug therapy in the following regimen: paclitaxel continuous intravenous infusion over 24 hours on day 1 followed by intraperitoneal cisplatin on day 2, and intraperitoneal paclitaxel on day 8. This combination is given every 3 weeks for a total of 6 cycles (National Comprehensive Cancer Network, 2010).

As stated earlier, for patients with suspected advanced OVCA, the general recommendation is primary surgical cytoreduction followed by chemotherapy. However, some patients are too medically ill to initially undergo any type of abdominal operation, whereas others have disease that is obviously too extensive to be resected by an experienced ovarian cancer surgical team. In these circumstances, neoadjuvant platinum and taxane-based chemotherapy is routinely used. Following a few courses of treatment, the feasibility of surgery can be reassessed (Schorge et al., 2010). In some cases, neoadjuvant therapy followed by interval tumor debulking has demonstrated comparable survival outcomes to those reported for primary surgery. In a study conducted by Schwartz et al. (1999), no statistical difference has been observed in OS ($P = 0.1578$) or in PFS between the group treated with neoadjuvant chemotherapy and the conventionally treated group ($P = 0.5327$), despite neoadjuvant chemotherapy patients being statistically older (median age 67 years [range 44 to 85 years] versus a median age of 60 years [range 19 to 79 years] for conventionally treated patients; $P < 0.001$), and having a statistically poorer performance status ($P < 0.001$) than the conventionally treated group.

Regardless the initial response to treatment (~80%), approximately 70% of OVCA cases will relapse after receiving the first-line platinum-based chemotherapy (even in combination with paclitaxel) (Galic et al., 2011; Ozols et al., 2003; Pfisterer et al., 2006). Platinum-sensitive OVCA cases are the ones that remain in remission for at least 6 months after completing the primary treatment regimen, whereas platinum refractory or resistant OVCA cases progress in less than 6 months from completion of the primary treatment scheme. Patients experiencing a durable response to platinum-based chemotherapy have a high probability of responding again to platinum-containing compounds. Then, patients with recurrent platinum sensitive OVCA have been retreated with platinum-based chemotherapy, often in combination with another chemotherapeutic agent. The preferred drug combination regimens to treat OVCA platinum-sensitive cases are: carboplatin and paclitaxel, carboplatin and docetaxel, carboplatin and gemcitabine, carboplatin and pegylated liposomal doxorubicin, cisplatin and gemcitabine (National Comprehensive Cancer Network, 2010).

In general, OVCA patients relapsing after receiving the first-line platinum-paclitaxel therapy are at risk of significant neurotoxicity when retreated with the same regimen within up to 12 months from the end of first chemotherapy round, due to the cumulative neurotoxicity of both carboplatin and paclitaxel. Therefore, combinations of other drugs with platinum have been explored. Among these possibilities are pegylated liposomal doxorubicin, epirubicin, ifosfamide (an alkylating agent), gemcitabine, topotecan, docetaxel, irinotecan (a topoisomerase I inhibitor), etoposide, hexamethylmelamine (an alkylating agent), vinorelbine, fluorouracil, capecitabine, pemetrexed (an antimetabolic drug that acts similarly to methotrexate), oxaliplatin (an alkylating agent), vinorelbine, tamoxifene or bevacizumab (National Comprehensive Cancer Network, 2010; National Cancer Institute, USA, 2011).

In patients with relapsed OVCA, the objectives of salvage therapy are considered aiming the maintenance of patients' quality of life, and prolongation of patient survival. Salvage chemotherapy in platinum-refractory patients typically results in low response rates and short survival, and the main goal of salvage therapy in this group of patients is palliation. Rechallenge with platinum-based treatments produces a response rate of approximately 10% and the response rate of drugs mentioned above with antitumor activity in paclitaxel-platinum-refractory disease is also of approximately 10% (Colombo et al., 2010).

The role of cytoreductive surgery at disease recurrence is controversial and guidelines are not standardized, but surgical management of recurrent ovarian cancer has been demonstrated to improve patients' survival in optimal surgical candidates, and several studies have suggested the importance of cytoreductive surgery prior to the initiation of second-line chemotherapy (Revised in Burton et al., 2010). Unfortunately, the conclusion is that OVCA remains a lethal disease for which improved screening and treatment strategies are urgently needed.

3. Cancer immunotherapy

3.1 Monoclonal antibodies in oncology

The idea of using specific antibodies to target tumors back over 100 years, when Paul Ehrlich hypothesized that "magic bullet" could selectively target a given disease. Nevertheless, it would only become possible with the development of the hybridoma technology by Kohler & Milstein (1975). This technology enabled the production of murine monoclonal antibodies (mAbs) with high specificity to a target. However, the applicability of these murine mAbs to human therapy was questioned, mainly because of their immunogenicity. The first studies involving cancer patients who received murine mAbs showed the production of human anti-globulin antibodies (HAGA) and human anti-mouse antibodies (HAMA). Among patients with solid tumors, 50-70% of them developed HAMA after the exposure to the Abs. The main problem from these responses was the implications it had on the effectiveness of the therapy (DeNardo et al., 2003), since subsequently administrated mAbs had different biodistribution patterns as well as altered pharmacokinetics. To overcome this curb, researches started looking for alternatives that would reduce this adversity. Actually, this has been accomplished by the development of chimeric and humanized mAbs, which carry the human Fc backbone and the murine variable region (or a part of it). More recently, modifications had been incorporated to the chimeric or humanized mAbs, so that they have an increased ability to bind to their targets or to recruit the immune system effectors components. These mAbs, also called "next generation mAbs" are still under development, and are expected to have a higher ability to penetrate solid tumors than the previous ones (Adams & Weiner, 2005).

Chimeric and humanized antibodies differ from each other by the extension of the murine segment that is incorporated to the human Fc portion: While chimeric antibodies contain the full murine variable region, humanized antibodies contain only the complementarity-determining regions (CDR) from mice. Besides diminishing the immunogenicity by assembling a chimeric or humanized mAb, this approach also enables choosing the IgG isotype according to the desired function. Antibodies designed to act by Fc domain-based functions, such as antibody-dependent-cellular cytotoxicity (ADCC), are produced as IgG1 or IgG3 antibodies. On the other hand, when antibody is expected to act by steric inactivation of its target, without recruitment of the immune system, they are usually IgG2 (Adams & Weiner, 2005).

As for the molecular target of therapeutic Abs, they could be an antigen presented in the cancer cell surface, or molecules presented in the cancer microenvironment. Cancer cell surface targets can be cancer-specific antigens or proteins that are overexpressed in the tumoral tissue. Antigens presented in the tumoral microenvironment, in turn, are usually growth-factors or molecules necessary for the tumor progression. The state-of-the art immunotherapy of cancer has revealed a remarkable progress in controlling cancer with monoclonal antibodies. Additionally, it has stimulated investigators worldwide to pursue with molecular studies aiming the identification of novel tumor markers that could ultimately serve as antigen to the development of other therapeutic antibodies (Adams & Weiner, 2005).

Mechanisms elicited by monoclonal antibodies against tumor are normally characterized by cytotoxic effects. Most of them are associated with ADCC or complement dependent cytotoxicity (CDC), both leading to the cytolysis of tumor cells (Adams & Weiner, 2005). ADCC happens after antibodies bind antigens on tumor cells, and the antibody Fc domain engages Fc receptor (FcR) on the surface of immune effector cells, principally natural killer (NK) cell, although neutrophils and eosinophils can also mediate ADCC. On the other hand, CDC is triggered by antibodies and complement system to induce cell killing. In addition to these classical methods, monoclonal antibodies can be used to target toxins or radioisotopes to kill tumor cells (Adams & Weiner, 2005; Challacombe et al, 2006; Rivolti et al, 1993).

Despite the optimism to fight cancer with molecular targets using antibodies, the therapeutic success is critically dependent on the proper selection of the antigen to be target. The current issue is related to the way that tumor-associated antigens (TAA) are expressed. Apart from the issue that some of them are not exclusive of the malignant cells, the ideal tumor targets need to be easily accessible, homogeneous, expressed in a vast majority of cancer cells, and would also need to be stationary. Although this may sound as utopia, in fact, up to now mAbs have been proven more effective against hematologic cancers than solid cancers, what is partially justified by the former best cellular accessibility (Nyberg, 2007). Nonetheless, an impressive progress in solid tumor control using antibodies has been noted. Therefore, important features of anti-tumor antibodies against BC and OVCA are herein presented, focusing on the current strategies and perspectives of new antibodies.

Since 1986, many platforms of antibodies have begun to be developed aiming the activation of effector pathways of immune response while avoiding immunogenicity. As a result, in 1997, the Food and Drug Administration (FDA) has approved the mAb rituximab, a chimeric IgG1 mAb developed to treat B cell non-Hodgkin lymphoma resistant to other chemotherapy regimens (Scott, 1998). This antibody binds CD20 protein, primarily found on the surface of B cells, and triggers cellular death by eliciting ADCC mechanisms.

In the next year, 1998, FDA approved trastuzumab, a recombinant humanized IgG1 antibody targeting HER2 (also known as Erb-2) encoded by the ERBB2 gene. This receptor belongs to the epidermal growth factor receptor (EGFR) family of receptors tyrosine kinases, which are involved in the control of gene expression of angiogenic factors. HER2, as well as HER1 and HER3, activation occurs by ligand binding followed by receptor dimerization and intracellular signaling initiation, leading to cellular proliferation. By binding to HER2, trastuzumab inhibits endogenous growth factor interaction, which, in turn, prevents the receptor dimerization and the activation of downstream proliferative pathways. The expression profile of HER2 has been widely studied in BC samples, and has been proven to be overexpressed in approximately 20 to 30% of invasive breast carcinomas. Besides, it has been observed that HER2-positive BC corresponds to poor prognosis disease when

compared to the HER2-negative counterpart (Hudis, 2007). Indeed, it has been well documented that the former presents a lower OS rate as well as different responses to chemotherapy and hormonal agents when compared to the later. Of clinical relevance, HER2 has been found to be overexpressed in several other tumor, including OVCA, in which case the profile has been observed in 11 to 16% of carriers. Also of interest, as noted for BC carriers, HER2-positive OVCA also correlates to poor prognosis disease (Hogdall et al., 2003; Camilleri-Broet et al., 2004). Importantly, trastuzumab has described synergistic and additive effects with several chemotherapeutic agents, including platinum, taxanes, cyclophosphamide, and anthracyclines, when treating BC (Florescu et al., 2011). Actually, the combination of trastuzumab and paclitaxel is recommended by FDA to treat patients with metastatic BC whose tumors overexpress HER2 protein.

More recently, bevacizumab has been also approved by FDA as the first therapeutic antibody targeting pro-angiogenic factors (Eskander & Randall, 2011). Among them, vascular endothelial growth factor (VEGF) deserves consideration. It acts by binding to the VEGF receptors VEGFR-1 and -2, eliciting biological effects that include induction of cellular proliferation and migration, remodeling of the extracellular matrix, increase of vascular permeability and contribution to newly formed blood vessels survival. Regarding its role in tumorigenesis, it is well known that VEGF is overexpressed in most of the human cancers, and increased levels of this growth factor is correlated with higher microvessels density and advanced disease stages. Bevacizumab recognizes the biologically active isoforms of VEGF and sterically inhibits their binding to the receptors, thus preventing angiogenesis. Originally, bevacizumab has been approved to treat metastatic carcinoma of the colon and rectum (Ferrara et al. 2004). Latter, its application has been expanded to other types of cancer including HER2-negative BC, and, in 2008, the combination of this hmAb with paclitaxel has been approved as a first-line metastatic BC treatment (Miller et al., 2007). Since then, many controversial data concerning the clinical benefits of bevacizumab-based treatment brought forth discussion whereas FDA approval should be kept or withdrawn. In 2010, the Office of New Drugs from the FDA decided for the withdrawn of bevacizumab as the first-line metastatic BC treatment, and has already started the removal of this indication from bevacizumab label (Jefferson, 2010). Regarding the bevacizumab use for OVCA treatment, several clinical trial are currently under development. Data from several phase II studies had shown 15 to 20% response rate with up to 50% PFS in 6 months, and some preliminary data from phase III studies had also indicated an increase in PFS when bevacizumab is combined with other treatments (Eskander & Randall, 2011). However, this hmAbs has not been FDA-approved to treat OVCA.

Also in 2004, FDA has approved the use of cetuximab for colorectal cancer which had spread to other tissues. This is a chimeric mAb which targets the epidermal growth factor receptor-1 (EGFR-1), a cell membrane receptor closely related to HER2. Activation of EGFR-1 occurs by ligand binding, leading to a signalization cascade that culminates with cell proliferation induction (Warksal, 1999). Similarly to what is observed with HER2, EGFR-1 has been found overexpressed in many tumor cells, including both OVCA and BC, among others. This antibody has recently been under clinical trials as a single agent and in combination with platinum-based chemotherapy regimes. Although these trials had shown quite modest results, with relatively low clinical relevant responses and some serious associated side-effects, it is believed that maybe OVCA with lower EGFR-1 expression could be more responsive to this treatment (Frederick et al., 2009).

Other therapeutic mAb that is currently under study is oregovomab, which targets cancer antigen 125 (CA-125). This is a surface glycoprotein antigen, known as a clinical relevant biomarker to OVCA, being elevated in 95% of patients with stage III and IV OVCA (Bast et al., 1983). However, CA-125 can also be found up-regulated in other malignancies, including BC, and in benign tumors and other inflammatory diseases. Oregovomab is a murine mAbs and its binding to CA-125 constitutes a complex that is recognized by the immune system as being foreign. Thereby, producing anti-mouse specific antibodies, anti-idiotypic antibodies, and antibodies specific to CA-125, inducing a humoral response to CA-125. A phase II clinical trial with 20 recurrent OVCA patients has evaluated the clinical value of the combination of oregovomab with cytotoxic chemotherapy (Gordon et al., 2004). Patients who have received the combined therapy have shown a good tolerance, presenting only mild side effects. Moreover, a significant longer survival span has been observed in patients who had developed a specific immune response. A randomized, double-blind, placebo-controlled stage III/IV clinical trial with stage III/VI OVCA patients has also been performed (Berek et al., 2008). Initially, the study failed to show a clinical advantage of oregovomab administration, once the median time to relapse (TTR) was unaltered between both groups of patients. However, a subgroup of patients with better prognosis features could be identified, and these, indeed, had a significant shorter TTR than other patients. On the other hand, patients who have received oregovomab and have failed to mount a specific immune response also had a worse prognosis than the placebo group. With respect to the 5-year disease-free survival, oregovomab-based treatment has led to a mean increased survival time of 58 months compared to 49 months among patients who received the placebo. Currently, oregovomab has not been FDA-approved yet, and it has the status of orphan product.

3.2 Perspectives for BC and OVCA immunotherapy

3.2.1 Cancer vaccines and cytokines

Whereas the use of Abs as therapeutic agents for OVCA and BC has already proven to be of remarkable clinical relevance, analog assessment has not become available as far as the utilization of vaccine- and cytokine-based strategies are concerned. Currently, no cancer vaccine has been approved by FDA, neither for BC, nor for OVCA; but results from many clinical trials using different vaccination approaches and immunogens have shown promising data towards the use of such therapeutic approaches for BC and OVCA (Floresco et al., 2010; Liu et al., 2010).

Many immunogens and vaccine compositions can be considered for cancer therapy. Firstly, it is possible to name vaccines using tumor antigens. A phase I/II study performed with BC, OVCA and lung cancer patients using a HER2 peptide-based vaccine showed that 92% of patients developed immunity to both HER2 peptide and protein (Disis et al., 2002). Besides that, several phase I trials of vaccination against HER2 have demonstrated safety and immunogenicity, with only rare grade 3 toxicity reports (Disis et al., 2004; Wiltshcke et al., 2008).

Professional antigen-presenting cells challenged with tumor antigens or fused to tumor cells also represent a vaccination option against cancer. Two small phase I studies have provided some promising data on a HER2 dendritic cell (DC) vaccine, lapuleucel-T (Peethambaram et al., 2009; Park et al., 2007); and a HER2-target DC vaccine could be used to eliminate HER2-overexpression cells in patients with ductal carcinoma in situ lesions (Czerniecki et al., 2007). In a similar study, patients with advanced BC or OVCA received autologous DCs

pulsed with HER2- or MUC1-derived peptides. After vaccination, 50% of patients developed peptide-specific cytotoxic T lymphocytes (Brossart et al., 2000).

Other approach for immunotherapy is the combinatory vaccination with the desired antigen and plasmid DNA (pDNA). The first induces antigen-specific antibody production, while the former efficiently promotes the generation of antigen specific T-cells, as well as antibody production (Donnelly et al, 1997). Encouraged by this scenario, some researchers have observed that HER2-pDNA vaccination in combination with cytokines granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-2 administration is safe, well tolerated and can induce long-lasting cellular and humoral immune responses against HER2 in patients with advanced BC (Norel et al., 2010). Yet, some studies, targeting metastatic BC, found evidence that vaccination with Mage-b DNA was effective against metastases in various metastatic mouse breast tumor models (Sypniewska et al., 2005; Gravekamp et al., 2008). Mage is an attractive tumor-associated antigen because it is expressed in >90% of all BC but not in normal cells (Park et al., 2002). To further improve the vaccine efficacy of Mage-b, investigators have used an attenuated *Listeria monocytogenes* (LM) as DNA delivery system. LM is an intracellular pathogen that delivers the vaccine antigen directly into APCs, such as macrophages, with high efficiency (Paterson & Maciag, 2005). Regarding OVCA, a phase II study has evaluated the toxicity of carboplatin, GM-CSF and recombinant interferon γ 1b in patients with recurrent, platinum sensitive ovarian, fallopian tube and primary peritoneal cancer. This study has revealed an overall reasonable response and improvement in patients' quality of life (Schemeler et al., 2009).

3.2.2 Novel targets

The identification of novel biomarkers and tumor associated antigens is a promising approach for the effective therapy of cancer. Nowadays, researches are looking for both the antigens and auto-antibodies to the tumoral tissue. The search for antigens can be done in many levels, using high throughput technologies that allow the identification of variations in the expression profile of either mRNA or protein; whilst the search for natural antibodies has been done base on display technologies and combined proteomic and immunological approaches.

Type IIb sodium-phosphate cotransporter (NaPi-IIb)

A correlation between NaPi-IIb and OVCA has been demonstrated even before either the protein or the gene that encodes it (SLC34A2) have been characterized. Latter on, both the gene and the protein have been found overexpressed in OVCA when compared to normal ovarian tissue; and a difference has been found among the histological OVCA types, and according to the differentiation grade of the tumor (Rangel et al., 2003; Yin et al., 2008; Gryshkova et al., 2009). Nowadays, NaPi-IIb is no longer considered an OVCA-specific marker, since differences in its expression profile have been observed in non-small cell lung cancer, papillary thyroid cancer and BC (Kopantzec et al., 2008; Jarbaz et al., 2005; Kim et al., 2010; Chen et al., 2010). With respect to BC, the same overexpression pattern described to OVCA has been observed, however no correlation between the NaPi-IIb expression level and the differentiation grade of the tumors have been established. Therefore, owing to the high specificity and differential expression patterns showed in cancer, SLC34A2/NaPi-IIb has been pointed as a clinical relevant tumoral biomarker thus representing a potential target for cancer immunotherapy.

Claudins

Claudins are a family of proteins encoded by the claudin gene (CLDN). These proteins are transmembrane proteins with crucial role in the formation and function of tight junctions (Lal-Nag & Morin, 2009). Several analyses had shown that the members of this family have their expression profile altered in various types of cancer. For instance, CLDN3 and CLDN4 have been found to be up-regulated in OVCA and BC, among others. Intriguingly, CLDN16 is up-regulated exclusively in OVCA (Rangel et al., 2003). Moreover, CLDN7 and CLDN1 are down-regulated in BC among others (Morin, 2007). The precise role of claudins in cancer is still unclear; however, it has been hypothesized that the lost of claudins and other tight junction proteins expression could be an important step in the progression of cancer to metastases, since it would lead to loss of cell adhesion. In OVCA, CLDN overexpression has been related to increased invasion, motility and cell survival, all important characteristics to metastasis, thus playing a positive effect on tumorigenesis (Agarwal et al., 2005). Due to the specificity of claudin expressions patterns in cancer, as well as its membrane localization, it has been suggested that members of this family of proteins can represent useful targets to cancer therapy.

4. Nanotechnology and the development of novel drugs

By the end of the last century, Nanomedicine has become a recurrent expression on medical literature, and conjures up the use of Nanotechnology has brought impressive advances in medicine, both in diagnosis and therapy. As for therapeutic exploitation of Nanotechnology, the focus of this chapter, one of the main advances in this area is the use of nanoproducts as carriers of chemotherapy agents, producing nanodrugs. These carriers may be of different origins, but one the most used ones for drug delivery are the lipid nanocarriers, which include liposomes and multilamellar, multivesicular liposomes, nanostructured lipid carries and solid lipids (Cattaneo et al., 2010). Yet, the carriers themselves may have some modifications to avoid the immune system, enhancing the drug availability to the organism. This encapsulation of drugs brings many advantages such as lowering systemic toxicity, enabling drug targeting, and prolongation of drug half-life; also overcoming the issue of drug solubility, among others (Jain, 2010).

These presented characteristics theoretically oppose to the main disadvantages of classical chemotherapy (low or no specificity to cancer cells; low accumulation of the drug at the tumor site and high systemic toxicity). Thus, the use of nanodrugs in oncology may be considered as one of the most significant breakthroughs in cancer therapy, bringing new alternatives for the treatment of advanced, refractory and relapsing tumors. Additionally, tumors also present some unique features that facilitate drug targeting by nanodrugs, namely the presence of cancer cell-specific and tumor vasculature markers.

Currently there are about 150 nanotechnology-based drug in development, and some of them had already been approved by FDA (Ruoslahti et al., 2010). Among these, two nanodrugs are indicated by FDA for the treatment of OVCA and/or BC.

Abraxane® (Abraxis Bioscience) is a paclitaxel albumin-bound injectable particle suspension approved by FDA in 2005 for the treatment of metastatic BC which failed to respond to combined chemotherapy or that relapse within 6 months of adjuvant chemotherapy. This is a novel solvent-free formulation of paclitaxel in which it is bound to albumin forming 130 nm stable particles. The classical solvent to paclitaxel, Cremophor-EL, causes severe hypersensitivity reaction, thus this new formulation eliminates solvent-related toxicity. The

approval of Abraxane® was performed based on a comparative trial versus taxol with a group of metastatic BC patients. In this study, patients who have received Abraxane® had better response and progression rates. Currently, the use of Abraxane® for the treatment of other solid tumors is under evaluation, and some beneficial effects of these treatments has been reported to treat refractory metastatic OVCA.

Doxyl®/Caelyx® (Ortho Biotech) is a doxorubicin hydrochloride (HCl) encapsulated in pegylated liposomes for intravenous administration. The liposomes used to encapsulate this drug are modified (pegylated) to avoid liposomes to be identified by the mononuclear phagocytosis system, thus increasing the compound half-life in the organism. It is hypothesized that this nanocapsules are able to penetrate the tumor vascular system, but not the normal one, due to its small diameter (approximately 100 nm). Because of this, Doxil® is much more specific to tumor tissues, and thus presents a lower cardiotoxic activity when compared to doxorubicin alone. Doxil is approved with 3 indications, including the treatment of OVCA patients whose disease has progressed or relapse after platinum-based chemotherapy. In 2009, Ortho Biotech requested the indication of Doxil® in combination with docetaxel for the treatment of locally advanced or metastatic BC based on the results from 3 large, international, randomized, controlled studies that enrolled more than 1,500 patients. Even though the conclusion of this studies were that patients with advanced BC would clinically benefit from Doxil® therapy with a favorable risk benefit rate, FDA has not stated its decision about this new application to Doxil®.

5. Conclusion

To put it briefly, the present chapter has compiled the state-of-the-art information regarding BC and OVCA treatment. Women's health matters remain a priority in public and private care systems, as female cancers remain lethal diseases for which improved screening and treatment strategies are urgently needed.

6. References

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Adjuvant Therapy for Resectable Colorectal Cancer Liver Metastases

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1. Introduction

The most effective treatment for liver metastases from colorectal cancer is surgical resection. When curative resection is possible, a 5-year survival rate of 25% to 58% can be expected; the mortality rate associated with surgery is as low as 1% to 5% (Adson et al., 1984; Huges et al., 1986; Scheele et al., 1995; Nordlinger et al., 1996; Jamison et al., 1997; Iwatsuki et al., 1999; Fong et al., 1999; Minagawa et al., 2000; Figueras et al., 2001; Choti et al., 2002; Kato et al., 2003; Abdalla et al., 2004; Fernandez et al., 2004; Wei et al., 2006; Rees et al., 2008) (Table 1).

Surgical procedures are improving for liver resection. In cases with synchronous liver metastases, resection of primary colorectal cancer with lymph node dissection and liver resection were indicated if tumors would be completely removed surgically. In cases with metachronous liver metastases, the indication for liver resection was the same as synchronous liver metastases if extrahepatic metastases were not detected. More recently, experience has demonstrated that patients with the traditional adverse factors can experience long-term survival following liver resection (Minagawa et al., 2000; Elias et al., 2003). Thus, a shift has occurred in the criteria used for assessing resectability, from morphologic criteria to new ones based on whether a macroscopically and microscopically complete resection of the liver can be achieved. These advancements were reported to improve the resectability and survivals of patients. Although evidence from cohort studies or randomized controlled trials are not available to support this conclusion, cases of long-term survival are almost nonexistent among patients who were eligible for surgical resection of liver metastases but did not undergo the procedure. Outcomes after hepatectomy are so good that studies comparing this treatment with other treatments may be difficult to accept. Despite this, recurrence is common after resection of liver metastases. Recurrence in the remaining liver occurs with a frequency of 50% to 60%, followed by lung metastasis at 20% to 30%, and they occur within the first 2 years after surgery. Based on these observations, liver metastasis can be thought of as a local or systemic disease. Thus, improving prognosis after resection of liver metastases likely involves controlling local recurrence (in the remaining liver) and extrahepatic metastases (primarily lung metastases). However, few clinical trials have focused on cases of colorectal cancer after hepatic resection; no adjuvant chemotherapy has yet been proven effective in this context.

Author	Year	Number of patients	Median survival, months	5 year overall survival rate	Mortality, percent
Adson	1984	141	-	25	4
Huges	1986	607	-	33	5
Scheele	1995	350	40	39	4
Nordlinger	1996	1568	-	28	2
Jamison	1997	280	33	27	4
Iwatsuki	1999	305	-	32	1
Fong	1999	1001	42	37	3
Minagawa	2000	235	37	38	-
Figueras	2001	235	46	36	4
Choti	2002	133	-	58	0
Kato	2003	585	-	32	-
Abdalla	2004	190	-	58	-
Fernandez	2004	100	-	58	-
Wei	2006	423	-	47	1
Rees	2008	929	42.5	36	1

Table 1. Results of hepatic resection for metastatic colorectal cancer

2. Evidence of adjuvant therapy for resectable liver metastases

2.1 Hepatic infusion therapy as adjuvant therapy

Liver metastasis is a local disease limited to the liver. Several comparative studies have evaluated the use of hepatic infusion therapy to prevent recurrence in the remaining liver before secondary metastasis (e.g., lung metastasis) can occur (Table 2). Lorenz et al. conducted a randomized controlled study in Germany with 226 colorectal cancer patients who underwent resection of liver metastasis, comparing the efficacy of surgical resection alone with that of postoperative adjuvant chemotherapy by hepatic arterial infusion of 5-fluorouracil (5-FU) (Lorenz et al., 1998). Results of the interim analysis indicated that the median survival time (MST) after resection alone was 40.8 months, whereas the MST after resection and hepatic arterial infusion chemotherapy was 34.5 months. Because the hepatic infusion group had a lower survival rate at the interim analysis, enrollment was terminated. This study used the “surgery only” group as the control. Hepatic infusion therapy appeared to suppress recurrence in the remaining liver but did not improve survival. With the objective of suppressing both recurrence in the remaining liver and extrahepatic recurrence, Kemeny et al. conducted a randomized controlled study in the United States with 109 colorectal cancer patients after resection of 1 to 3 liver metastases.

They compared results of surgical resection alone with those of postoperative hepatic arterial infusion with floxuridine (FUDR) in combination with systemic chemotherapy with 5-FU (Kemeny et al., 2002). The 4-year disease-free survival rate was 25% for the surgery alone group and 46% for the postoperative adjuvant chemotherapy group. However, MST did not differ significantly between the two groups (surgery alone, 49.0 months; postoperative adjuvant chemotherapy, 63.7 months; $p=0.60$), i.e., adjuvant chemotherapy did not prolong survival in this study population. Using a somewhat different approach, Kemeny et al. compared two systemic chemotherapies: 5-FU with or without leucovorin versus hepatic arterial infusion of FUDR plus 5-FU with or without leucovorin. The investigators reported that FUDR combination chemotherapy resulted in better 2-year disease-free survival and 2-year survival rates (Kemeny et al., 1999). However, none of the studies shown in Table 2 clearly demonstrated that adjuvant hepatic arterial infusion chemotherapy is more effective than surgery alone.

Author	Year	N	Regimen	DFS MST, months	DFS, percent	P value	OS MST, monts	OS, percent	P value
Lorenz	1998	226	no	13.7		0.75	40.8		0.15
			HAI 5FU/LV	14.2			34.5		
Kemeny	2002	109	no		4 year, 25%	0.04	49		0.6
			5FU+HAI FUDR		4 year, 46%		63.7		
Kemeny	1999	156	5FU/LV		2 year, 42%	0.07		2 year, 76%	0.03
			5FU/LV+HAI FUDR		2 year, 57%			2 year, 86%	

Table 2. Randomized adjuvant studies comparing HAI with surgery \pm systemic chemotherapy for resectable liver metastases. HAI=hepatic arterial infusion, DFS=disease-free survival, OS=overall survival, NS=not significant

2.2 Systemic chemotherapy as adjuvant therapy

Few high-quality studies have evaluated the efficacy of systemic chemotherapy as a postoperative adjuvant therapy for cases of liver metastasis resection in colorectal cancer. One was a randomized controlled study (Portier et al., 2006), and the other was a pooled analysis (Mitry et al., 2008) (Table 3).

Using cases of curative resection, Portier et al. compared outcomes after surgery alone with outcomes after 6-month systemic chemotherapy using 5-FU and leucovorin. The 5-year recurrence-free survival rate was higher after adjuvant chemotherapy, but overall survival did not differ significantly (Portier et al., 2006). Mitry et al. re-analyzed the data from this study and an intergroup study (ENG trial) that had been terminated prematurely due to slow subject accumulation. Multivariate analysis showed that chemotherapy consisting of 5-FU and leucovorin improved life prognosis, but survival time did not differ significantly compared with surgery alone (Mitry et al., 2008).

Author	year	Number of patients	Regimen	DFS MST, months	<i>P</i> value	OS MST, months	<i>P</i> value
Portier	2006	171	no	17.6	0.028	46.4	0.13
			5FU/LV	24.4		62.1	
Mitry	2008	302	no	18.8	0.058	47.3	0.095
			5FU/LV	27.9		62.2	

Table 3. Randomized adjuvant studies comparing systemic chemotherapy with surgery alone for resectable liver metastases. DFS=disease-free survival, OS=overall survival

The European Organization for Research and Treatment of Cancer (EORTC) conducted a randomized controlled study (EORTC40983) that enrolled 364 colorectal cancer patients with liver metastasis eligible for curative resection (Table 4). The study compared results of surgical resection alone with results of surgical resection combined with leucovorin, 5-FU, and oxaliplatin (FOLFOX4) chemotherapy administered before and after surgery. The results were published in 2008 in *The Lancet* (Nordlinger et al., 2008). In the subgroup that underwent hepatectomy, the 3-year recurrence-free survival rate after hepatectomy was 33% for patients who underwent surgery alone and 42% for those who also received adjuvant chemotherapy ($p=0.025$). However, the trial design has been criticized. Although the secondary analysis showed significantly improved 3-year progression-free survival with adjuvant chemotherapy, the intention-to-treat analysis, which was the main analysis, did not show a significant difference. Overall survival time, the true endpoint, has not been published. The secondary analysis purportedly demonstrated a significant difference in progression-free survival time, but questions have been raised as to how this endpoint was defined (Nakamura et al., 2008). For example, to counteract a clear bias during the hepatectomy period for each group, they calculated early events by combining them at a single point; the chemotherapy group included cases in which hepatectomy was not indicated due to progression of the hepatic lesion or appearance of extrahepatic lesions after chemotherapy was initiated. For that reason, interpretation of the results is difficult.

Analysis by treatment group	chemotherapy group N	surgery group N	difference in rate of PFS at 3 years	HR	<i>P</i> value
All assigned patients (ITT analysis)	182	182	7.2	0.79	0.058
All eligible patients	171	171	8.1	0.77	0.041
All resected patients	151	152	9.2	0.73	0.025

Table 4. Results of EORTC40983 trial. ITT=intention-to-treat, PFS=progression-free survival, HR=hazard ratio

These data were not sufficient to establish preoperative and postoperative chemotherapy as a new standard treatment. Further, the subjects enrolled in EORTC40983 were limited to cases with no more than four liver metastases, corresponding to the H1 stage of the liver metastasis classification of the Japanese Society for Cancer of the Colon and Rectum (Kanahara Shuppan, 2009). The H1 stage is the earliest stage of metastasis, comprising fewer than half of liver metastasis resection cases encountered in clinical practice ("Study on liver metastasis in colorectal cancer," Project Study by Japanese Society for Cancer of the Colon and Rectum, 2004).

Thus, results of EORTC40983 demonstrated that the perioperative addition of FOLFOX4, a potent chemotherapy regimen, could improve prognosis after liver metastasis resection. Nevertheless, the optimal method of administering adjuvant therapy has not yet been established for liver metastasis resection in colorectal cancer.

2.3 Preoperative adjuvant chemotherapy for resectable liver metastases

The National Comprehensive Cancer Network guidelines (<http://www.nccn.org>) recommend multimodality therapy involving hepatectomy and chemotherapy for liver metastasis. For resectable liver metastasis, it recommends FOLFOX with or without bevacizumab or combined systemic and hepatic arterial infusion therapies after hepatectomy. Alternatively, systemic chemotherapy (FOLFOX, FOLFIRI, bevacizumab, cetuximab) should be administered before and after hepatectomy.

2.3.1 Clinical trials of preoperative adjuvant chemotherapy

To date, no study has reported the effectiveness of preoperative adjuvant chemotherapy for cases in which curative hepatectomy is suitable. An aforementioned EORTC40983 study (Nordlinger et al., 2008) shows a questionable survival benefit with chemotherapy; however, the EORTC40983 trial can be used as a reference.

2.3.2 Advantage of administering adjuvant chemotherapy before hepatectomy

What are the advantages of preoperative adjuvant chemotherapy compared with postoperative chemotherapy?

2.3.2.1 Increase in complete (R0) resection due to the shrinkage of liver metastases

Compared with prognosis after R0 resection, prognosis after incomplete resection (R1, R2) is significantly poorer. Charnasangavej et al. reported that cases with positive margins at hepatectomy frequently result in local recurrence at the site of resection, leading to poor prognosis (Charnasangavej et al., 2006). When liver metastasis foci are large or near the root of the hepatic vein or hepatic portal vessel, there is little room to obtain wide margins of normal tissue. A diameter greater than 5 cm for liver metastasis is an adverse prognostic factor (Kato et al., 2003). If neoadjuvant chemotherapy shrinks the liver metastasis, an adequate resection margin is easier to ensure, increasing the likelihood of R0 resection.

2.3.2.2 Preoperative chemotherapy is more effective at targeting micrometastatic foci and suppressing recurrence

Compared with healthy individuals, patients with liver metastasis from colorectal cancer have significantly higher levels of tumor growth factors, including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF). Levels of HGF and bFGF further increase after

hepatectomy to promote regeneration of the liver (Yoon et al., 2006). HGF enhances the movement and proliferation potential of colorectal cancer cells (Fukuura et al., 1998), and other growth factors likely stimulate and expand cancer microfoci. Elimination of cancer microfoci with preoperative chemotherapy would prevent recurrence after hepatectomy, even in the presence of high levels of growth factors. Tanaka et al. reported that preoperative hepatic arterial infusion chemotherapy suppressed microvascular invasion near liver metastasis foci in cases of multiple liver metastases in bilateral lobes (Tanaka et al., 2004). Therefore, preoperative chemotherapy is expected to exert some suppressive effects on micrometastatic foci.

2.3.2.3 Preoperative chemotherapy can be used to evaluate drug sensitivities

Sensitivity to chemotherapy can be evaluated by computed tomography, magnetic resonance imaging studies, or by histologic evaluation of the excised specimen. The most effective chemotherapy regimen is thus a strong candidate for postoperative adjuvant therapy. Ineffective regimens should not be used for later treatment.

2.3.2.4 Tolerance to chemotherapy is higher in the preoperative period than the postoperative period

For effective adjuvant chemotherapy, the planned dose should generally be administered without dose reduction. Preoperative chemoradiation for rectal cancer is thought to produce milder adverse events and a higher completion rate than postoperative treatment (Sauer et al., 2004). Similarly, liver metastasis tolerance to chemotherapy may be higher before hepatectomy.

2.3.3 Issues in neoadjuvant chemotherapy

2.3.3.1 Does neoadjuvant chemotherapy increase postoperative complications due to toxicity in normal liver tissue?

Neoadjuvant chemotherapy combining FOLFOX or FOLFIRI with bevacizumab and/or cetuximab exerts a strong tumor cell-killing effect; however, chemotherapy also damages normal liver tissue. A high degree of liver damage induced by chemotherapy may cause serious postoperative complications after hepatectomy. For example, the EORTC40983 trial (Nordlinger et al., 2008) reported that six courses of FOLFOX4 significantly increased postoperative complications. When choosing drugs and planning the duration of preoperative chemotherapy, a balance must be made between the tumor cell-killing effects of treatment and liver damage. Many cases of steatohepatitis were observed after treatment with the CPT-11 regimen, and the death rate soon after surgery was high (Vauthery et al., 2006); therefore FOLFOX is preferred. Results of the EFC2962 trial conducted by De Gramont et al., which evaluated progressive and recurrent colorectal cancer not treated with chemotherapy, and the V308 trial conducted by Tournigand et al. indicate that it takes about 2 to 3 months before the effects of FOLFOX therapy begin to appear (de Gramont et al., 2000; Tournigand et al., 2004). Neoadjuvant FOLFOX chemotherapy likely requires at least 4 to 6 courses.

2.3.3.2 When complete remission (CR) is achieved, resection of liver metastasis foci is difficult

Benoist et al. administered chemotherapy to 586 patients with liver metastasis, and 38 cases (6%), or 66 foci, were determined to have achieved CR based on imaging. Post-hepatectomy

pathologic examination revealed that 55 of the 66 (83%) foci were viable tumor foci (Benoist et al., 2006). Thus, metastatic foci may contain residual viable cancer cells, even if CR is noted on imaging; if not removed, residual cancer cells are likely to cause recurrence. Further, the lesion that demonstrated CR on imaging after preoperative chemotherapy cannot always be detected during surgery. While it is desirable to shrink cancer foci with preoperative chemotherapy, the danger is that the lesion cannot be later identified and resected.

At this point, neither safety nor effectiveness has been established for preoperative chemotherapy for resectable liver metastasis. Additional clinical trials will be needed to address this issue.

2.4 Future research

Surgery and chemotherapy combined can reduce the risk of relapse. However, until recently the role of adjuvant chemotherapy in the perioperative setting has been of unclear benefit. EORTC40983 trial (Nordlinger et al., 2008) fulfill a profound need for a well done randomized trial to compare surgery alone with surgery and chemotherapy for patients with resectable colorectal liver metastases. However, their decision to give preoperative chemotherapy to patients with resectable colorectal liver metastases, thereby postponing a possible curative treatment, can be disputed. Patients who receive preoperative chemotherapy increase their chance of postoperative complications, as stated in their report (25% vs 16%, $p=0.04$). Postoperative chemotherapy should theoretically be effective in dormant cancer cells in the remnant liver or body. Until the report of the trial by Portier *et al* (Portier et al., 2006), there has been no clear evidence that adjuvant chemotherapy, either systemic or by hepatic arterial infusion, added benefit over surgery alone from a randomized trial. The results of the trial led by Portier *et al* (Portier et al., 2006) represents that patients receiving postoperative systemic fluorouracil (FU) plus leucovorin (LV) fared significantly better than those receiving surgery alone (24.4 months vs 17.6 months, respectively) in disease-free survival. However, enrollment to the trial was suspended after 173 patients due to slow accrual and this trial did not have sufficient statistic power. When such trial took 10 years to finish accrual, the original question became outdated since the chemotherapy used in it is now considered inferior to currently available regimens containing potentially more active agents such as oxaliplatin, irinotecan, bevacizumab or cetuximab. Thus, there remains a need for clear evidence for whether combined treatment with chemotherapy is better than surgery alone in patients with resectable liver metastases from colorectal cancer. We, therefore, conducted a phase II/III randomized controlled trial to evaluate modified FOLFOX (mFOLFOX) as adjuvant chemotherapy for patients with curatively resected liver metastases from colorectal cancer.

The study protocol was designed by the Colorectal Cancer Study Group (CCSG) of the Japan Clinical Oncology Group (JCOG), approved by the Protocol Review Committee of JCOG on 15 February 2007. This trial was registered as JCOG0603 study (Kanemitsu et al., 2009) at the UMIN Clinical Trials Registry as UMIN000000653 (<http://www.umin.ac.jp/ctr/index.htm>).

2.4.1 Digest of the JCOG0603 study protocol

2.4.1.1 Purpose

To evaluate systemic intravenous adjuvant chemotherapy in comparison with observation alone after curative resection of liver metastases from colorectal cancer (Fig. 1).

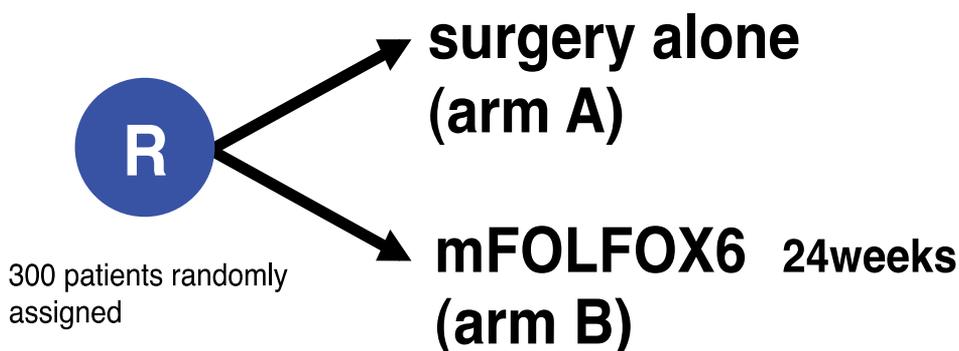


Fig. 1. JCOG0603 study scheme

2.4.1.2 Study setting

A multi-institutional prospective randomized controlled trial, with participating institutions including 39 specialized centers as of 4 September 2008.

2.4.1.3 Resources

Health and Labour Sciences Research Grants for Clinical Research for Evidenced Based Medicine, Clinical Cancer Research and Grants-in-Aid for Cancer Research (17S-3, 17S-5), from the Ministry of Health, Labour and Welfare, Japan.

2.4.1.4 Endpoints

The primary endpoint is treatment compliance at 9 courses after beginning mFOLFOX (bolus and infusion FU and LV with oxaliplatin) in phase II and is disease-free survival in phase III, respectively. Secondary endpoints are overall survival, incidence of adverse events defined by Common Terminology Criteria for Adverse Events (CTCAE) v. 3.0 and mode of recurrence after liver resection.

2.4.1.5 Eligibility criteria

Primary tumors are staged according to the sixth edition of the tumor-nodes-metastasis (TNM) classification system of the Union International Contre Cancer (UICC).

2.4.1.6 Inclusion criteria

Prior to entry to the study, the patients must fulfill the following criteria:

- i. histologically proven adenocarcinoma of the colorectum.
- ii. Complete macroscopic and microscopic (R0) resection of both primary and secondary lesions.
- iii. No extrahepatic disease.
- iv. No prior chemotherapy except oxaliplatin or radiotherapy within 3 months preceding registration.
- v. No prior radiofrequency ablation or cryotherapy for liver metastasis.
- vi. At 42 to 70 days after hepatectomy.
- vii. Age ranging between 20 and 75 years old.

- viii. Preoperative ECOG performance status 0-1.
- ix. Sufficient organ functions before chemotherapy.
- x. Written informed consent.

2.4.1.7 Exclusion criteria

Exclusion criteria are as follows: (i) synchronous or metachronous cancer; (ii) pregnancy; (iii) psychological disorder; (iv) steroid administration; (v) patients must use flucytosine, phenytoin or warfarin potassium; (vi) insulin dependent or uncontrollable diabetes mellitus; (vii) diarrhea or peripheral neuropathy greater than grade 1 and (viii) medical history of allergy or hypersensitivity to any drug.

2.4.1.8 Registration

Using telephone or fax to the JCOG Data Center, eligible patients between 42 and 70 days after liver surgery are registered centrally and assigned randomly by the minimization method of balancing the arm according to the synchronous or metachronous metastases to the liver, the number of metastases, the largest size of metastases, number of metastatic lymph nodes in the primary lesion and institution.

2.4.1.9 Treatment Methods (Fig. 2)

Enrolled patients are assigned to surgery alone (arm A, control group) or to mFOLFOX6 and surgery (arm B, treatment group). In arm B, adjuvant treatment is started between 56 and 84 days after liver surgery. Chemotherapy consisted of an intravenous injection of oxaliplatin 85mg/m² with I-LV 200mg over 2 hours plus 5FU 400mg/m² bolus and 2400mg/m² continuous infusion over 46 hours every 2 weeks. This cycle is repeated for 12 courses in the absence of disease progression or unacceptable toxicity.

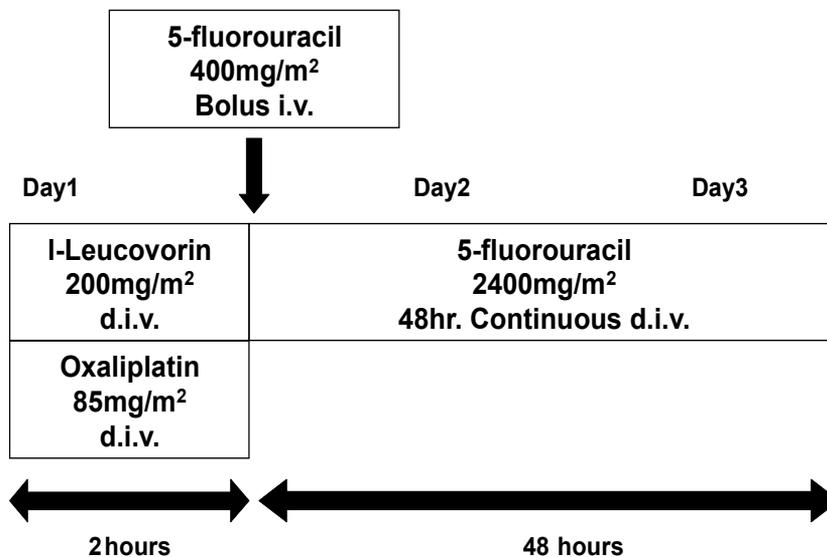


Fig. 2. Treatment schedule in postoperative chemotherapy arm

3. Conclusion

To date, no studies have demonstrated that adjuvant chemotherapy before or after radical resection clearly improves life prognosis for patients with liver metastasis from colorectal cancer. After EORTC40983, regimens involving strong preoperative chemotherapy attracted attention. However, preoperative chemotherapy increases the risk of hepatic dysfunction and postoperative complications. Thus, doubt has been raised regarding the use of preoperative adjuvant chemotherapy for resectable liver metastasis. Furthermore, hepatectomy may become impossible if the tumor grows because preoperative chemotherapy is ineffective or if target lesions disappear in cases of complete response. To improve outcomes for cases of resectable liver metastasis, postoperative adjuvant therapy strategies may be useful; however, there is insufficient evidence to recommend postoperative adjuvant therapy in clinical practice. This therapy should be considered experimental, and participation in a clinical trial is recommended to obtain high-level evidence. The Japan Clinical Oncology Group initiated a comparative study of surgery alone versus FOLFOX6 after curative resection of liver metastasis from colorectal cancer (JCOG0603). The active drugs (5-FU, CPT-11, oxaliplatin, and the molecular-targeted drugs bevacizumab and cetuximab) are available for use. In addition, the EORTC40983 trial suggested the usefulness of perioperative chemotherapy. Although the efficacy of these treatments has not yet been fully established, the use of adjuvant therapy before and/or after hepatectomy may be increasing. Opinion leaders in the world acknowledge that a no-treatment group control arm is necessary to obtain highly accurate study results (Alberts et al., 2006); however, the difficulty of enrolling patients in past trials (Langer et al., 2002; Portier et al., 2006) has led to study designs that no longer include a no-treatment group. This has hampered the accumulation of evidence supporting adjuvant chemotherapy in liver metastasis resection in colorectal cancer (Alberts et al., 2006). Combination chemotherapy has improved outcomes for colorectal cancer, but studies of adjuvant therapy for cases of liver metastasis resection in colorectal cancer are increasingly designed with technical rather than scientific considerations in mind. The no-treatment control group in the JCOG0603 trial allowed high-level evidence to be obtained, and the significance of these results is unquestionable. On the other hand, EORTC40983 presented ambiguous results. We conclude that the JCOG0603 trial is the only randomized controlled trial that may be able to clarify the effectiveness of postoperative adjuvant chemotherapy when liver metastasis is completely resected. We eagerly await the results of this trial.

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The Treatment of Metastatic Liver Disease of Colorectal Origin

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1. Introduction

Colorectal carcinoma (CRC) is one of the most frequent cancers in western societies, with an incidence of approximately 700 per million people. Every year there are approximately 125,000 new cases of colon cancer in the U.S. Fifteen percent of these patients will have liver metastases at the time of diagnosis, and another 50% will develop liver metastatic disease during the course of their disease (Kemeny et al., 2004). Although median survival of patients with untreated metastatic CRC is around 6 months, recent advances in adjuvant therapy after the colon resection offer the promise of a decrease in the number of cases with metastatic disease (Andre et al., 2004). Just as important, for the patients with liver metastatic disease, newer chemotherapeutic agents such as irinotecan and oxaliplatin, as well as new targeted agents such as cetuximab and bevacizumab in the current protocols have improved response rates and survival (Cunningham et al., 2004; Douillard et al., 2000; De Gramont et al., 2000; Hurwitz et al., 2004; Tournigand et al., 2004). Unfortunately, even with these combinations, the two-year survival is limited to 40% at best for patients with metastatic disease and only about 10% of patients with metastatic CRC survive beyond 5 years (McCarter & Fong, 2000).

These findings make surgical treatment the cornerstone of the therapeutic approach to this disease. Although only 10-25% of patients with liver metastatic disease are candidates for surgical resection, a combined therapeutic approach has shown the most promise, especially since it has been possible to convert around 15%-30% of previously considered unresectable patients and achieve survivals similar to the ones deemed resectable from the beginning (Bismuth et al., 1996). The majority of patients who undergo liver resection for metastases will experience intra- and/or extra-hepatic relapse of the disease. Even so, it has been demonstrated that resection of liver metastases increases survival, with 5-year survival rates of 30-50% in patients undergoing curative resections for their metastatic disease compared to 5-10% for non-operated patients (Fong et al., 1997; Kemeny et al., 2004; Scheele et al., 1990).

2. Defining resectable disease

The first question that has to be answered is what is considered resectable disease, a concept that has evolved significantly over time. Originally, it was felt that more than three metastatic liver lesions or patients with bilobar disease were not appropriate for resection.

However, more recent studies have shown that even in patients with poor prognostic signs, 5-year survival can be achieved after curative liver resection (Poston et al., 2005; Fong et al., 1999; Nordlinger et al., 1996). Despite the potential for cure, formal staging for liver metastases has not changed and remains stage IV along with incurable metastatic disease. The effort has thus been to identify appropriate selection criteria that allow discrimination of patients that would or would not benefit from surgical intervention. The value of these prognostic scoring systems is based on a combination of ability to predict outcome, as well as simplicity. Different studies have used a variety of prognostic features including age, number of metastases, size of the largest lesion, carcino-embryonic antigen (CEA) level, primary tumor stage, positive tumor resection margins, disease-free interval, positive lymph nodes from the primary, and have attempted to provide risk scores based on these factors (Fong et al., 1999; Nordlinger et al., 1996; Poston et al., 2005). Normograms have also been proposed as a potential improvement upon previous scoring systems. Rather than count risk factors, a normogram takes the specific value for each factor into account and calculates a specific score for each patient (Kattan et al., 2008). This leads to a potentially more accurate prediction, as it is specific to each patient. There is common agreement, however, in all these studies that although poor characteristics and high risk scores will definitely decrease survival, 5-year survival was still better compared to those patients with liver metastatic disease that had not undergone resection. As a result, none of these series suggested that patients with poor prognostic signs should not undergo surgery.

The indications have changed over time to the extent that currently a surgical resection would be of benefit if it is possible after resection to get an R0 resection, leave behind at least two contiguous segments and functional liver volume >20%. As it will be discussed later, even extra-hepatic metastatic disease is not necessarily a contraindication and more patients are being considered for resection (Khatri et al., 2005). Advances that have played a central role in this include preoperative portal vein embolization to induce hypertrophy of the nondiseased part of the liver that would remain behind, better vascular clamping techniques, controlled anatomic resection, the use of radiofrequency and microwave ablation for small lesions that may remain in the liver left behind after a resection, and more recently the use of image-guided liver surgery (Cash et al., 2007; Couinaud 1957; Curley et al., 1999; Fong & Wong 2009; Makuuchi et al., 1987, 1990).

Portal vein embolization (Figure 1) has allowed surgeons to be more aggressive in the treatment of CRC hepatic metastases, as one of the contraindications was a small future liver remnant (FLR) in patients with a small left lateral lobe who require an extended right hepatectomy. The small residual liver volume increases the risk of postoperative hepatic failure, and so in patients without cirrhosis a FLR of non-tumor volume of 25-30% is considered safe for hepatic resection. Selective portal vein embolization can produce atrophy of the segments affected by the cancer and compensatory hypertrophy of the contralateral segments, providing an increase of 10-30% in the FLR. Overall, there is no agreement that any specific substance is significantly better for the embolization. As helpful as this technique is, there have been several concerns raised. One potential difficulty for patients with metastatic CRC is that portal vein embolization may end up promoting tumor growth, thereby increasing the incidence of recurrence following liver resection. However, in a study of the long term survival following portal vein embolization, with 41 patients with CRC liver metastases, there was no evidence to suggest that patients whose surgery had been made possible by this technique were associated with a poorer long term survival (Elias et al., 2002). Given that the waiting time between embolization and resection is

usually around 6 weeks, an additional concern is the effect of chemotherapy administered during the peri-procedural period on the FLR hypertrophy and on the tumor growth in embolized segments in patients with CRC liver metastases. In a study of patients receiving chemotherapy after the portal vein embolization, FLR hypertrophied whether the patient had received chemotherapy or not; however, the hypertrophy was significantly less in those patients that had received post-embolization chemotherapy suggesting that although chemotherapy is not contraindicated, it should be considered carefully in those patients requiring a large compensatory hypertrophy (Beal et al., 2006).

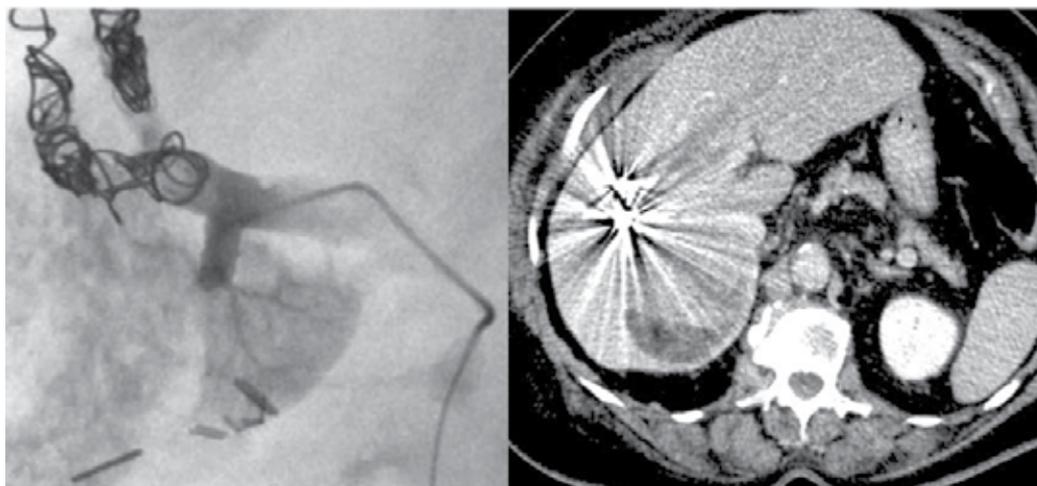
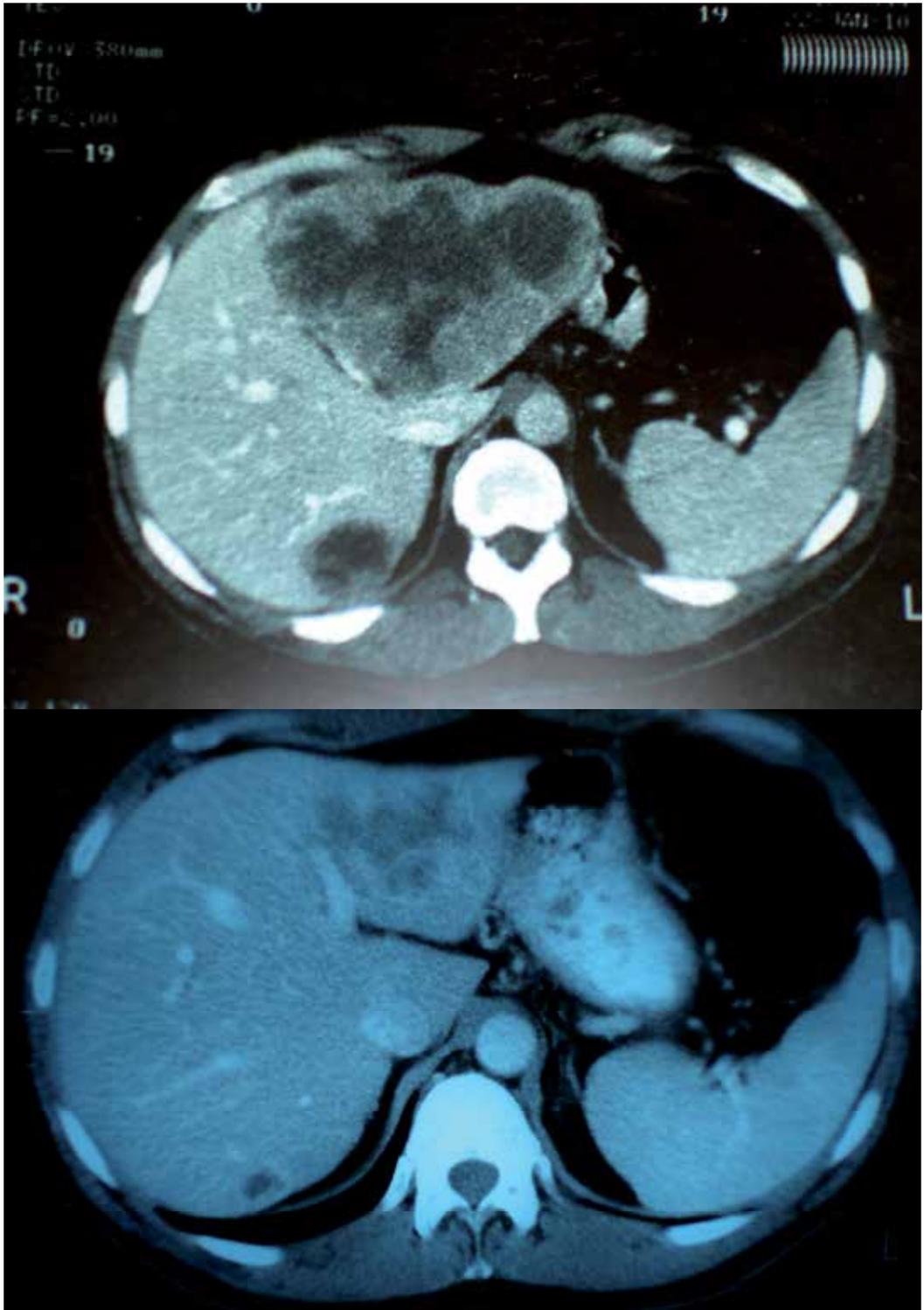


Fig. 1. Embolization of right portal vein branch to induce hypertrophy of the future left lobe remnant in anticipation of planned hepatectomy.

3. The role of chemotherapy

The targeted use of chemotherapy, such as 5-fluorouracil, Leucovorin and Oxaliplatin (FOLFOX) and 5-fluorouracil, Leucovorin and Irinotecan (FOLFIRI) has been critical in increasing resectability with rates ranging from 10% to 35% (Alberts et al., 2005; Ho et al., 2005; Masi et al., 2006; Wein et al., 2003). Comparisons have been somewhat difficult because of the different ways that unresectability is perceived or defined. Some studies include number of lesions or bilobar disease, whereas others look at more technical issues, such as involvement of all three hepatic veins, both portal veins, or the retrohepatic vena cava, or that resection would leave less than two segments or not an adequate liver reserve. Even with these limitations, there is definitely a role for neoadjuvant treatment in moving from unresectable disease to surgical cure (Figure 2 a, b, c).

More interesting is the question of the use of chemotherapy prior to resection in the case of resectable lesions. Arguments in favor include the decrease in tumor size, the potential control of micrometastatic disease, the assessment of the activity of chemotherapy as a method of *in vivo* chemosensitivity, improved chemotherapy tolerance, and a potential marker for the success of liver surgery (Adam et al., 2004; Fong et al., 1999; Nordlinger et al., 1996; Poston et al., 2005; Sauer et al., 2004). Arguments against preoperative chemotherapy use in resectable patients include liver toxicity (chemotherapy-associated steatohepatitis or CASH), risk of progression or growth in other sites, selection of resistant clones and the fact



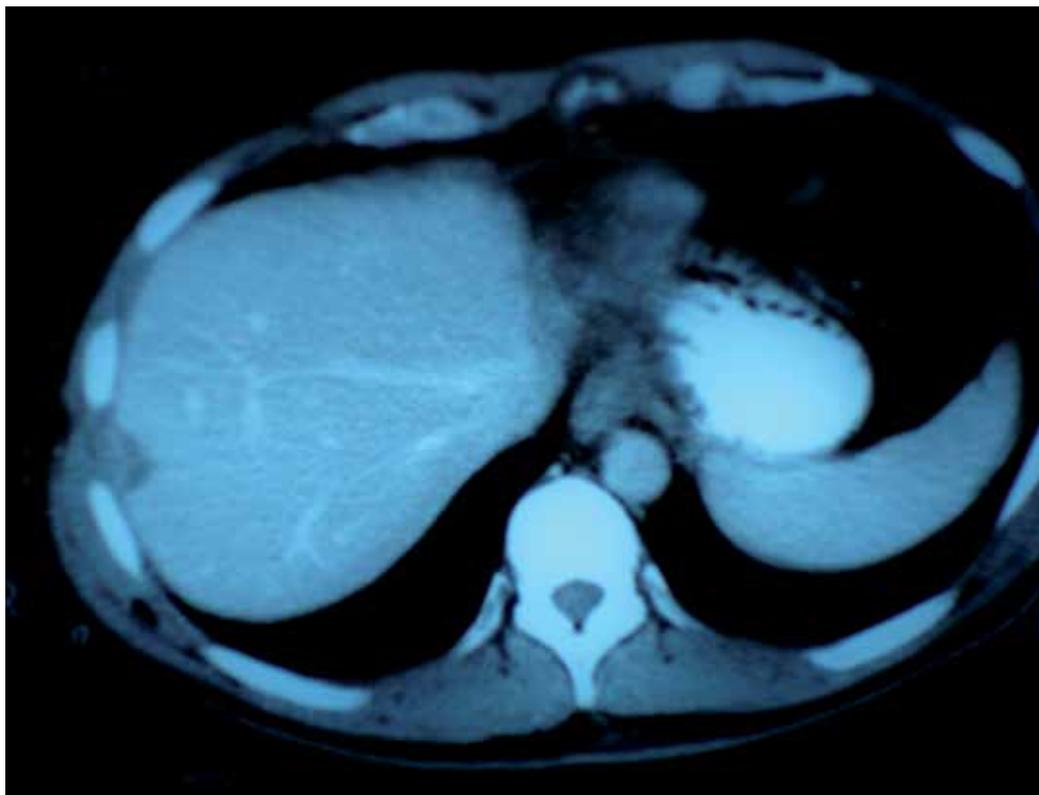


Fig. 2. a, b, c: Example of a good response to chemotherapy leading to resection of the metastatic disease. (a) CT of a patient with a large left lobe liver metastatic lesion from CRC. (b) Decrease in size of metastatic lesion after a full course of chemotherapy. (c) CT scan after the patient underwent a left hepatectomy and a radiofrequency ablation of a lesion on the right.

that response may make the surgery more difficult (Fernandez et al., 2005; Karakousis & Fong, 2009; Kooby et al., 2003). Specifically, as 70% of patients respond to cetuximab and FOLFOX chemotherapy and another 25% have stable disease, it is a very small percentage of 5-10% who may experience disease progression while receiving first line chemotherapy (Nordlinger et al., 2008; Tabernero et al., 2007). As a result, it is only after failure of first line chemotherapy that the concept of *in vivo* chemosensitivity makes sense. More importantly, advances in identifying molecular patterns and predictors of response have increased the value of resection, as such a strategy would make it possible to interrogate the tissues completely and potentially choose the best chemotherapy. One such example is the finding that tumor analysis for K-ras mutation status can be used as a predictor of response to cetuximab and oxaliplatin (Bibeau et al., 2009). Another problem with neoadjuvant chemotherapy for resectable lesions is that in a recent study only 66% of disappearing liver metastases following chemotherapy were complete responses, which means that a significant percentage of metastatic lesions were still present as the result of a reduction in the sensitivity of imaging during chemotherapy (Auer et al., 2010). This could lead to incomplete, noncurative resections. Overall, the prevailing opinion appears to be that,

unless the lesions are metachronous and of borderline resectability, they should be resected first with chemotherapy to follow (Figure 3).

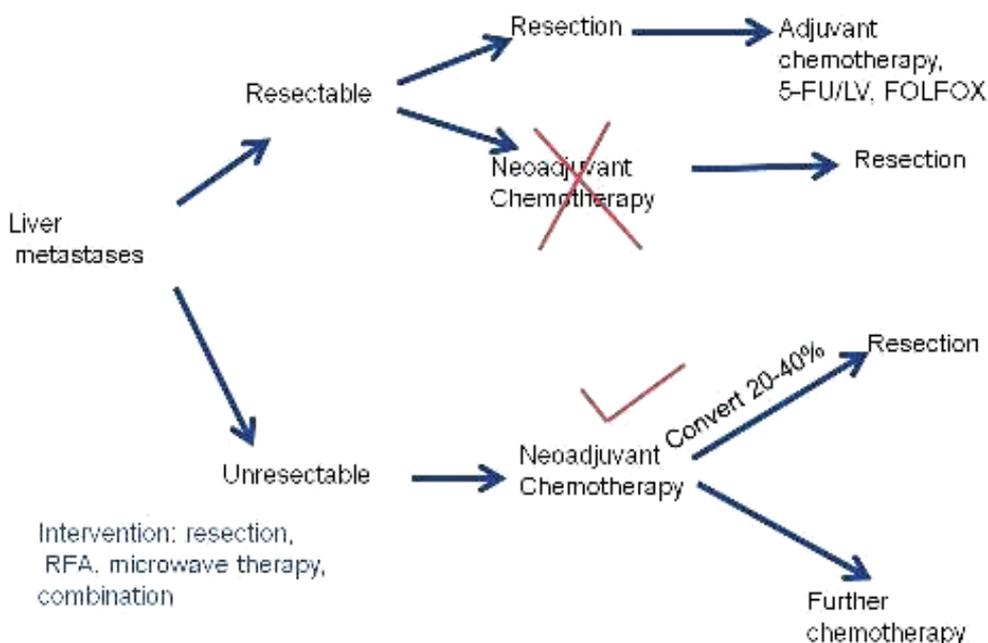


Fig. 3. Proposed algorithm for the treatment of liver metastases from CRC regarding the role of surgery and chemotherapy.

Regarding the question of adjuvant treatment of choice, there is agreement that adjuvant therapy after liver resection is useful. If a patient has not received any prior chemotherapy, treatment with 5-fluorouracil/leucovorin or FOLFOX are both reasonable choices, although there is definitely a need for further trials in the adjuvant setting (Park et al., 2007; Portier et al., 2006). For patients that fail first line adjuvant therapy molecular profiles, when available, may help in determining the optimal treatment. If a patient's disease has failed to respond to FOLFOX or FOLFIRI, then there exist options such as irinotecan/cetuximab or Xeloda/bevacizumab, treatments with a high cost of about \$100,000 for 6 months. These patients need to be enrolled in registries and treated under protocols, so that decisions can be made based on available data.

4. Pre- and intra-operative plan

4.1 Pre-operative planning

It was not unusual having percentages as high as 40% of patients found to be unresectable during surgery, mainly because of the difficulties in properly assessing the location and number of metastatic lesions (Steele et al., 1991). Progress has been made and in order to determine the resectability of the lesion preoperatively, radiologic studies can offer valuable information. Specifically, a triple phase CT with volumetry can be used to identify the location and vascular supply of the lesions and serve as a road map, as well as an estimate

of whether a resection would leave behind an adequate liver remnant. More advanced versions of this are the computer-generated models that allow an image-guided approach to the resection of these lesions, even in real time (Cash et al., 2007; Fong & Wong, 2009). One example is the MeVis software package from HepaVision (Bremen, Germany) which allows a computer-assisted 3D surgery planning. This leads to improved pre-operative planning on how to gain better access to the metastatic lesions even in regions at risk for devascularization or impaired drainage. Also, the volume of the remaining liver parenchyma is calculated separately for each resection proposal (Figure 4).

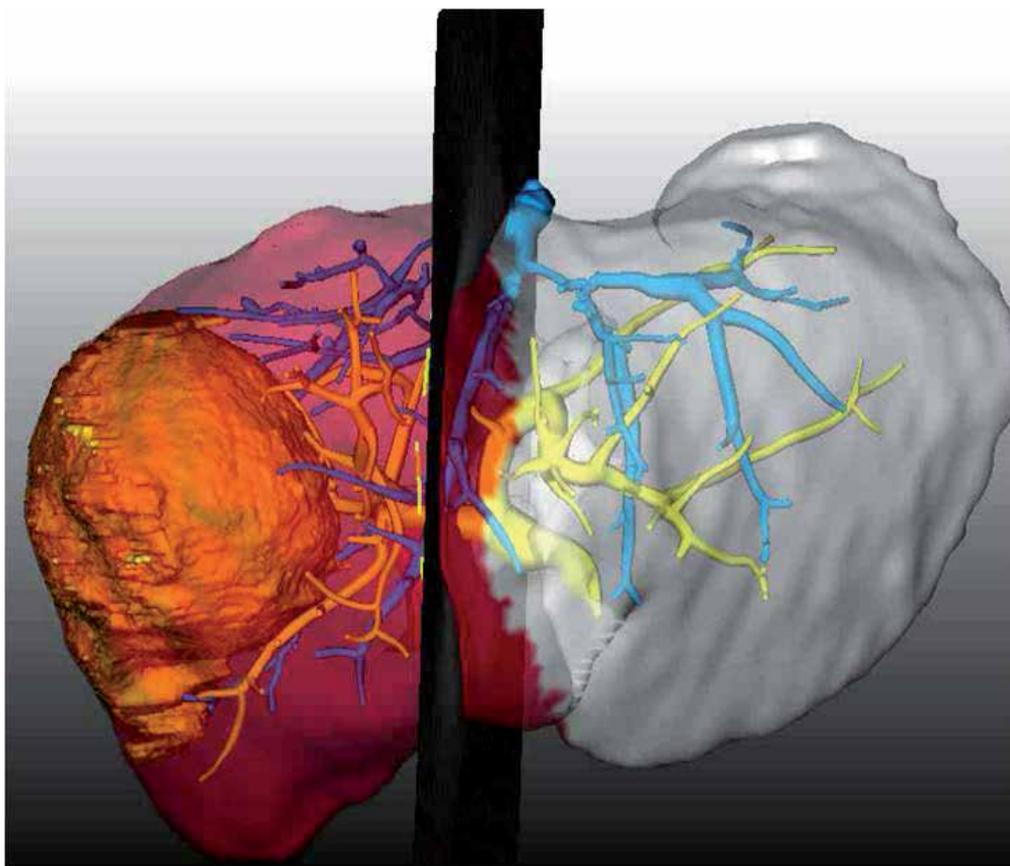


Fig. 4. 3D modelling of a large right lobe metastatic lesion based on CT data obtained using the MeVis system (HepaVision, Bremen, Germany), that allows visualization of the vasculature of the mass, as well as its relation to the venous drainage system of the liver and an estimation of the remaining liver volume based on the proposed resection plane.

It is also important to perform a thorough search for extrahepatic disease, to either exclude the patient from resection, or at least have a plan that would address the different sites of metastatic disease. For a patient with isolated liver metastatic disease, a CT of the chest, abdomen and pelvis is performed at first to identify the full extent of the hepatic disease, as well as discover any extrahepatic disease. FDG-PET has also been used to both identify the presence of hepatic colorectal metastases and to improve the staging of patients under

consideration for resection (Truant et al., 2005). Although PET can be a valuable resource in helping define whether a lesion is metastatic or not, there are limitations, such as the fact that it may miss small lesions, it is expensive, and that it may be affected by recent administration of chemotherapy. Hepatic lesions identified should not be biopsied as there is a real risk of extrahepatic dissemination of tumor with percutaneous biopsies (Metcalfe et al., 2004).

4.2 Intra-operative planning

This diligence should continue intraoperatively, where laparoscopy at the beginning of the surgery may identify occult metastatic disease that may prevent an unnecessary laparotomy. Furthermore, the use of intraoperative ultrasound is almost mandatory, both to identify the known lesions and their location in relation to the surrounding vessels, as well as to look for other lesions that may not have been detected preoperatively (Figure 5). Intraoperative ultrasound allows confirmation of expected sites of disease and may detect additional lesions in 10-50% of cases (Machi et al., 1991; Makuuchi et al., 1991). Confirmation of the hepatic vascular anatomy in relation to the lesion and identification of specific segmental pedicles give the surgeon the opportunity to obtain a clear demarcation line in the parenchyma by occluding the vascular pedicle responsible. This allows resection of only the involved parenchyma with an exact transection plane.

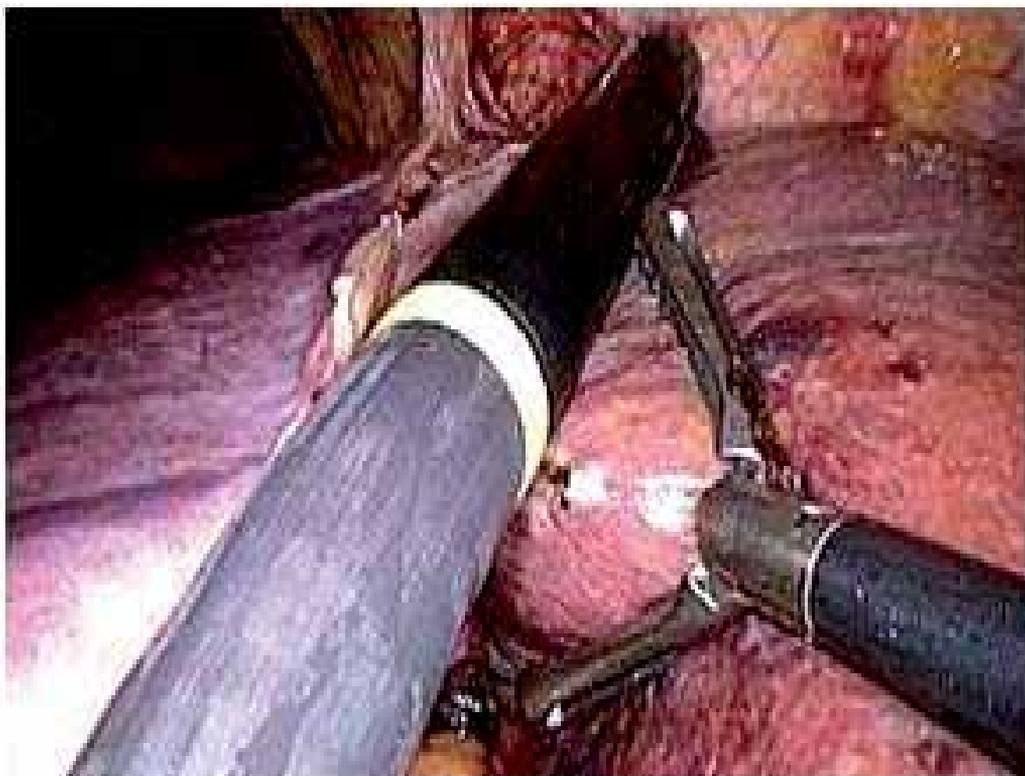


Fig. 5. Intraoperative US of the liver during a laparoscopic liver resection.

Regarding the technical part of the resection, the aim is to resect the liver parenchyma with minimal bleeding, avoiding biliary leaks and leaving adequate functional liver. Various techniques can be used according to surgeon preference to achieve this, including the use of hemostatic clamps, the handle of a scalpel, or finger fracture. More recently, there is widespread use of ultrasonic dissection using ultrasonic aspirators (an acoustic vibrator, perfused with saline, which disrupts the liver parenchyma by producing a cavitation force), or other instruments such as water-jet dissection or ultrasonic cutting. Prior to division of the parenchyma, whenever possible, vascular occlusion can be attempted to minimize bleeding, and which is subsequently released at the end of the parenchymal dissection.

Over the last several years there has been a more aggressive approach undertaken by many surgeons in the treatment of CRC liver metastases, based on the significant improvements in surgical techniques, adjunctive treatments such as portal vein embolization and radiofrequency ablation, and the effectiveness of newer chemotherapeutic regimens. This has led to a change in surgical approach with an increase of nonanatomical resections (Gold et al., 2008). This technique maximizes the amount of residual parenchyma, which is important for patients at risk for hepatic insufficiency, as well as in those that have received chemotherapy (Figure 6 a,b).



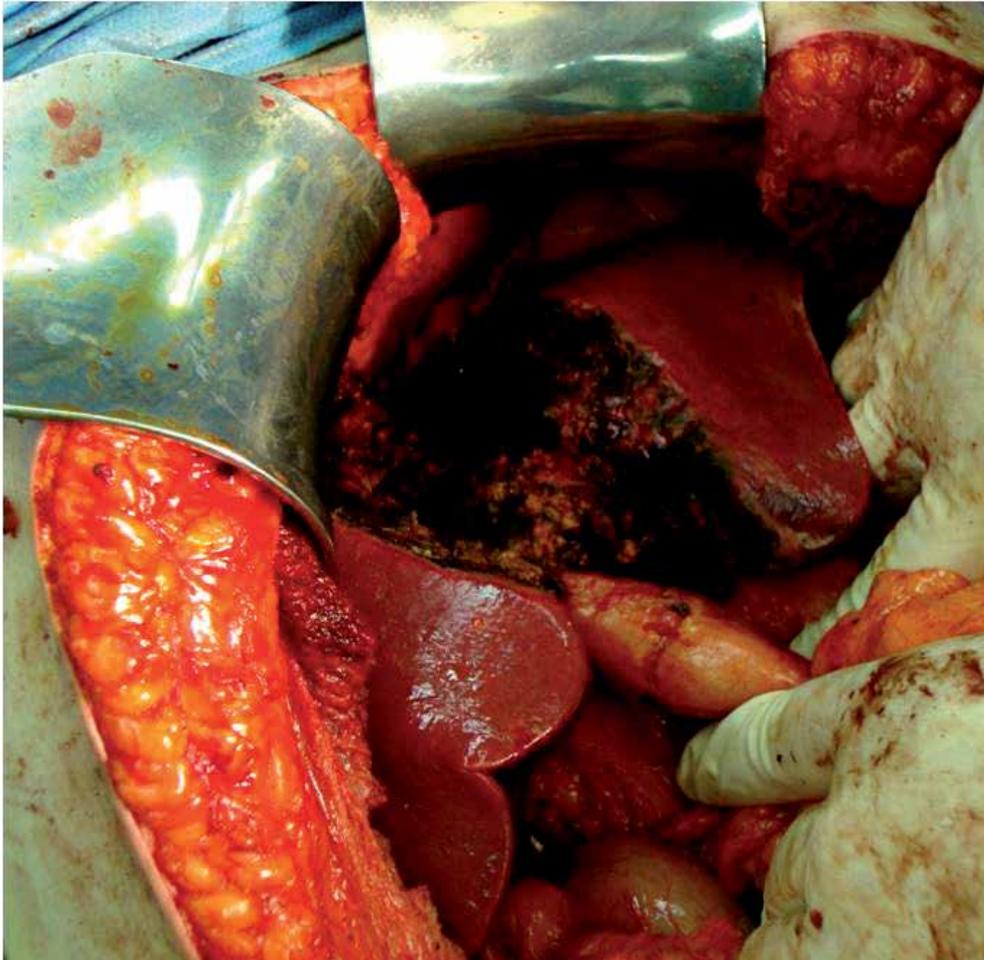


Fig. 6. a, b: (a) CT of a large CRC hepatic metastatic lesion. (b) Nonanatomical resection of central CRC hepatic metastatic lesion.

Additionally, in case of intrahepatic recurrences after partial liver resection in patients with liver metastatic disease, a sufficient liver remnant allows the possibility of further surgical or ablative treatments (Figure 7 a,b). Sparing liver parenchyma may also mean minimizing the surgical stress involved for the patients as the surgery becomes more targeted, something which can translate to shorter operating times and decreased blood loss (Stewart et al., 2004).

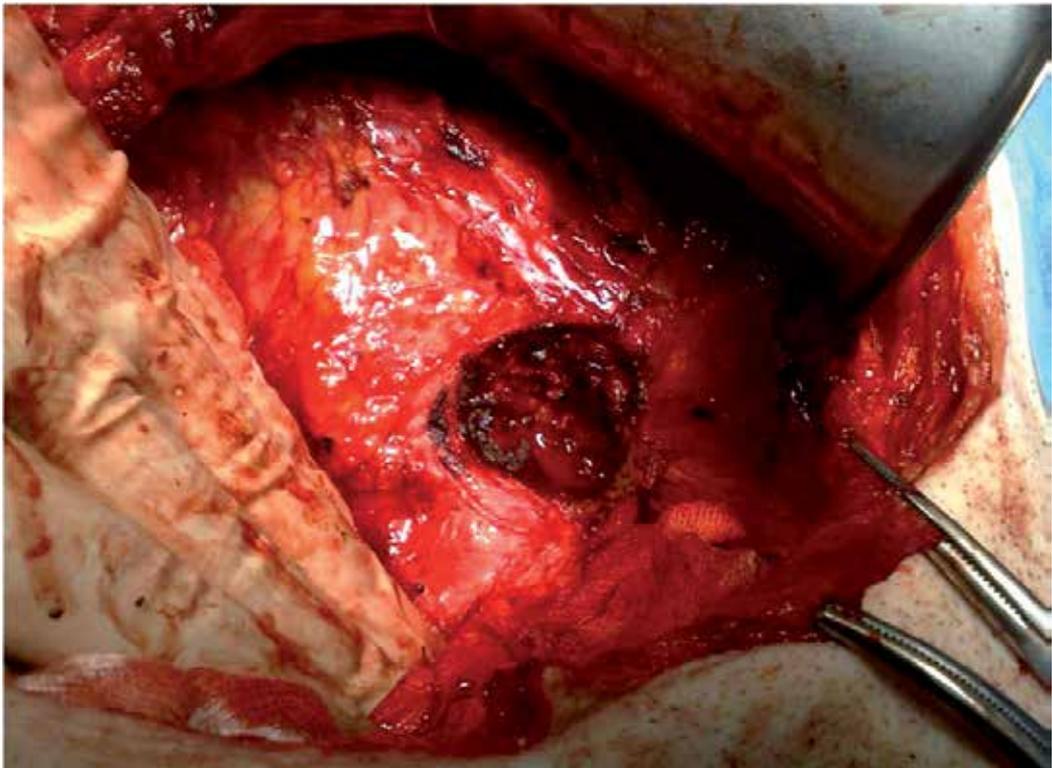
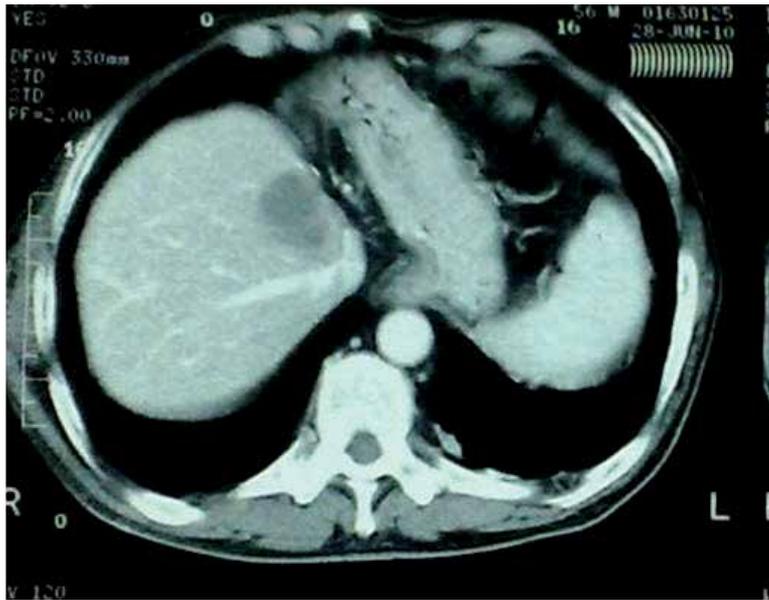


Fig. 7. a, b: (a) CT of a CRC hepatic metastatic lesion in a patient that had undergone a previous left hepatectomy for another metastatic lesion. (b) Nonanatomical repeat resection of metastatic lesion in close proximity to the hepatic veins.

5. Technical considerations & controversies

5.1 Laparoscopic resection

Since the first laparoscopic liver resection was reported in 1992, the number has increased significantly, numbering in the several thousands, as the technique offers the advantages over open surgery of reduced postoperative pain, less operative morbidity, shorter hospital stay, and faster recovery (Buell et al., 2008; Koffron et al., 2007). These lesions can be resected using a totally laparoscopic technique, a robotic-assisted minimally invasive technique, or a hand-assisted laparoscopic approach. The latter is considered as a safe first step in the learning curve, as it offers the surgeon the safety of having a hand inside the abdomen, thus making it easier to obtain vascular control, should that become necessary (Figure 8).



Fig. 8. Port placement for the performance of a hand-assisted laparoscopic liver resection.

Despite the early introduction of laparoscopic liver resection, the technique has not gained widespread acceptance. Most hepatobiliary centers perform only open liver surgery, and even in centers performing laparoscopic liver resections, the open procedures are still the majority. One reason is the technical challenge of the procedure, as it is time-consuming and difficult to master laparoscopic liver resection. Especially for senior surgeons skilled in traditional hepatobiliary techniques, achieving mastery with a skill as complicated as laparoscopic liver resection may seem as a challenge not worth the effort. This can restrain the rapid development of laparoscopic hepatic resections in hepatobiliary centers. Another reason for the limited use of this technique is the fear that it represents a less oncologically sound method, by being less radical than the open procedure. This has not been shown to be the case, as laparoscopic liver resection does not appear to compromise oncological measures, such as margin status, disease-free survival and overall survival (Nguyen et al., 2011). In a multicenter, international series of laparoscopic resection for colorectal carcinoma metastases in 109 patients there were no perioperative deaths and a complication rate of 12% (Nguyen et al., 2009). The series included a significant number of major resections (45% were more than 3 segments) and negative margins were achieved in 94% of patients, with

overall survivals at 1-, 3- and 5-years of 88%, 69% and 50% respectively. These numbers are highly comparable to the open procedure regarding outcome.

Although the outcome between open and laparoscopic liver resection may be similar, there are areas where the laparoscopic procedure appears to have the upper hand, including median hospital stay and morbidity (Buell et al., 2008; Koffron et al., 2007). Another increasingly relevant question is that of the cost. It has been found that patients undergoing laparoscopic surgery have higher operating room costs compared to patients undergoing an open liver resection; however, the total hospital costs are not different between the two groups, mainly because the laparoscopic group of patients are able to leave the hospital sooner (Nguyen et al., 2011).

5.2 Treatment of synchronous metastases

Synchronous liver metastases, commonly defined as liver metastases occurring within 12 months of the colon primary, offer the challenge of the optimal timing for surgical resection. The original paradigm of the staged resection (colon primary first with the liver metastatic resection 2 to 3 months later), has begun to change and has evolved to one where good results can be achieved with simultaneous resection (Martin et al., 2003; Lyass et al., 2001). A study of 230 patients (70 undergoing simultaneous resection and 130 staged) revealed no difference in morbidity and mortality, but a significantly shorter hospital stay for the group undergoing simultaneous resection (Martin et al., 2009). The main limitation of this strategy is that it can only be offered to a limited number of patients with synchronous disease and that it is associated with an increased risk of postoperative complications as the patient undergoes two major surgeries in the same setting (Reddy et al., 2007). Recently, a “reverse strategy” has been advocated, in which preoperative chemotherapy is followed by resection of the CRC liver metastases first, followed by resection of the colorectal primary at a second stage (Mentha et al., 2006). This has been proposed for patients with advanced CRC liver metastases and a stable primary cancer, especially if it is a rectal one. The rationale is that complications (bleeding, perforation, obstruction) from the primary are rare in patients with stage IV CRC being treated with chemotherapy, and also that this way treatment of the metastatic disease is not delayed till completion of the treatment of the primary. A recent study has shown that all 3 strategies, namely the classic, the combined and the reverse in patients with synchronous presentation of liver metastases have similar outcomes (Brouquet et al., 2010). For this reason it is important to individualize the strategy according to the extent of the disease of both the primary and the metastases for each patient.

5.3 Management of extrahepatic disease

What was previously not an option, has gained significant ground as an aggressive multidisciplinary approach leads to long term survival in cases of serial metastasectomy of hepatic and pulmonary metastases from colon cancer. Studies have reported 5-year survivals of 51%, with the key being an aggressive approach where every time a metastatic lesion is identified, it is resected (Mineo et al., 2003; Nagakura et al., 2001; Shah et al., 2006). Although there have been reports of resection of portal lymph-nodes and peritoneal metastatic lesions, in addition to the hepatic ones, these should be viewed very cautiously as the results are rather disappointing (Adam et al., 2008; Yan et al., 2006). However, the possibility does remain that in time as our experience grows it may be possible to better

define long-term outcomes and identify biological markers that will predict tumor behavior, and thus enable a more aggressive approach.

5.4 Is there a role for liver transplantation?

The past experiences of liver transplantation for colorectal cancer liver metastases have led to long-term survival and even cure in some cases (Hoti & Adam, 2008; Kappel et al., 2006). This is not surprising since liver transplantation for liver-only metastatic disease is by definition an R0 resection and as such from an oncological perspective acceptable. However, from the perspective of liver transplantation, given the organ shortage, the outcome has to be comparable to other indications for liver transplantation. If we add to this the fact that overall survival following liver transplantation has dramatically improved and that patients with hepatic metastatic disease of colorectal origin present less of a technical challenge, given the lack of cirrhosis and portal hypertension, these patients should be considered relatively low-risk for liver transplantation. An additional argument is the use of a class of immunosuppressive medications, the mTOR inhibitors that have shown clinical effect and stabilization of disease for a variety of cancers, in their role as antiproliferative agents (Atkins et al., 2004; Chan et al., 2005; Fung et al., 2005).

Based on these premises, a group from Norway, taking advantage of the surplus of donor organs in that country, initiated a study where 16 patients underwent liver transplantation for isolated hepatic metastatic colorectal disease (Foss et al., 2010). Although 2-year survival was 94%, there was a high recurrence rate of 63%, with an excellent quality of life. These preliminary data seem promising; however, it is too early to tell whether this is a beneficial strategy. Specifically, it is important to evaluate the 5-year survival in order to see whether it is comparable to other indications for liver transplantation so as to justify the use of a limited organ supply. In addition, selection criteria for the candidates need to be refined to be able to decrease the high recurrence rate.

5.5 The role of combination therapy

The same aggressive approach to the treatment of colorectal liver metastases, has led to the use of several treatment modalities in combination, which in turn has led to renewed efforts towards dealing with more advanced lesions. In a series of 224 patients, where a very high number had multiple (five or more) bilateral liver lesions, treatment consisted of a combination of hepatic arterial chemotherapy, cryotherapy and resection (Yan et al., 2006). This led to 1-, 3-, and 5-year survival rates of 87%, 43% and 23% respectively in this high risk group of patients. The surgeon and the medical team have a wide armamentarium in their hands and it is up to them to find the right treatment modality for the right patient.

5.6 The role of ablative therapy

A special mention should be made regarding the role of ablative therapy in the treatment of liver metastatic disease from colorectal cancer, and especially that of radiofrequency ablation (RFA). With this technique a probe is inserted into the lesion and through the transfer of current the lesion is heated to the level of 90 to 100 degrees centigrade (Figure 9). The use of this method has grown exponentially with the combined use of intraoperative ultrasound as smaller lesions can be localized more accurately. Additionally, advances such as the use of multiple tines on these probes have made possible the ablation of bigger lesions up to 5 or 6cm.

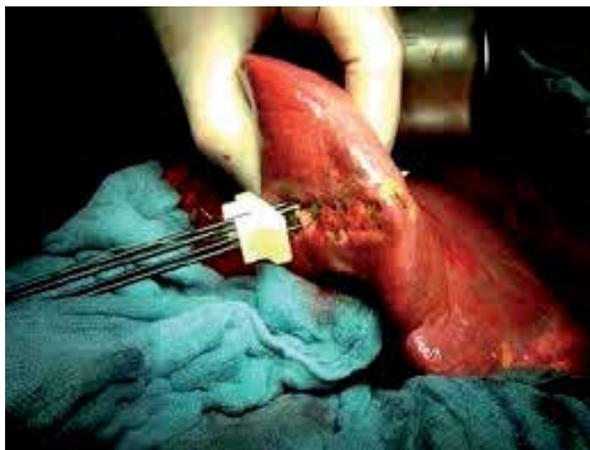


Fig. 9. Radiofrequency ablation of hepatic metastatic lesion from colorectal cancer.

As experience with RFA has increased over the last several years, there has been an effort to comprehensively evaluate the results. The American Society of Clinical Oncology in 2009 in a Clinical Evidence Review regarding RFA of hepatic metastases from colorectal cancer, suggests that, based on the existing evidence, hepatic resection improves overall survival compared to RFA, especially for patients with resectable tumors without extrahepatic disease (Wong et al., 2010). RFA investigators report a wide variability in the 5-yr survival rate (14% to 55%) and local tumor recurrence rate (3.6% to 60%). The reported mortality rate was low (0% to 2%), and the rate of major complications was commonly reported to be 6% to 9%. In another systematic review of the clinical benefit and role of radiofrequency ablation as treatment of colorectal liver metastases, the authors found that comparative studies indicated significantly improved overall survival after RFA versus chemotherapy alone, RFA plus chemotherapy versus RFA alone and up-front RFA versus RFA following second-line chemotherapy (Stang et al., 2009). The findings of these authors support the notion that RFA prolongs time without toxicity and survival as an adjunct to hepatectomy and/or chemotherapy in well-selected patients, but not as an alternative to resection.

6. Outcomes and keys to success

Treatment outcomes for patients with hepatic metastatic lesions from CRC have improved significantly over the last decade. This has been a result of a variety of factors, including improvements in surgical techniques and instrumentation, advances in chemotherapy, our understanding of tumor biology, but more importantly the use of multidisciplinary teams where the combined expertise of different specialties is used for the patient's benefit.

6.1 Outcome and recurrence after liver resection

Five-year overall survival for patients with hepatic metastases from CRC treated with the combination of surgery and chemotherapy ranges anywhere from 45% to 65% for both open and laparoscopic liver resections (Castaing et al., 2009; Ito et al., 2008; Kazaryan et al., 2010). Recurrence can occur in as many as 60% of patients following liver resection of colorectal metastatic disease, with the most frequent site of recurrence being the liver; in approximately 20% of these patients the liver may be the only site of recurrence and as a

result these patients may be suitable for re-resection (Wong et al., 2010). The vast majorities of these recurrences occur in the first two years and for that reason frequent surveillance with CT is critical for early detection. This becomes even more important if we consider that the reported morbidity and mortality rates, as well as overall survival rates after re-resection, despite the potential greater technical difficulty, are similar to those reported for the initial hepatectomy (Stang et al., 2009; Wong et al., 2010). In the current cost-conscious environment, the fact that intensive 3-monthly CT surveillance detects recurrence that is amenable to further resection in a considerable number of patients, leads to significantly better survival for these patients with a reasonable cost per life-year gained (Wanebo et al., 1996).

6.2 Use of multidisciplinary teams

A key component for a successful outcome for patients with hepatic metastases from colorectal cancer is the close cooperation between the colorectal and the hepatobiliary team. Both of these should consist of specialist surgeons, in addition to an oncologist, gastroenterologist, diagnostic and interventional radiologist, histopathologist and clinical nurse specialist. The goal is to achieve a multidisciplinary input, as well as develop protocols that will be the cornerstone of developing a “best practices” approach. The improved outcomes that we are witnessing in the management of liver metastatic lesions from colorectal cancer are most likely the result of this concerted effort, as well as possibly a volume effect.

7. Conclusion

Patients with hepatic metastases from colorectal cancer represent a difficult challenge for the medical and surgical team caring for them, as we are dealing with an advanced stage of a disease. However, the coordinated effort of the different specialties has made it possible to achieve 5-year survivals of 50% and in certain cases even talk about a cure. Central to this effort are the surgical advances and techniques that have allowed resection of these metastatic lesions in a safe and precise manner and have transformed the essential question from “what can be removed” to “what needs to be left behind”. Patients and physicians undertaking this endeavour need to be prepared for a “marathon”, as when dealing with metastatic lesions one has to be prepared for recurrences and find ways to address them. However, with a combination of proper patient selection, choice of the appropriate strategy in terms of combining surgery with chemotherapy, and application of the right mixture of resection and ablation techniques, it is possible to achieve optimal oncologic results in this very challenging group of patients.

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Th1 Cytokine-Secreting Recombinant Bacillus Calmette-Guérin: Prospective Use in Immunotherapy of Bladder Cancer

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1. Introduction

1.1 Clinical use of BCG in bladder cancer treatment

Urothelial carcinoma of the bladder is the second most common urologic neoplasm after prostate carcinoma in the United States, with an estimated 70,530 new cases and 14,680 deaths in 2010 (Jemal et al., 2010). Global prevalence of bladder cancer is estimated at >1 million and is steadily increasing. At the time of diagnosis, 20-25% of cases are muscle invasive (stage T2 or higher) and are typically treated with surgical resection (radical cystectomy) (Williams et al., 2010). The remainders are nonmuscle invasive bladder cancer (NMIBC) including tumors confined to the epithelial mucosa (Ta), tumors invading the lamina propria (T1), and carcinoma *in situ* (Tis). Transurethral resection of bladder tumor (TURBT) is the primary treatment for Ta and T1 lesions. Intravesical therapy is used as adjuvant treatment to prevent recurrence and progression of the disease after TURBT and is also the treatment of choice for carcinoma *in situ*. Intravesical administration of bacillus Calmette-Guérin (BCG), a live attenuated strain of *Mycobacterium bovis* widely used as a vaccine against tuberculosis, is currently the most common therapy employed for NMIBC. Since its advent in 1976 (Morales et al., 1976), BCG has been extensively used to reduce recurrence and progression of NMIBC in an attempt to preserve the bladder. BCG therapy results in 50-60% effectiveness against small residual tumors and a 70-75% complete response rate for carcinoma *in situ*. Adjuvant intravesical therapy was noted by the 2007 American Urological Association (AUA) panel to reduce recurrences by 24% and treatment with BCG was recommended by the panel. Unfortunately, a high percentage of patients fail initial BCG therapy and 40-50% of BCG responders develop recurrent tumors within the first 5 years (Williams et al., 2010). In addition, up to 90% of patients experience some sort of side effects including, although rare, life-threatening complications such as sepsis.

According to the AUA's 2007 clinical practice guidelines, BCG therapy should be initiated two to three weeks following TURBT with a classic course consisting of six weekly intravesical installations. Lyophilized powder BCG (81 mg corresponding to $1-5 \times 10^8$ colony-forming units of viable mycobacteria) is reconstituted in 50 ml of saline and administered via urethral catheter into an empty bladder with a dwell time of 2 hours. Maintenance BCG is more effective in decreasing recurrence as compared to induction therapy alone. Multiple meta-analyses support BCG maintenance and it is now firmly established in clinical practice. The European Association of Urology (EAU) and the AUA

recommend one year of maintenance for high risk patients (Hall et al., 2007; Babjuk et al., 2008). An optimal schedule/duration of therapy has yet to be determined; however, most who use maintenance follow some permutation of the Southwest Oncology Group (SWOG) program, a 3-week “mini” series given at intervals of 3, 6, 12, 18, 24, 30 and 36 months (Lamm et al., 2000). At our own institution, induction (first BCG therapy) is initiated 2 to 3 weeks following TURBT with 6 weekly installations and a 1-2 hour dwell time. For patients with carcinoma *in situ*, severe dysplasia, Grade 3/high grade or poorly differentiated pathology, and/or stage T1 disease, formal restaging under anesthesia is performed 6 weeks later including obtaining bilateral upper tract cytology, retrograde pyelograms, 4-5 random bladder biopsies, and prostatic urethral biopsies. If this pathology and restaging is negative, maintenance cycles may be initiated in 6 weeks. We classify three maintenance cycles A, B and C. Maintenance A consists of 3 weekly instillations followed by cystoscopy 6 weeks later. Cytology and fluorescence *in situ* hybridization (FISH) in urine specimens may be obtained at this time. If cystoscopy/cytology is negative, maintenance B may be initiated 6 months after the conclusion of cycle A, again for 3 weekly treatments. Maintenance C is initiated 6 months after the conclusion of cycle B. Following cycle C, cystoscopy/cytology is repeated every 3 months for 2 years from the original diagnosis at which time it is extended to every 6 months for 1 year, and then annually.

1.2 Mechanism of BCG action

Since its first therapeutic application in 1976, major efforts have been made to decipher the mechanisms through which BCG mediates anti-bladder cancer immunity (Brandau & Suttman, 2007; Alexandroff et al., 2010). During the past decades, many details of the molecular and cellular mechanisms involved have been discovered although the exact mechanisms of BCG action still remain elusive. It is now accepted that a functional host immune system is a necessary prerequisite to successful BCG immunotherapy. It has also become clear that the effects of intravesical BCG depend on the induction of a complex inflammatory cascade event in the bladder mucosa reflecting activation of multiple types of immune cells and bladder tissue cells (Brandau & Suttman, 2007; Alexandroff et al., 2010). After instillation, BCG adheres to fibronectin on the urothelial lining through a fibronectin attachment protein (FAP) on BCG (Kavoussi et al., 1990). This interaction between BCG and the urothelium is one of the first and most crucial steps. Attached BCG is then internalized and processed by urothelial cells including urothelial carcinoma cells (UCC), resulting in secretion of an array of proinflammatory cytokines and chemokines such as interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)- α , and granulocyte-macrophage colony stimulating factor (GM-CSF) (Becich et al., 1991; Bevers et al., 2004). Following urothelial cell activation, an influx of various leukocyte types into the bladder wall occurs including neutrophils, monocytes/macrophages, lymphocytes, natural killer (NK) cells, and dendritic cells (DC) (Böhle et al., 1990; Prescott et al., 1992; Saban et al., 2007). These infiltrating leukocytes are activated and produce a variety of additional proinflammatory cytokines and chemokines and also form BCG-induced granuloma structures in the bladder wall (Böhle et al., 1990; Saban et al., 2007). Subsequently, a large number of leukocyte types such as neutrophils, T cells and macrophages are expelled into the bladder lumen and appear in patients' voided urine (De Boer et al., 1991; Simons et al., 2008). In addition, transient massive cytokines and chemokines can be detected in voided urine including IL-1 β , IL-2, IL-6, IL-10, IL-12, IL-18, interferon (IFN)- γ , TNF- α , GM-CSF, macrophage colony-stimulating factor (M-CSF), macrophage-derived chemokine (MDC), monocyte chemoattractant protein (MCP)-1,

macrophage inflammatory protein (MIP)-1 α , interferon-inducible protein (IP)-10, monokine induced by γ -interferon (MIG), and eosinophil chemoattractant activity (Eotaxin) (^bBöhle et al., 1990; ^cDe Boer et al., 1991; De Reijke et al., 1996; Taniguchi et al., 1999; Saint et al., 2002; Nadler et al., 2003; Luo et al., 2007). The urine of animals treated with intravesical BCG also showed increased IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IFN- γ , TNF- α , GM-CSF, M-CSF, MIP-1 α , regulated on activation normal T cell expressed and secreted (RANTES), and keratinocyte-derived chemokine (KC) (^aSaban et al., 2007). It has been noted that the development of a predominant Th1 cytokine profile (e.g. IFN- γ , IL-2 and IL-12) is associated with the therapeutic effects of BCG, whereas the presence of a high level of Th2 cytokines (e.g. IL-10) is associated with BCG failure (De Reijke et al., 1996; Saint et al., 2002; Nadler et al., 2003). Thus, a shift of the cytokines produced towards a Th1 milieu is necessary for successful BCG immunotherapy of bladder cancer. To support this, it has been observed that both IFN- γ and IL-12 but not IL-10 are required for local tumor surveillance in an animal model of bladder cancer (Riemensherger et al., 2002). Mice deficient in IL-10 genetically (IL-10^{-/-}) or functionally via antibody neutralization can also develop enhanced anti-bladder cancer immunity in response to intravesical BCG (Nadler et al., 2003).

Multiple immune cell types participate in the inflammatory response induced by BCG in the bladder. It is well accepted that macrophages, an indispensable cellular component of the innate immune system, serve as the first line of defense in mycobacterial infection. Activation, maturation and cytokine production of macrophages are primarily induced by Toll-like receptor (TLR) 2 ligation (Heldwein et al., 2003). Following BCG instillation, an increased number of macrophages can be observed in bladder cancer infiltrates and the peritumoral bladder wall. Voided urine after BCG instillation also contains an increased number of macrophages and the cytokines and chemokines predominantly produced by macrophages such as TNF- α , IL-6, IL-10, IL-12 and IL-18 (^bBöhle et al., 1990; ^{a,c}De Boer et al., 1991; Saint et al., 2002; Nadler et al., 2003; Luo et al., 2007). In addition to presenting BCG antigens, both human and murine macrophages are capable of functioning as tumoricidal cells toward bladder cancer cells upon activation by BCG *in vitro* (Pryor et al., 1995; Yamada et al., 2000; Luo et al., 2004, 2006, 2010). The killing of bladder cancer cells by macrophages relies on direct cell-to-cell contact and release of various soluble effector factors such as cytotoxic cytokines TNF- α and IFN- γ and apoptotic mediators such as nitric oxide (NO) (Jansson et al., 1998; Luo et al., 2004, 2006, 2010). Th1 cytokines (e.g. IFN- γ) enhance the induction of macrophage cytotoxicity whereas Th2 cytokines (e.g. IL-10) inhibit the induction of macrophage cytotoxicity (Luo et al., 2006, 2010).

Neutrophils also compose the early responding cells to BCG instillation of the bladder and can be observed in the bladder wall and urine shortly after BCG instillation (^{a,c}De Boer et al., 1991; ^aSaban et al., 2007; Simons et al., 2008). Neutrophils are central mediators of the innate immunity in BCG infection and are activated by signalling through TLR2 and TLR4 in conjunction with the adaptor protein myeloid differentiation factor 88 (MyD88) (Godaly & Young, 2005). In addition to secretion of proinflammatory cytokines and chemokines (e.g. IL-1 α , IL-1 β , IL-8, MIP-1 α , MIP-1 β , MCP-1, transforming growth factor (TGF)- β , and growth-related oncogene (GRO)- α) that lead to the recruitment of other immune cells (Suttman et al., 2006), recent studies revealed that neutrophils are the primary source of TNF-related apoptosis-inducing ligand (TRAIL) found in the urine after BCG instillation (Ludwig et al., 2004; Kemp et al., 2005). TRAIL is a member of the TNF family that induces apoptosis in malignant cells but not in normal cells. Studies have indicated that the

neutrophil TRAIL response is specific to BCG stimulation rather than nonspecific immune activation. Studies have also revealed a positive correlation between urinary TRAIL level and the therapeutic effects of BCG, as BCG responders contained a significant higher amount of urinary TRAIL than BCG nonresponders (Ludwig et al., 2004). These observations suggest an important role of neutrophils in BCG-induced anti-bladder cancer immunity. Indeed, it has been observed that depletion of neutrophils resulted in a reduced BCG-induced anti-bladder cancer response in a mouse model of bladder cancer (Suttman et al., 2006).

Following the activation of macrophages and neutrophils in the bladder wall, driven by chemoattractants, recruitment of other immune cell types including CD4⁺ T cells, CD8⁺ T cells, NK cells, and DC takes place (aBöhle et al., 1990; Prescott et al., 1992). As for neutrophils and macrophages, these cell types can be found in the voided urine of patients after BCG instillation (a-cDe Boer et al., 1991). These effector cells produce various cytokines and chemokines to further promote BCG-induced anti-bladder cancer immune responses in the local milieu. In addition, DC, together with macrophages, trigger an anti-BCG specific immune response via antigen presentation to T cells that also amplifies the BCG-induced antitumor immunity. Like neutrophils and macrophages, both T cells and NK cells are cytotoxic toward bladder cancer cells upon activation. They kill target cells via major histocompatibility complex (MHC) restricted (e.g. for cytotoxic T lymphocytes (CTL)) and/or MHC non-restricted pathways (e.g. for NK cells) (Pryor et al., 1995; Suttman et al., 2004; Liu et al., 2009). Perforin-mediated lysis and apoptosis-associated killing (e.g. via Fas ligand and TRAIL) have been implicated as the major molecular effector mechanisms underlying the eradication of bladder cancer cells. These effector cell types are crucial for BCG immunotherapy of bladder cancer, as depletion of these cell types failed to develop effective anti-bladder cancer responses *in vivo* and kill bladder cancer cells *in vitro* (Ratcliff et al., 1993; Brandau et al., 2001).

It has been shown that stimulation of human peripheral blood mononuclear cells (PBMC) by viable BCG *in vitro* leads to the generation of a specialized cell population called BCG-activated killer (BAK) cells (Böhle et al., 1993; aBrandau et al., 2000). BAK cells are a CD3⁺CD8⁺CD56⁺ cell population whose cytotoxicity is MHC non-restricted (a,bBrandau et al., 2000). BAK cells kill bladder cancer cells through the perforin-mediated lysis pathway and are effective on lysing NK cell-resistant bladder cancer cells (Böhle et al., 1993; a,bBrandau et al., 2000). Macrophages and CD4⁺ T cells have been found to be indispensable for the induction of BAK cell killing activity but have no such activity by themselves (aBrandau et al., 2000). Th1 cytokines IFN- γ and IL-2 have also been found to be required for the induction of BAK cell cytotoxicity, as neutralizing antibodies specific to these cytokines could inhibit BCG-induced cytotoxicity (aBrandau et al., 2000). BAK cells, together with lymphokine-activated killer (LAK) cells, a diverse population with NK or T cell phenotypes that are generated by IL-2 (Jackson et al., 1992; Shemtov et al., 1995), have been suggested to be the major effector cells during intravesical BCG immunotherapy of bladder cancer. Other potential cytotoxic effector cells include CD1 restricted CD8⁺ T cells (Kawashima et al., 2003), $\gamma\delta$ T cells (Higuchi et al., 2009), and natural killer T (NKT) cells (Emoto et al., 1999; Higuchi et al., 2009).

Activation of the innate immune system is a prerequisite for the BCG-induced inflammatory responses and the subsequent eradication of bladder cancer by intravesical BCG. In BCG instillation, TLRs participate in neutrophil, macrophage and DC maturation and activation.

Both TLR2 and TLR4 appear to serve important but distinct roles in the induction of host immune responses to BCG or BCG cell-wall skeleton (Heldwein et al., 2003). Like other microbes, BCG has surface components called pathogen-associated molecular patterns (PAMPs) that are recognized by cells of the innate immune system through TLRs during infection (Aderem & Ulevitch, 2000). It is this interaction between TLRs and PAMPs that activates the cells of the innate immune system, leading to BCG-induced inflammatory responses and subsequent eradication of bladder cancer. It is known that the antitumor effect of intravesical BCG depends on its proper induction of a localized Th1 immune response. However, a systemic immune response also appears involved in intravesical BCG therapy. It has been reported that purified protein derivative (PPD) skin test often converts from negative to positive after BCG instillation and the effective treatment is associated with the development of delayed-type hypersensitivity (DTH) reaction to PPD (Bilen et al., 2003). Animal studies have also demonstrated the importance of DTH in the antitumor activity of intravesical BCG therapy (Nadler et al., 2003). Moreover, studies have shown increased levels of cytokines and chemokines in the serum (e.g. IL-2, IFN- γ , MCP-1 and RANTES), along with production of these cytokines and chemokines in the urine and/or bladder, during the course of BCG instillation (Taniguchi et al., 1999; Reale et al., 2002). Furthermore, studies have also shown an increase in PBMC cytotoxicity against UCC after BCG instillation (Taniguchi et al., 1999).

In addition to the ability of BCG to elicit host immune responses, evidence supports a direct effect of BCG on the biology of UCC. *In vitro* studies have shown that BCG is anti-proliferative and even cytotoxic to UCC (Pryor et al., 1995; Pook et al., 2007). However, this direct cytotoxic effect of BCG is not convincing under physiological conditions *in vivo*, as intravesical BCG showed no therapeutic effect on bladder cancer in animal models with different immunodeficiencies (Ratliff et al., 1993; Brandau et al., 2001; Suttman et al., 2006). *In vitro* studies have also shown that BCG can induce UCC expression of cytokines and chemokines (e.g. IL-1 β , IL-6, IL-8, TNF- α and GM-CSF) (Bever et al., 2004), antigen-presenting molecules (e.g. MHC class II, CD1 and B7-1) (Ikeda et al., 2002), and intercellular adhesion molecules (e.g. ICAM-1) (Ikeda et al., 2002). Analysis of tumor biopsy specimens from bladder cancer patients who underwent intravesical BCG therapy further supported the ability of BCG to induce UCC expression of these molecules *in vivo* (Prescott et al., 1992). Moreover, the bladder urothelium of animals treated with intravesical BCG showed upregulation of HLA antigens (e.g. MHC class I and II) and IFN- γ -induced small GTPase families (e.g. GBPs and p47GTPases) as well as activation of canonical signaling pathways (e.g. nuclear factor (NF) κ B, axonal guidance, aryl hydrocarbon receptor, Wnt/ β -catenin, and cAMP) (Saban et al., 2007). However, intravesical BCG treatment also down-regulated urothelial expression of certain molecules (e.g. single-spanning uroplakins, SPRR2G, GSTM5, and RSP 19) (Saban et al., 2007). Recent studies have revealed that by cross-linking α 5 β 1 integrin receptors BCG exerts its direct biological effects on UCC, including activation of the signal transduction pathways involving activator protein (AP) 1, NF κ B and CCAAT-enhancer-binding protein (C/EBP) (Chen et al., 2002), upregulation of gene expressions such as IL-6 and cyclin dependant kinase inhibitor p21 (Chen et al., 2002; Zhang et al., 2007), and cell cycle arrest at the G1/S transition (Chen et al., 2005). Although some studies showed the ability of BCG to induce apoptosis in UCC (Ping et al., 2010), other studies demonstrated that BCG induced no apoptosis or even caused apoptotic resistance in UCC (See et al., 2009).

Further studies revealed that BCG induced UCC death in a caspase-independent manner (See et al., 2009) and that p21 played an important role in modulating the direct effects of BCG on UCC (See et al., 2010). During the past decade, studies have also demonstrated that peroxisome proliferator-activated receptor gamma (PPAR γ), a member of the steroid receptor superfamily of ligand-activated transcription factors, is involved in the pathogenesis of bladder cancer (Mylona et al., 2009). PPAR γ is a key regulator of adipogenic differentiation and its ligands have been found to induce terminal differentiation or growth inhibition of various cancer cell types including bladder cancer (Mansure et al., 2009). Although there is a discrepancy with regard to its actual role in bladder cancer, it appears that the lack of PPAR γ expression is associated with bladder cancer progression. Recent studies have demonstrated that BCG could directly induce PPAR γ in UCC both *in vitro* and *in vivo*, which may contribute to the antitumor activity of BCG (Lodillinsky et al., 2006).

1.3 Combination of BCG with Th1 cytokines for bladder cancer treatment

The proper induction of Th1 immunity is required for successful BCG immunotherapy of bladder cancer. Since a high percentage of patients do not respond to BCG and the effect of BCG is associated with significant toxicity, strategies to combine BCG with recombinant (τ) Th1 cytokines to enhance BCG therapeutic efficacy while reducing BCG toxicity have been employed and studied. Among Th1 cytokines, rIFN- α is most extensively studied and has been shown to be safe and tolerable when used intravesically, alone or in combination with BCG, in many controlled studies (O'Donnell et al., 2001; Lam et al., 2003; Joudi et al., 2006; Nepple et al., 2010; Bazarbashi et al., 2011). The side-effect profile of combination therapy is similar to BCG monotherapy including lower urinary tract symptoms such as frequency, urgency, dysuria, bladder spasm and hematuria. Systemic fever, flu-like symptoms, and myalgias were found in <25% of patients and were self-limited. Benefits have been seen in patients with BCG failures (O'Donnell et al., 2001; Lam et al., 2003; Joudi et al., 2006). Treatment with low-dose BCG (1/3 or 1/10 the standard dose) combined with rIFN- α resulted in 45-53% of patients who had failed prior BCG monotherapy to remain disease free at 24-month median follow-up (O'Donnell et al., 2001; Joudi et al., 2006). The benefit in naïve patients is currently in question with recent studies showing mixed results. A Phase III study suggested no benefit in BCG naïve patients (Nepple et al., 2010). However, no subgroup analysis was performed for carcinoma *in situ* or high risk patients. Therefore, it can still be concluded that the BCG-rIFN- α combination therapy may provide a benefit to patients with high risk disease or carcinoma *in situ*. Data since the release of the Phase III study supports the combination therapy with BCG and rIFN- α in BCG naïve patients (Bazarbashi et al., 2011). Thus, more studies are needed to formally determine the effect of the combination therapy for BCG naïve patients. To date, a combination therapy with BCG and rIFN- α 2B has been employed, particularly for patients with previous BCG failures, those with carcinoma *in situ*, and the elderly (Joudi et al., 2006). Optimal dose and schedule have yet to be defined in controlled trials and debate continues on the subject. At our institution, we use the standard dose of TICE BCG plus 50 million units (MU) of rIFN- α 2B intravesically as induction therapy for BCG naïve patients. For BCG exposed patients, 1/3 the standard dose of BCG plus 50 MU of rIFN- α 2B is utilized. The dose may be lowered for those patients experiencing lower urinary tract symptoms or low grade fever. For

maintenance cycle A, we adjust the BCG dose for week 1 consisting of 1/3 the standard dose of BCG plus 50 MU of rIFN- α 2B. For weeks 2 and 3, the BCG dose is lowered to 1/10 the standard dose plus 50 MU of rIFN- α 2B. Maintenance cycles B and C utilize similar dosing.

Other cytokines that have been used intravesically include rIL-2, rIL-12, rIFN- γ and rGM-CSF. A study demonstrated that intravesical rIL-2 was beneficial for patients with T1 papillary bladder carcinoma after TURBT showing regression of marker lesions and lack of major toxic effects (Den Otter et al., 1998). Other studies also demonstrated intravesical rIL-2 to be feasible, safe and effective in patients with NMIBC who were untreated or had failed prior intravesical therapy with other agents (Tubaro et al., 1995; Ferlazzo et al., 1996). A study demonstrated that intravesical rIL-12 was well tolerated by patients with recurrent NMIBC but showed no clinically relevant antitumor and immunologic effects (Weiss et al., 2003). However, the maximum tolerated dose of rIL-12 was not reached in the study. Different from human studies, animal studies showed encouraging results. A survival advantage of intravesical rIL-12 was observed in a mouse orthotopic bladder cancer model (Zaharoff et al., 2009). Further studies for intravesical rIL-12 use are warranted. For intravesical rIFN- γ , a study showed the absence of major toxicity and the therapeutic effect superior to mitomycin C for patients with NMIBC who underwent TURBT (Giannopoulos et al., 2003). In addition, populations of leukocytes in the urothelium were significantly increased in rIFN- γ treated patients confirming its induction of localized cellular immune responses. Other studies also supported the safety and antitumor activity of intravesical rIFN- γ monotherapy (Stavropoulos et al., 2002). Studies also demonstrated that intravesical rGM-CSF was effective as a prophylactic therapy for patients with NMIBC after TURBT (Stravoravdi et al., 1999; Theano et al., 2002). In correlation with regression of marker lesions, intravesical rGM-CSF induced leukocyte migration and activation in the bladder mucosa. Despite all these observations, however, single cytokine therapy has only been evaluated in small numbers of patients and has not yet shown compelling results in general. Indeed, *in vitro* studies have demonstrated that cytokines IL-2, IL-12 and TNF- α , like IFN- α , can enhance BCG for the induction of Th1 immune responses in human PBMC (Luo et al., 1999, 2003; O'Donnell et al., 1999). Thus, addition of these cytokines to BCG may provide benefits for BCG therapy particularly for BCG nonresponders or relapsers. Studies are absolutely needed to examine the combination of BCG with these cytokines for the treatment of bladder cancer.

2. Advances in genetic engineering of BCG for cytokine delivery

2.1 BCG as a heterologous gene delivery vehicle

Because of its unique characteristics, such as adjuvant potential, low toxicity and potent immunogenicity, BCG has long been considered to be an attractive live vaccine delivery vehicle with which to deliver protective antigens of multiple pathogens. During the past 2 decades, with advances in knowledge of mycobacterial genetics and molecular biology, a wide range of recombinant BCG (rBCG) vaccine candidates expressing bacterial, viral, parasitic antigens have been developed including those for *Mycobacterium tuberculosis* (*M.tb*), human immunodeficiency virus (HIV), and hepatitis B and C viruses (Bastos et al., 2009). As early as in the 1980s, studies showed that mycobacteria were capable of delivering foreign genes that were introduced into the microbes (Jacobs et al., 1987; Snapper et al., 1988). In the early 1990s, vectors carrying strong promoters from the mycobacterial major

heat-shock protein genes (e.g. *hsp60* and *hsp70*) and unique cloning sites, which allowed extrachromosomal or integrative expression of foreign antigens, were developed (Stover et al., 1991; Lee et al., 1991). Using these expression vectors, BCG was further demonstrated to be an effective live delivery vehicle for foreign antigens (Stover et al., 1991, 1993; Aldovini & Young, 1991; Connell et al., 1993; ^{a,b}Langermann et al., 1994). These rBCG strains constitutively expressed foreign antigens and elicited long-lasting specific humoral and/or cellular immune responses in mice. Some of these rBCG strains even generated protective immunity against respective pathogens whose antigens were expressed by mycobacteria such as the outer surface protein A (OspA, *Borrelia burgdorferi*) (Stover et al., 1993), surface proteinase gp63 (*Leishmania spp*) (Connell et al., 1993), and surface protein A (*Streptococcus pneumoniae*) (^aLangermann et al., 1994). Since the vectors used contained no signal sequence, the foreign antigens were expressed in the cytoplasm of mycobacteria. During that time period, vectors permitting surface expression of foreign antigens in mycobacteria or secretion from mycobacteria were developed (Matsuo et al., 1990; Stover et al., 1993). Infection with these rBCG strains led to enhanced immune responses to some antigens in mice (Stover et al., 1993; ^aLangermann et al., 1994; Grode et al., 2002). Meanwhile, vectors with various mycobacterial gene promoters, such as α -antigen, P_{AN}, ag85b, 18kDa and furA (among many others), were also developed and demonstrated to be effective to elicit specific immune responses and/or protective immunity in different animal species including mouse, guinea pig, hamster, pig, sheep, rabbit, and monkey (Matsuo et al., 1990; Murray et al., 1992; Honda et al., 1995; Horwitz et al., 2000; Bastos et al., 2009). In addition, progress has continued in the refinement of the safety and efficacy of the rBCG vaccine vehicles. To date, numerous improved systems employed to express heterologous genes in BCG are available. Among them are vectors with limited replication or auxotrophic complementation for safe use in HIV-infected individuals, capability to replicate at a high-copy number for increased antigen delivery, dual expression cassettes for multivalent antigen delivery, capability to integrate into the genome at multiple sites for differential antigen expression, inducible elements for controlled gene expression, and expression of perfringolysin or listeriolysin (with or without urease C gene deletion) for increased CD8⁺ T cell stimulation. Although clinical use of rBCG vaccines is still in an early stage, studies have already demonstrated that rBCG is safe and effective in humans such as those expressing OspA and *M.tb* antigen 85B (Ag85B). In the years to come, more rBCG vaccines will be evaluated clinically and their usefulness in preventing human infectious diseases will become clear. In addition to a wide range of bacterial, viral and parasitic antigens, BCG has also been engineered to deliver tumor-associated antigens. For example, BCG expressing prostate specific molecules such as prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA) have been developed. Mice immunized with the rBCG-PSA or rBCG-PSMA strain developed antigen-specific immune responses, primarily a cellular immune response (Geliebter, 2010). We also independently developed a rBCG strain that secretes the full-length PSA. We observed that mice immunized with the rBCG-PSA strain, but not a control BCG strain carrying an empty vector, developed a potent specific CTL activity against PSA expressing RM11psa cells (our unpublished observations). In addition, we further observed that mice primed with the rBCG-PSA strain and boosted with Ad-PSA, a replication-defective adenoviral vector carrying the full-length PSA coding sequence (Elzey et al., 2001), developed enhanced PSA-specific CTL activity and IFN- γ expressing CD4⁺ and CD8⁺ T cells (our unpublished observations). Several studies including ours have also demonstrated that BCG could be engineered to express mucin-1 (MUC1), a candidate tumor-associated

antigen for breast cancer and other epithelial adenocarcinomas, in a manner of multiple tandem repeats with coexpression of IL-2, GM-CSF or CD80 (He et al., 2002; Chung et al., 2003; ^{a,b}Yuan et al., 2009, 2010). Severe combined immunodeficient (SCID) mice reconstituted with human peripheral blood lymphocytes (PBL) followed by immunization with these MUC1 expressing rBCG strains developed specific protective immunity against MUC1-positive human breast cancer xenografts. These observations warrant further studies in rBCG delivering tumor antigens for the treatment of malignant diseases.

Studies have shown that BCG delivery of certain biologically active molecules can induce enhanced immune responses. A study demonstrated that a rBCG strain secreting cathepsin S, a cysteine endoprotease involving in MHC class II antigen presentation, could restore intracellular cathepsin S activity and improve the capacity of BCG-infected macrophages to stimulate CD4⁺ T cells (Soualhine et al., 2007). A study also demonstrated that mice simultaneously immunized with intraperitoneal ovalbumin (OVA) and intranasal rBCG secreting the assembled pentameric cholera toxin B subunit developed a long-lasting OVA-specific mucosal IgA response as well as a systemic IgG response (Biet et al., 2003). Remarkably, a rBCG strain expressing the genetically detoxified S1 subunit of pertussis toxin (SIPT) showed enhanced BCG adjuvant potential and, when administered intravesically, resulted in bladder weight reduction and increased survival time in a mouse syngeneic orthotopic tumor model (Chade et al., 2008; Andrade et al., 2010). Moreover, BCG has also been engineered to express the model antigen OVA for studies of the mechanisms underlying BCG induction of antigen-specific immune responses (van Faassen et al., 2004). These studies revealed that the ability of BCG to induce a delayed but persistent immune response was due to its chronicity in infection that led to a long effector phase and reduced immune cell attrition compared to *Listeria monocytogenes* (an acute pathogen). Furthermore, we and others have also engineered BCG to express green fluorescent protein (GFP), either alone or in combination with antigenic molecules (e.g. OVA) or cytokines (e.g. IL-2), for the studies of BCG trafficking, antigen deliver, and anti-mycobacterial infection (Luo et al., 1996, 2000; Hulseberg et al., 2010).

2.2 Th1 cytokine-secreting rBCG

In our early studies, we developed a panel of rBCG strains that secreted mouse IL-2 or rat IL-2 under the control of the mycobacterial *hsp60* promoter and α -antigen signal sequence (O'Donnell et al., 1994). We demonstrated that the IL-2 secreting rBCG strains induced enhanced IFN- γ production by mouse splenocytes *in vitro* compared to wild-type BCG. Since then, numerous rBCG strains secreting different mouse and human cytokines, primarily Th1 cytokines (e.g. IL-2, IL-18, IFN- γ and IFN- α), have been developed (Table 1). In addition, rBCG strains secreting other cytokines or chemokines (e.g. GM-CSF, IL-15, TNF- α and MCP-3) have also emerged. Most of these cytokine- and chemokine-secreting rBCG strains showed their abilities to enhance BCG-induced cellular immune responses including Th1 cytokine production, cellular cytotoxicity, DC activation, and anti-BCG or anti-*M.tb* infection. Some of them even showed their antitumor effects in animal models of melanoma (Duda et al., 1995), breast cancer (Chung et al., 2003; ^aYuan et al., 2009, Yuan et al., 2010), and bladder cancer (Arnold et al., 2004). Certain cytokine-secreting rBCG strains also induced humoral immune responses and Th2 cytokine production other than cellular immune responses *in vitro* and *in vivo*.

<u>Strain</u>	<u>Cytokine</u>	<u>Species</u>	<u>Immunological Effect</u>	<u>Reference</u>
IL-2 BCG (RBD)	IL-2	m	Th1 cyt prod, Antitumor, Cytotoxicity	O'Donnell et al., 1994; Duda et al., 1995; Luo et al., 2006
IL-2 BCG (MAO)	IL-2	r	Th1 cyt prod	O'Donnell et al., 1994
BCG-CI	IL-2	h	Anti-BCG	Kong & Kunimoto 1995
BCG-CII	IL-2	h	Anti-BCG	Kong & Kunimoto 1995
BCG-IL-2	IL-2	m	CI, Th1 & Th2 cyt prod	Murray et al., 1996
BCG-GM-CSF	GM-CSF	m	CI, Th1 & Th2 cyt prod, DC act, Anti- <i>M.tb</i>	Murray et al., 1996; ^a Ryan et al., 2007
BCG-IFN- γ	IFN- γ	m	CI, Th1 & Th2 cyt prod, Anti-BCG	Murray et al., 1996; Wangoo et al., 2000
rBCG/IL-2	IL-2	m	CI, Th1 cyt prod, Anti-BCG	Slobbe et al., 1999; ^{a,b} Young et al., 2002
rBCG-IL-2/GFP	IL-2	m	CI, Th1 cyt prod, Anti-BCG	Luo et al., 2000
rBCG(α -Ag-IL-2)	IL-2	m	Th1 cyt prod, Cytotoxicity	Yamada et al., 2000
BCG-IFN- γ	IFN- γ	m	Th1 cyt prod, Anti-BCG	Moreira et al., 2000
rBCG-IFN- α	IFN- α 2B	h	Th1 cyt prod, Cytotoxicity	Luo et al., 2001; Chen et al., 2007; Liu et al., 2009
rBCG/IL-18	IL-18	m	no clear effect	^b Young et al., 2002
BCG IL-18	IL-18	m	Th1 & Th2 cyt prod	Biet et al., 2002; Biet et al., 2005
BCG-hIL2MUC1	IL-2	h	CI, Th1 cyt prod, Antitumor	He et al., 2002; Chung et al., 2003
rBCG-IFN- γ	IFN- γ	m	CI, Th1 cyt prod, Antitumor	Arnold et al., 2004
rBCG-IL-18	IL-18	m	Th1 cyt prod, Anti-BCG, Cytotoxicity	Luo et al., 2004; Luo et al., 2006
rBCG-huIL-2-ESAT6	IL-2	h	CI, Th1 cyt prod, Cytotoxicity, HI	Fan et al., 2006
rBCG-IL-2	IL-2	h	Th1 cyt prod	Chen et al., 2007
BCG _{MCP-3}	MCP-3	m	CI, Anti-BCG	^b Ryan et al., 2007
rBCG-AEI	IFN- γ	m	CI, HI, Anti- <i>M.tb</i>	Xu et al., 2007
rBCG-Ag85B-IL15	IL-15	m	CI, Th1 cyt prod, Anti- <i>M.tb</i>	Tang et al., 2008
rBCG-MVNTR4-CSF	GM-CSF	h	CI, Th1 cyt prod, Antitumor	^a Yuan et al., 2009; Yuan et al., 2010
rBCG-MVNTR8-CSF	GM-CSF	h	CI, Th1 cyt prod, Antitumor	^a Yuan et al., 2009; Yuan et al., 2010
rBCG-Ag85B-Esat6-TNF- α	TNF- α	m	CI, HI	Shen et al., 2010

Table 1. Cytokine- and chemokine-expressing rBCG strains (Anti-BCG: anti-BCG infection; Anti-*M.tb*: anti-*Mycobacterium tuberculosis* infection; CI: cellular immunity; DC act: dendritic cell activation; h: human; HI: humoral immunity; m: mouse; r: rat; Th1 cyt prod: T helper type 1 cytokine production; Th2 cyt prod: T helper type 2 cytokine production)

3. Application of Th1 cytokine-secreting rBCG

3.1 Anti-tuberculosis studies

Tuberculosis (TB), an airborne transmitted disease caused by *M.tb*, remains a leading cause of mortality and morbidity worldwide. Currently, BCG is the only available vaccine for prophylaxis against TB. Evidence indicates that BCG vaccine is effective for childhood disseminated TB but shows variable efficacy in adult pulmonary TB, which accounts for most TB worldwide. Additional challenges to TB control include the recent emergences of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) as well as TB co-infection with HIV. Therefore, a new and more effective vaccine against TB is urgently needed. Studies have demonstrated that cellular immunity rather than humoral immunity is critical for TB protection (Flynn & Chan, 2001; Mittrücker et al., 2007). CD4⁺ T cells of Th1 type, which is a major source of IFN- γ , are crucial for protection against TB. In addition, CD8⁺ T cells also play an indispensable role in control of TB, particularly as the infection progresses. With the advances in mycobacterial genome research, many strategies have become available for improving BCG's safety, immunogenicity and vaccine efficacy. Of them, genetic manipulation of BCG has gained a great momentum. To date, BCG has been engineered to express *M.tb* antigens, such as the early secreted antigen target-6 kDa (ESAT-6), Ag85B and culture filtrate protein (CFP), to evoke anti-TB specific immune responses (Horwitz et al., 2000; Pym et al., 2003). In addition, rBCG strains secreting cytokines (e.g. IL-2, IL-15, IL-18, GM-CSF and IFN- γ) or chemokines (e.g. MCP-3), alone or in combination with *M.tb* antigens, have also been developed (Table 1). These cytokine- and chemokine-secreting rBCG strains can induce a more rapid and more effective mobilization of T cells as well as other immune cell types at the site of bacterial multiplication and thus may reduce the risk of developing active TB.

Several studies including ours demonstrated that mice infected intravenously with rBCG strains secreting mouse IL-2 exhibited significantly reduced bacterial growth *in vivo* compared to control mice infected with non-cytokine secreting BCG strains (Luo et al., 2000; Young et al., 2002). This anti-mycobacterial infection was correlated with the induction of enhanced Th1 immune responses in the former mice. Mice vaccinated subcutaneously with a mouse IL-2 secreting rBCG strain also showed significantly increased clearance of BCG delivered intranasally compared to control mice vaccinated with wild-type BCG (Young et al., 2002). The same rBCG strain also induced a Th1 biased immune response in deer; however, it failed to induce enhanced protection against BCG infection in this animal species (Slobbe et al., 1999). Interestingly, human IL-2 secreting rBCG strains are also capable of inducing protective immunity against BCG infection in mice, as mice infected with the rBCG strains exhibited increased bacterial clearance *in vivo* (Kong & Kunimoto, 1995; Fan et al., 2006). In addition, mice infected with a rBCG strain coexpressing human IL-2 and *M.tb* antigen ESAT-6 developed enhanced antigen-specific Th1 immune responses including CTL activity against ESAT-6 expressing target cells (Fan et al., 2006). The same rBCG-infected mice also developed increased ESAT-6 specific antibody titer. Similar to IL-2 secreting rBCG strains, mouse IFN- γ secreting rBCG strains showed their abilities to induce enhanced protective immunity in rBCG-infected mice compared to non-cytokine secreting BCG (Wangoo et al., 2000; Moreira et al., 2000). Studies also demonstrated that infection of IFN- γ gene disrupted mice (IFN- γ ^{-/-}) via aerosol with a mouse IFN- γ secreting rBCG strain could result in reduced bacterial load and better differentiated granulomas as well as reduced levels of IL-10 mRNA (Moreira et al., 2000). However, the same rBCG-infected IFN-

γ^- mice failed to develop effective protection against subsequent aerosol challenge with *M.tb*. Similarly, a rBCG strain secreting mouse MCP-3 also afforded effective protection against BCG infection in both immunocompetent and recombinase-activating gene-1 deficient (RAG-1^{-/-}) mice but failed to provide protection against subsequent aerosol challenge with *M.tb* (^aRyan et al., 2007). There is a discrepancy with regard to the effect of mouse IL-18 secreting rBCG strains on the induction of protective immunity against BCG infection. Although studies including ours have demonstrated that mouse IL-18 secreting rBCG strains could induce enhanced Th1 immune responses (Biet et al., 2002, 2005; Luo et al., 2004, 2006), their ability to induce protection against BCG infection was not consistent among the studies (Biet et al., 2002, 2005; ^bYoung et al., 2002; Luo et al., 2004).

Studies have demonstrated that certain cytokine-secreting rBCG strains can provide enhanced protection against dissemination of *M.tb* infection in mice including those secreting mouse GM-CSF (^aRyan et al., 2007), mouse IFN- γ fused to *M.tb* antigens Ag85B-ESAT-6 (Xu et al., 2007), and mouse IL-15 fused to *M.tb* antigen Ag85B (Tang et al., 2008). In correlation with enhanced protection against aerosol challenge with *M.tb*, mice vaccinated with the mouse GM-CSF secreting rBCG strain showed increased activation of antigen-presenting cells (e.g. DC) and frequency of anti-mycobacterial IFN- γ producing T cells compared to control mice vaccinated with non-cytokine secreting BCG (^aRyan et al., 2007). Mice vaccinated with the rBCG strain coexpressing mouse IFN- γ and the highly immunogenic antigens Ag85B and ESAT-6 of *M.tb* developed significantly increased both cellular and humoral immune responses as well as enhanced protection against intravenous challenge of *M.tb* compared to control mice vaccinated with rBCG strains expressing Ag85B and/or ESAT-6 alone (Xu et al., 2007). Similarly, mice vaccinated with the rBCG strain coexpressing mouse IL-15 and Ag85B showed increased induction of antigen-specific CD4⁺ and CD8⁺ T cells as well as enhanced protection against intratracheal challenge of *M.tb* compared to control mice vaccinated with a rBCG strain expressing Ag85B alone (Tang et al., 2008). These cytokine-secreting rBCG strains merit further appraisal as vaccine candidates for the control of TB in humans.

3.2 Anti-tumor studies

BCG is a potent immunoadjuvant and induces a Th1 predominant immune response that is required for effective tumor eradication in most cancer types. Genetic manipulation of BCG to secrete Th1-stimulating cytokines with simultaneous coexpression of tumor-associated antigens may therefore potentiate the induction of specific antitumor immune responses. This strategy has been approached since the emergence of cytokine-secreting rBCG strains in the 1990s. Early studies demonstrated that mouse IL-2 secreting rBCG was at least equally effective to wild-type BCG when used as an intratumoral injection or a vaccine therapy in conjunction with irradiated tumor cells in a mouse melanoma model (Duda et al., 1995). However, it was not until recently that the potential of rBCG for treating cancer has gained further appreciation. We and others have developed rBCG strains that deliver the breast cancer-associated antigen MUC1 in a form of multiple tandem repeats with coexpression of human IL-2 or human GM-CSF (He et al., 2002; Chung et al., 2003; ^aYuan et al., 2009, 2010). SCID mice reconstituted with human PBL followed by immunization with the rBCG strains developed MUC1-specific cellular immune responses and enhanced protection against MUC1-positive human breast cancer xenografts compared to control mice reconstituted with human PBL and immunized with non-cytokine secreting BCG. Studies have also

demonstrated that the antitumor effects of the rBCG strains were correlated with the number of MUC1 tandem repeats delivered by BCG (Yuan et al., 2009, 2010). These results suggest that these MUC1 rBCG strains coexpressing Th1-stimulating cytokines are promising candidates as breast cancer vaccines and thus deserve further investigation.

3.3 Anti-bladder cancer studies

Intravesical BCG is currently the treatment of choice for NMIBC. As for most other cancer types, the proper induction of a cellular immune response is required for successful BCG immunotherapy of bladder cancer. As described in sections 3.1 and 3.2, Th1 cytokine-secreting rBCG strains can induce enhanced cellular immune responses, leading to effective protection against mycobacterial infection (e.g. *M.tb*) and tumor progression (e.g. breast cancer) in various animal models. Unfortunately, studies on rBCG for treating bladder cancer are currently underdeveloped and, up to date, only a few reports have been available. However, studies have demonstrated that Th1 cytokine-secreting rBCG strains are superior to non-cytokine secreting BCG for the induction of anti-bladder cancer immune responses *in vitro* and *in vivo*.

3.3.1 In vitro studies

It has been known that BCG stimulation of human PBMC leads to the generation of effector cells cytotoxic to bladder cancer cells *in vitro* (Böhle et al., 1993; Brandau et al., 2000). We recently demonstrated that stimulation of human PBMC with rBCG-IFN- α , a rBCG strain secreting human IFN- α 2B (Luo et al., 2001), *in vitro* for 7 days induced enhanced PBMC cytotoxicity toward human bladder cancer cell lines T24, J82, 5637, TCCSUP and UMUC-3 by up to 2-fold compared to control BCG carrying an empty vector (Liu et al., 2009). This induction of enhanced PBMC cytotoxicity was correlated with increased production of IFN- γ and IL-2 by rBCG-stimulated PBMC. Studies further revealed that this enhancement in PBMC cytotoxicity was dependent on BCG secreted rIFN- α as well as endogenously expressed IFN- γ and IL-2, as blockage of IFN- α , IFN- γ or IL-2 by neutralizing antibodies during BCG stimulation reduced or abolished the induction of this enhanced PBMC cytotoxicity. Studies using NK and CD8⁺ T cells isolated from human PBMC revealed that both cell types were responsible for the enhanced PBMC cytotoxicity induced by rBCG-IFN- α with the former cell type being more predominant.

An early study demonstrated that human peripheral monocytes/macrophages were capable of functioning as tumoricidal cells toward bladder cancer UCRU-BL-17 cells upon activation by BCG *in vitro* (Pryor et al., 1995). It was observed that the cytotoxic activity of human monocytes/macrophages was significantly enhanced after BCG stimulation, while the naïve cells exhibited only minimum cytotoxicity. Later, more studies including ours further demonstrated that mouse macrophages could also function as tumoricidal cells toward bladder cancer cells upon activation by BCG *in vitro* (Yamada et al., 2000; Luo et al., 2004, 2006, 2010). Stimulation of thioglycollate-elicited peritoneal macrophages by BCG for 24 hour resulted in macrophage-mediated killing of bladder cancer MBT-2 (C3H background) and MB49 (C57BL/6 background) cells in a dose-dependent manner (Luo et al., 2006, 2010). Studies also revealed that endogenous Th1 cytokines (e.g. IL-12, IL-18, IFN- γ and TNF- α) played an important role in BCG-induced macrophage cytotoxicity, as blockage of these cytokines during BCG stimulation led to substantially reduced macrophage cytotoxicity toward bladder cancer cells (Luo et al., 2006). In contrast, supplementation of BCG with Th1

cytokines (e.g. rIL-2, rIL-12 or rIL-18) increased macrophage cytotoxicity by approximately 2-fold. Consistent with these observations, rBCG strains secreting mouse IL-2 or mouse IL-18 showed enhanced macrophage-mediated killing on bladder cancer MBT-2 cells, which was correlated with increased expression of IFN- γ , TNF- α and IL-6 by rBCG-stimulated macrophages (Luo et al., 2006). The effect of mouse IL-2 secreting rBCG strain on the induction of macrophage cytotoxicity toward bladder cancer MBT-2 cells was also demonstrated by a separate study (Yamada et al., 2000).

3.3.2 In vivo studies

Although the *in vitro* studies have suggested the potential usefulness of Th1 cytokine-secreting rBCG strains for the treatment of bladder cancer, the effect of rBCG on treating bladder cancer *in vivo* has not well been studied. Up to date, only an rBCG strain secreting mouse IFN- γ (rBCG-IFN- γ) has been studied in a mouse MB49 syngeneic orthotopic tumor model (Arnold et al., 2004). This study showed that, with a low-dose treatment regimen, intravesical administration of rBCG-IFN- γ significantly prolonged animal survival compared to medium-treated controls, whereas BCG carrying an empty vector only slightly increased survival. In a similar experiment using the MB49 syngeneic orthotopic tumor model in IFN- γ knockout mice, intravesical treatment with rBCG-IFN- γ failed to prolong survival of mice, indicating that rBCG-derived IFN- γ had no measurable antitumor effect in the absence of endogenous IFN- γ . Studies also provided the mechanisms underlying the effect of rBCG-IFN- γ on treating bladder cancer. As demonstrated, this rBCG-IFN- γ strain could specifically upregulate the expression of MHC class I molecules on MB49 cells *in vitro* compared to control BCG, as the MHC class I upregulation could be blocked by an inhibitory antibody to IFN- γ . This rBCG strain also enhanced recruitment of CD4⁺ T cells into the bladder and further induced the local expression of IL-2 and IL-4 mRNA compared to control BCG. In addition, we have also evaluated the effects of rBCG strains secreting mouse IL-2 or mouse IP-10 (a Th1 chemokine) on treating bladder cancer in the MB49 syngeneic orthotopic tumor model and observed survival benefits of these rBCG strains (our unpublished observations). All these observations suggest that rBCG strains secreting Th1 cytokines or chemokines possess improved antitumor properties and may offer new opportunities for the treatment of bladder cancer.

Supporting Th1 cytokine-secreting rBCG, *Mycobacterium smegmatis* (*M. smegmatis*), a closely related non-pathogenic mycobacterial organism, has been engineered to secrete mouse TNF- α (*M. smegmatis*/TNF- α) and tested in a transplantable MB49 tumor model (Young et al., 2004). Studies demonstrated that lymphocytes from tumor-bearing mice vaccinated with *M. smegmatis*/TNF- α produced elevated and prolonged IFN- γ but no IL-10 in response to mycobacterial antigen or tumor lysate stimulation *in vitro*. Histopathology revealed significantly increased infiltrating CD3⁺ lymphocytes in the tumor nodules of mice receiving the recombinant vaccine compared to those of mice receiving wild-type bacteria. These observations indicated that *M. smegmatis*/TNF- α induced cell-mediated immunity. Importantly, mice implanted subcutaneously with MB49 tumor and treated at an adjacent site with the recombinant vaccine exhibited significantly reduced tumor growth with a 70% durable tumor-free survival compared to those treated with wild-type bacteria or BCG (a 10-20% long-term survival). Interestingly, treatment with *M. smegmatis*/TNF- α also resulted in similar tumor growth inhibition in T cell-deficient athymic nude mice and reduced but not abolished tumor growth inhibition in NK cell-deficient Beige mice. These observations

indicated that NK cells contribute to the antitumor effect of *M. smegmatis*/TNF- α but are not solely responsible for the eradication of tumor. Like immunocompetent mice, Beige mice also developed tumor specific memory after treatment with *M. smegmatis*/TNF- α . A study also demonstrated enhanced immunotherapeutic potential of a human TNF- α secreting recombinant *M. smegmatis* for treating bladder cancer (Haley et al., 1999). The ability to deliver immunomodulatory cytokines with no pathogenic effects makes *M. smegmatis* attractive as an alternative intravesical mycobacterial agent for bladder cancer treatment.

4. Future perspectives

Numerous rBCG strains secreting Th1 cytokines (e.g. IL-2, IL-18, IFN- γ and IFN- α) have been developed and studied. Most of them have been shown to be capable of enhancing BCG-induced cellular immune responses, leading to effective protection against mycobacterial infection in animal models. Some of them have also been shown to induce enhanced antitumor immunity in animal models including bladder cancer. However, up to date, studies on rBCG for bladder cancer treatment are limited and have not well been developed. Currently, a number of bladder cancer models simulating human NMIBC are available and have been used in anti-bladder cancer studies including BCG immunotherapy. These animal models provide very useful tools for the evaluation of Th1 cytokine-secreting rBCG strains. Since intravesical administration of IFN- γ secreting rBCG strain has been demonstrated to prolong survival of animals bearing bladder orthotopic tumor, other Th1 cytokine-secreting rBCG strains are also likely effective on treating bladder cancer and should be evaluated in the animal models. Clinically relevant therapeutic and prophylactic effects of the rBCG strains relative to each other should be determined through analysis of the induction of antitumor responses in both effector and memory phases. The rBCG dosing should be optimized and the treatment schedule refined for each rBCG strain. Application of multiple rBCG strains for treatment should be tested and the toxic effects evaluated. Moreover, development of new rBCG strains will continue. We have been constructing BCG to secrete IL-12 (p35/p40 heterodimer) or mutant IL-10 with an intention to develop more potent rBCG strains for bladder cancer treatment. Furthermore, the mechanisms underlying rBCG actions need to be explored. In addition to classical effector cells, influence of the rBCG strains on Th17 and regulatory T (Treg) cells should be evaluated as the importance of these cell types in bladder cancer has being emerged. All these efforts will afford us a better understanding of Th1 cytokine-secreting rBCG strains and the steps necessary for use of these rBCG strains for treating bladder cancer. The pace of this research must be maintained if we are to improve this gold standard therapy for bladder cancer.

5. Conclusion

Intravesical administration of live BCG for superficial bladder cancer is the most successful immunotherapy for solid malignancy. However, BCG therapy is associated with significant toxicity and is ineffective in approximately 30-40% of cases. During the past 2 decades, the advances in mycobacterial genetics and molecular biology have offered unprecedented opportunities for the development of genetically modified BCG strains that possess improved safety profile, immunogenicity, and protective efficacy. Of them, manipulation of BCG to secrete Th1 cytokines, alone or in combination with coexpression of bacterial or tumor antigens, represents one of the most attractive strategies for the development of

improved vaccines. This type of rBCG strains has shown their potential to induce enhanced cellular immunity, leading to effective protection against mycobacterial infection (e.g. *M.tb*) and tumor progression (e.g. breast cancer) in various animal models. In bladder cancer treatment, BCG is administered intravesically; therefore, rBCG strains secreting Th1 cytokines can augment a localized cellular immune response that is crucial for effective BCG immunotherapy of bladder cancer. Since intravesical BCG in combination with local administration of Th1 cytokines such as rIFN- α has already been used in humans and demonstrated to be beneficial for bladder cancer patients, Th1 cytokine-secreting rBCG strains could be very useful as improved BCG agents. Indeed, these rBCG strains have been demonstrated to be capable of inducing anti-bladder cancer immune responses both *in vitro* and *in vivo* in animal studies. Because of their enhanced immunogenicity, Th1 cytokine-secreting rBCG strains can be used at a low dose, causing reduced side effects. These rBCG strains merit further appraisal as improved BCG immunotherapeutic agents for the treatment of bladder cancer.

6. References

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The Role of Irradiation in the Treatment of Chordoma of the Base of Skull and Spine

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1. Introduction

Chordoma is an uncommon neoplasm of the bone arising from embryonic remnants of the notochord. The overall age-adjusted incidence is about 8 per 10 million, but this figure is dependent on age, sex, and race (Jemal et al., 2007). This tumor typically occurs in the axial skeleton mainly involving the sacrococcygeal region and the base of the skull (Mirra et al., 2002). The natural history of such malignancy is of a slow but progressive growth ultimately translating into a local aggressive behaviour. Overall, five-year survival rates are near 60% to 70%, although 10-year survival drops to 35% to 40% (Dorfman, 1998). The rate of distant metastases (to lung, bone, soft tissue, lymph nodes, liver, and skin) varies in a range between 0% and 40% (Chambers et al., 1979) even though usually late detected with most patients succumbing to their local disease. Based on these considerations the control of primary disease remains the major therapeutic challenge.

Given the rarity of this tumor, data on efficacy and safety of the treatments are limited and mainly based on few, small sized, retrospective series. The standard of care is considered surgery, when feasible, with the aim of establishing a definitive diagnosis and obtaining the maximal debulking of the lesion. Surgical outcomes depend on tumor location and size at diagnosis. Considering the large size of most sacral lesions and the proximity to critical healthy structures of skull base and vertebral chordomas, maximal resection usually entails a relevant morbidity with poor functional outcome in a significant proportion of patients. Therefore, even if local control and survival rates strictly depend on the achievement of negative margins, radical surgery can be rarely obtained (Cotler et al., 1983). In such scenario recurrence rates can approach 70%.

This situation clearly supports the interest for radiation therapy as an adjuvant modality after residual disease even though the irregular and infiltrative nature of this tumor makes it difficult to be targeted.

The role of irradiation either as a postoperative treatment or as a curative measure in inoperable lesions is widely debated. Chordomas have been historically considered radio resistant tumors requiring high doses of radiation (> 60 Gy) to respond best. However, a dose-response relationship has not been clearly reported across all series (Tai et al., 1995) and the doses needed to control the tumor in general exceed the tolerance dose levels of nearby normal structures (Pai, 2001; Slater, 1988).

Several irradiation modalities have been proposed (particle therapy, intensity-modulated radiation therapy, stereotactic irradiation) without a clearly established superiority of one

technique over the others. No randomized studies are reported in the literature on this topic and the current available evidence is based on mono-institutional series using different treatment techniques over a long period of time, thus limiting the strength of the corresponding findings.

Technological progress has made it possible to improve the quality of irradiation in an attempt to safely deliver high doses to the target volume while sparing organs at risk.

Since the seventies, particles (administered either alone or in combination with conventional photon beam therapy) have been used with the aim to improve the clinical results. Thank to the rapid dose fall-off beyond the target and the corresponding sparing of surrounding tissues, proton beam irradiation shows a distinct dosimetric advantage over conventional external beam radiotherapy. Ions can exploit the same physical advantage along with a superior radiobiological effect.

At the same time, the recent development of new radiation delivery modalities (such as intensity-modulated radiation therapy) has improved the use of conventional photon radiotherapy. Stereotactic radiosurgery has been used as an effective adjunct in the management of small tumors. Fractionated stereotactic radiotherapy with the use of micro-multileaf collimators may help to optimize radiation delivery. As a consequence, hadron-based radiation therapy and best photon-based techniques deserve comparative evaluations. To date, current data suggests that the optimal treatment strategy includes maximal safe resection and shaping of residual disease to a very limited volume (if any) in order to optimize postoperative proton or modern external photon beam radiation therapy (Crockard et al., 2001).

The purpose of this chapter is to review the literature and the developments in the multimodal approach to chordoma with particular regard to the role of radiation therapy.

2. Chordoma of the base of the skull

Base of the skull presentation represents about one third of all chordomas. This tumor usually affects younger individuals, even children and adolescents (Tai et al., 2002), and is diagnosed more frequently in males. In adults, skull base chordomas occur close to the sphenoccipital area while craniocervical lesions most often involve dorsum sellae, clivus, and nasopharynx. Chordoma is the only tumor that can present with dysfunction of any cranial nerve due to its location. Patients with skull base chordomas can also develop endocrinological dysfunction due to involvement of the pituitary gland within the sella turcica.

The standard treatment is surgery with the aim to assess the pathological diagnosis and to perform the maximal resection even though a radical removal of the lesion is infrequent due to the critical location and the infiltrative pattern of these lesions. Gross total resection is accomplished in three quarter of the patients and 10-year recurrence free survival is about 30% (Tzorzydizis et al., 2006). As a consequence, the likelihood of tumor control is low after surgery alone, even after gross total removal (Menezes et al., 1997). For this reasons, in an attempt to accomplish radical resection and improve the overall outcome, advanced microsurgical techniques have been developed and applied into skull base surgery (Scholz et al., 2010). However, the possibility of complete resection, even with modern surgical techniques, has been associated with still high morbidity and mortality rates (Monfared et al., 2007) as well as with the risk of permanent neurological deficits in 25% of patients (Gay et al., 1995).

The tumor can keep stable even after subtotal resection but the patients ultimately experience local recurrence and need to repeat surgery during the course of their disease. Moreover, recurrent tumors are generally more challenging for surgical interventions and have worse overall outcomes.

In order to avoid the potential evolution of the residual disease, the use of conventional photon radiotherapy was introduced in the eighties in the postoperative setting without being able to increase survival rates but showing longer local control in comparison to surgery alone (Table 1).

Author	Year	Pts	TD range in Gy (med)	% OS (years)		% LC (years)		Med F/U in months
				5	10	5	10	
Cummings et al.	1983	10	25-60 (50)	62	28	41 (3.5)		40
Chetiyawardana et al.	1984	14	30-40	45	23	NA		12-240
Raffel et al.	1985	17	36-69.36 (54.54)	70	--	47	--	60
Amendola et al.	1986	11	53.2-66.3 (60)	30	--	40 (3)		48
Fuller & Bloom	1986	13	47-65 (55)	44	17	23	16	31
Forsyth et al.	1993	39	22.93-67.42 (50)	51	35	39	31	99
Watkins et al.	1993	38	50-60	63	59	34	--	84
Catton et al.	1996	20	25-60 (50)	54	20	23	15	62
Zorlu et al.	2000	18	50-64 (60)	35	--	23	--	42
Cho et al.	2008	11	50.4-69.3 (59.4)	72	--	40	--	55

Legend: Pts: patients; Gy: Gray; TD: total dose; NA: not available; OS: overall survival; LC: local control; Med: median; F/U: follow-up.

Table 1. Published studies on photon beam conventional radiation therapy of skull base chordoma

In general, the series using conventional radiotherapy report on a limited number of patients, treated with median total doses between 50 and 60 Gy, far from the needed high dose level to control such a tumor. As a consequence, the rates of long-term response and survival resulted limited.

High doses (in the range of 70-75 Gy) of radiation are considered necessary for treating chordoma, but, unfortunately, nearby critical neurologic structures (spinal cord, brainstem, optic nerves and chiasm) limit the doses that can be delivered with conventional techniques. Charged particles, alone or in combination with photons, have been used since long time after surgical excision providing adequate support to their use for their peculiar physical/dosimetric advantage (protons and ions) and radiobiological features (ions) over conventional photon radiotherapy. The estimated overall survival rates obtained with protons range between 62% and 80.5% at 5 years and are of 54% at 10 years (see Table 2). Several types of ions (Helium, Neon, Carbon) have been also used with comparable results (see Table 3).

Author	Pts	Rad. type	TD in CGE (range)	% LC at 5 years	% OS at 5 years	Med F/U in months
Hug et al.	33	P	71.9 (66.6-79.2)	59	79	33.2
Munzenrider & Liebsch	169	P + Ph	66-83	73 (10-year: 54)	80 (10-year: 54)	41
Igaki et al.	13	P	72 (63-95)	46	66.7	69.3
Weber et al.	18	P	74 (67-74)	87.5 (3-year)	93.8 (3-year)	29
Noël et al.	100	P + Ph	67 (60-71)	53.8 (4-year)	80.5	31
Ares et al.	42	P (+Ph 4 pts)	73.5 (67-74)	81	62	38

Legend: Pts: patients; Rad.: radiation; P: protons; Ph: photons; LC: local control; OS: overall survival; Med: median; F/U: follow-up; TD: Total dose; CGE: Cobalt Gray equivalent.

Table 2. Series of skull base chordoma treated with protons

Author	Pts	TD in CGE	% LC at 5 years	% OS at 5 years	Med F/U in months
Berson et al.	32	59.4-80	classical Ch 55, chondroid Ch 36	classical Ch 89, chondroid Ch 80	min. 12
Castro et al.	53	60-80 (mean 65)	63	75	51
Schulz-Ertner et al.	96	60-70 (med 60)	70	88.5	31
Tsuji et al.	25	48-60.8	88 (3-year)	86	NA
Mizoe et al.	34	48-60.8	85.1	87.7	53

Legend: Pts: patients; LC: local control; OS: overall survival; med: median; F/U: follow-up; TD: total dose; NA: not available; CGE: Cobalt Gray equivalent; Ch: chordoma; min: minimum.

Table 3. Series of skull base chordoma treated with ions

The debate on the use of this wide set of irradiation techniques is still open in the radiation therapy community (Brada, 2007, Lodge, 2007, Goitein, 2008). Proton therapy is now widely considered the best radiotherapeutic approach but high level of evidence is still lacking. Hence, this treatment modality probably deserves comparative evaluations with the other available conformal technologies in order to optimize the management of the patients, tailoring the radiation treatment to each specific clinical presentation.

From this standpoint, newer methods of delivering photon-based radiation therapy, including fractionated stereotactic radiation therapy, radiosurgery and intensity-modulated radiation therapy have allowed to deliver the dose with better conformity. In particular, despite the limitation concerning the small size of the suitable target, gamma-knife surgery is the most frequently used radiosurgical machine and it has been employed also in the treatment of skull base chordomas. However, data of the most recent literature on this argument (see Table 4) show not consistent results in terms of local control.

Author	Pts	Mean treated volume	Type of radiation treatment	Med dose in Gy	% LC (years)		% OS (years)		Med F/U in months
					5	10	5	10	
Chang et al.	10	1.1-21.5 mL	5 CyberK, 5 LINAC	19.4	2	PD	NA		4
Crockard et al.	26	40.8 cm ³ (pre-op.)	GK	15	NA		65	-	51
Krishnan et al.	25	14.4 cm ³	GK	15	32	-	88	-	56
Martin et al.	18	9.8 cm ³ (average)	GK	16.5	63	- (+ 10.4)	63	-	88
Hasegawa et al.	30	19.7 mL	GK	14.0	72	67	80	56	59
Kano et al.	71	7.1 cm ³	GK	15.0	66	-	80	-	60

Legend: Pts: patients; NA: not available; Gy: Gray; LC: local control; med: median; F/U: follow-up; pre-op.: preoperative; GK: gamma knife; CyberK: cyberknife; LC: local control; OS: overall survival; PD: progression disease.

Table 4. Data of patients with base of the skull chordoma treated with radiosurgery

2.1 Pediatric chordoma

The median age at presentation of chordomas is around 60 years; however, such skull base tumors may occur also at a younger age and has been reported in children and adolescents (Tai et al., 1995).

Special techniques such as intensity-modulated radiation therapy, brachitherapy or intraoperative radiotherapy have been introduced in the management of childhood tumors (Saran, 2004). Proton therapy is treating an increasing proportion of patients (DeLaney et al., 2005) and there is a general agreement that protons will play a major role in the future in treating childhood cancer (Wilson et al., 2005) for its peculiar properties in the potential reduction of secondary cancer risk and reducing rates of late side effects (Miralbell, 2002; Schneider, 2008). Table 5 summarizes some data on the use of particle therapy in chordoma presenting during childhood. In general it is possible to observe that patients with cervical chordoma had a significant worse survival than those with base of the skull presentation, that survival in males was significant superior than in females, and that the reported rate of Grade 3-4 late side effects is very low.

Author	Pts	Radiation	TD in CGE	% LC at 5 years	% OS at 5 years	Med F/U in months
Hug et al	10	P	73.7 (70-78.6)	60	60	30
Hoch et al.	73	P	NA	NA	81	86.5
Habrand et al.	26	P + Ph	69.1	77	100	26.5
Rombi et al.	19	P	74.0 (73.8-75.6)	81	89	46
Combs et al.	7	I	60-66.6	1 progression	-	49

Legend: Pts: patients; P: protons; Ph: photons; I: ions; CGE: Cobalt Gray equivalent; LC: local control; OS: overall survival; med: median; F/U: follow-up; TD: Total dose; NA: not available.

Table 5. Series of skull base pediatric chordoma treated with particles

3. Chordoma of the spinal axis

Overall, chordoma of the spine represents more than half of all chordomas. Along the spinal axis, the most common site of origin is the sacrococcygeal region. The distribution of the remaining vertebral group, in a decreasing order of frequency, is cervical, lumbar and thoracic, respectively. Bjornsson et al. did report that 325 chordomas were diagnosed at the Mayo Clinic since 1902 (Bjornsson et al., 1993). One hundred fifty-six patients (48%) had tumors involving the sacrococcygeal region and 44 (13.5%) had chordomas of the mobile spine.

Because of their slow growth rate, the onset of symptomatology is gradual and long lasting with early symptoms differing according to the anatomical location. At the time of diagnosis, most patients experience pain secondary to bone destruction. However, sacral chordomas may cause rectal and urinary dysfunctions as well as deficient motor function of the lower extremities, whereas lesions involving the rest of the spine usually compress the nerve roots, the spinal cord or adjacent organs mainly translating into sensory deficits, motor disturbances or organ-specific symptoms.

The above-mentioned variability according to the anatomical localization of the tumor along the spinal axis also concerns the tumor size at diagnosis. In fact, sacrococcygeal chordomas can grow filling up the pelvic spaces so that they are usually huge, whereas the limited space availability along the mobile spine translate into an earlier diagnosis of smaller lesions.

The treatment mostly advocated in the literature is surgery. However, the impossibility to achieve an oncologically adequate tumor resection at least in a certain amount of patients has increased the use of radiation therapy as well. Unfortunately, because of the low incidence rate of this malignancy only few centers have achieved extensive experience in the management of chordomas. Nevertheless, the relative rarity of chordomas also explains why the patients collected in clinical series were treated over a long period of time and even managed according to different strategies. Overall, such drawbacks hampered the attainment of robust evidence able to lead the therapeutic strategies.

The present section addresses the main issues dealing with each treatment modality and provides an overview of the main clinical series reported in the literature.

3.1 Surgical management

The spine has a very complex anatomy due to its relationship with vessels (e.g. vertebral arteries in the cervical region), joints, nerve roots, and nearby organs. Besides, structural peculiarities featuring each spinal segment itself add further difficulty. Overall, this makes it tough to accomplish an oncologically proper tumor resection and increases the surgical morbidity as well as mortality. Hence, the best surgical care must include an experienced multidisciplinary team with an oncologic orthopedist, a spine surgeon, a plastic one and a vascular surgeon as well. In fact, several authors noted that patients who received their original surgical procedures outside of recognized centres had worse local control (Bergh, 2000, Schwab, 2009) and/or overall survival (Choi et al., 2010), emphasizing the critical role of experience and clinical expertise in managing this rare malignancy.

Early studies on spinal chordomas reported that the very high local recurrence rates following conventional surgical debulking entailed a very poor survival (Eriksson et al., 1981). Clinical outcomes are considerably improved by the means of better surgical techniques that allowed wide resections and complete removal of the tumors (Boriani et al., 2009). From this standpoint, several series with long enough follow-up have demonstrated that radical resection with adequate surgical margins translates into high local control rate, which ultimately prolongs overall survival. So far, patients amenable by wide resection with adequate margins range between 23% (Yonemoto et al., 1999) and 82% (Ozger et al., 2010) mainly depending on tumor location along the spine and size of the lesion. In fact, the number of vertebral chordomas suitable for radical resection is usually smaller than that occurring in the sacrococcygeal area (Sundaresan, 1979, Bjornsson, 1993, Boriani, 2006). Accomplishing this type of surgery contributes to high absolute local control rates that are very consistent and vary mainly between 72% (Kaiser et al., 1984) and 87% (Hsieh et al., 2009). Few series pointed out even the absence of local relapse (Yonemoto, 1999, Osaka, 2006). Concerning overall survival, radical resection can achieve absolute values in the range of even 90-100% even though these values are reported by a very limited number of studies (Hsieh, 2009, Fuchs 2005). It is noteworthy, that while in some series inadequate surgical margins were an adverse prognostic factor for local recurrence (York, 1999, Bergh, 2000) or for both local recurrence and overall survival (Fuchs et al., 2005), other authors pointed out the lack of such a role (Hulen, 2006, Schwab, 2009). However, ensuring adequate margins can be at the expense of relevant surgical morbidity and mortality. In the management of vertebral chordomas, neurologic deficit and early postoperative deaths have been reported till 55% (Bergh et al., 2000) and 12% of the patients respectively (Boriani et al., 2006). In sacrococcygeal surgical procedures, neurologic deficit correlate with the number of sacrificed nerve roots and the rate of bowel, bladder and sexual dysfunctions can score 89%, 74% and 67%, respectively (Schwab et al., 2009). Besides, fatigues fractures can occur up to 20% of the patients (Bergh et al., 2000), ambulatory deficits till 10% (Hsieh et al., 2009) and wound complications up to 50% of the cases (Hulen et al., 2006) while mortality can achieve 18% (Ozger et al., 2010).

Finally, it is proper to remark that despite apparently macroscopic total resection, local recurrence of disease is not a rare event with most series reporting a rate between 20% (Boriani et al., 2006) and 29% (Ozger et al., 2010).

3.2 Radiotherapy

Local recurrence and progression are inevitable in case of suboptimal surgery. Hence, postoperative adjuvant radiotherapy has been widely employed in the attempt to achieve local control and possibly improving overall survival.

Since the seventies radiotherapy has been applied both as a curative and adjuvant treatment of spinal chordomas. However, for tumors in this location, it is to note that radiation oncologists face the same constraints hampering an adequate surgical excision. In fact, the tolerance dose of most organs nearby the spine is widely below that providing effective treatment.

From this standpoint, it is not surprising that most of the series employing photon radiotherapy (the main series are reported in Table 6) were not able to deliver average doses exceeding 60 Gy. However, the most recent studies pointed out that the evolutionary developments of photon techniques such as three-dimensional conformal radiation therapy and intensity-modulated radiation therapy allowed the delivery of more than 70 Gy though employed only in a limited number of patients.

Author	N. Irr. Pts	Site	Surg	Dose in Gy Mean/Range	Results (%)	Med F/U in months (range)
<i>Conventional photon radiotherapy</i>						
Cummings et al.	11	11 S	2 ST, 9 B	48/24-66	OS 5y 62 10y 28	NR
O'Neill et al.	11	11 S-Cx	3 MT, 8 ST	-/10-60	ST+RT° 5y OS 55 10y OS 20	(12-240)
Fuller & Bloom	12	9 S, 3 SP	5 ST, 7 B	52/30-70	LC° 5y 42 10y 0 OS° 5y 50 10y 0	Min 60
Romero et al.	10	5 S-Cx, 5 SP	8 ST, 2 B	Conv 60/56-65 Hyper 40/30-59	5y PFS° 0 5y OS° 20	Mean 54 (12-102)
Samson et al.	16	21 S	NR	-/50-65	LC° 5y 77 10y 77	Mean 54
Cheng et al.	13	13 S-SP	13 M/I	54/40-70	LC° 5y 72 10y 44 OS° 5y 84 10y 43	Mean 84 (18-288)
York et al.	18	18 S	8 MT, 10 ST	53/30-74	ST+RT Med TtR 25 months	43 (4-408)

Baratti et al.	10	10 S-Cx	10 M/I	-/50-60	Ab. LC 50	71 (15-200)
Atalar et al.	10	10 S-Cx	7 M/I, 3 B	52/50-62	3y LC° 60 3y OS° 78	Mean 65 (7-152)
Boriani et al.	34	34 SP	8 E-b I/C, 16 I, 10 B/P	40-44/-	I surg +RT Ab LC 25 E-b surg +RT Ab LC 50	(3-155)
Stacchiotti et al.	42	42 S-SP	4 W, 13 M, 25 I	79% pts <60 Gy 21% pts ≥60 Gy	LC 5y 52 10y 33 OS 5y 85 10y 58	142 (76-210)
Chen et al.	15	15 S	15 M/I	50/30-60	Cont. DFS 5y 59 10y 42	Mean 74 (16-182)
<i>"High-tech" photon radiotherapy</i>						
Zabel-du Bois et al.	34	34 S	4 R0, 4 R1, 16 R2, 10 B	PTV1 -/40-66 PTV2 -/60-72	5y LC 27 5y OS 70	54 (4-109)

Legend: N.: number; irr.: irradiated; pts: patients; surg.: surgery; Gy: Gray; med: median; F/U: follow-up; S: sacral; Cx: coccygeal; SP: spinal; MT: macroscopically total resection; ST: subtotal resection; B: biopsy; M: marginal; I: intralesional; W: wide; C: contaminated; P: palliative; E-b: en bloc; R0: complete resection; R1: microscopic residual tumor; R2: macroscopic residual tumor; conv: conventional fractionation; hyper: hyperfractionated regimen; PTV: planning target volume; RT: adjuvant radiotherapy; OS: overall survival; LC: local control; y: year; TtR: time to recurrence; ab.: absolute; cont.: continuous; DFS: disease-free survival; °: data from article's graphics; NR: not reported; min: minimum.

Table 6. Main series concerning vertebral chordomas treated with photon radiotherapy

The analysis of the results dealing with photon radiotherapy shows that there was a great variability among the radiation regimens in terms of both total dose and dose per fraction. Probably, this feature can explain why data are not consistent. Overall, the use of a postoperative dose usually less than 60 Gy improved local control compared to subtotal resection only (Cummings, 1983, O'Neill, 1985, Fuller, 1988, Romero, 1993). However, such a dose level does not provide long lasting results. The resulting 5-year local control is only 42% (Fuller & Bloom, 1988) and 10-year overall survival ranges between 0% (Fuller & Bloom, 1988) and 28% (Cummings et al., 1983). At the same time, there are also some series providing better results. Probably, this is because these studies delivered a slightly higher dose to most patients. The corresponding 5-year local control varied between 50% (Baratti et al., 2003) and 77% (Samson et al., 1993) while 10-year overall survival increased up to 43% (Cheng et al., 1999). However, data on long-term local control appear still disappointing especially in comparison with the results of wide radical resection.

It is to note that two authors (Cheng, 1999, Baratti, 2003) pointed out no significant differences in terms of local control and overall survival comparing patients with positive margins treated with adjuvant radiotherapy with those having negative margins who did

Author	N. Irr. Pts	Site	Surg.	Dose in CGE Mean/Range	Results (%)	Med F/U in months (range)
Nowakowsky et al.	12	12 SP	NR	He-Ne +/- Ph 72/-	3y LC 33 3y OS 48	28 (18-89)
Schoenthaler et al.	14	14 S	4 MT, 8 ST, 2 B	He +/- Ne-Ph 75/70-80	LC 5y 55 10y 23 OS 5y 85 10y 22	Mean 65 (22-164)
Breteau et al.	12	11 S Cx	2 B; 10 ID	N + Ph -/55-65 N only -/10 and 17.6	4y LC 54 4y OS 61	NR
Munzenrider & Liebsch	85	85 SP	NR	Ph + P -/66-83	LC 5y 69 10y 48 OS 5y 80 10y 33	36 (1-172)
Hug et al.	14	8 S, 6 SP	4 MT, 8 ST, 2 B	Ph + P 75/67-82	5y LC 53 5y OS 50	Mean 38 (6-136)
Schulz-Ertner et al.	16	8 S, 8 SP	NR	C +/- Ph Ovrl med 68	Ab. LC 87 Ab. OS 87	NR
Park et al.	27	27 S	5 NM, 16 PM, 6 B/ID	Ph +/- P 72/59-84	LC 5y 72 10y 57 OS 5y 82 10y 62	Mean 91 (26-261)
Rutz et al.	26	7 S-Cx, 19 SP	18 MT, 8 ST	P +/- Ph -/59-74	LC 3y 86 5y 69 OS 3y 84	35 (13-72)
Wagner et al.	25	25 S-SP	NR	P, Ph, P + Ph -/70-77	5y LC 73 5y OS 64	32
Imai et al.	95	95 S	95 ID	70/53-74 (70 CGE in 90% of pts)	5y LC 88 5y OS 86	NR

Legend: N.: number; irr.: irradiated; pts: patients; surg.: surgery; CGE: Cobalt Gray equivalent; S: sacral; SP: spinal; Cx: coccygeal; NR: not reported; MT: macroscopically total resection; ST: subtotal resection; B: biopsy; ID: inoperable disease but pathologically proved; NM: negative margins; PM: positive margins; He: helium ions; Ne: neon ions; Ph: photons; N: neutrons; P: protons; C: carbon ions; ovrl: overall; y: year; LC: local control; OS: overall survival; ab.: absolute.

Table 7. Main series concerning vertebral chordomas treated with particle radiotherapy

not receive adjuvant irradiation. Finally, the only series reporting the use of intensity-modulated radiation therapy (Zabel-du Bois et al., 2010) highlighted that dose higher than 60 Gy significantly improved local control and overall survival as well as the radiation delivery at the time of initial diagnosis. The disappointing local control rate reported in this study could be explained considering the wide range of dose delivered to the gross tumor volume with only a limited number of patients receiving 72 Gy.

The significant advantage related to the different energy deposition in tissues supported the use of charged particles (protons, ions, neutrons) also in spinal chordomas. As above mentioned, protons do not have a significant biologic advantage over conventional photon irradiation and only their physical properties make attractive this treatment modality. Conversely, concerning ions, the favorable physical features coexist with a radiobiological advantage that could further increase the tumor control probability.

Such evolutionary technique has been applied to vertebral chordomas only recently. Therefore, only a limited number of studies are reported in literature (main series are summarized in Table 7).

Likewise to photon radiotherapy, mainly mono-institutional retrospective series enrolling a limited number of patients over many years are reported. However, most series employing particle radiotherapy were able to deliver average doses exceeding 70 Gy as well as total doses even higher than 80 Gy.

Therefore, analyzing the results, it is not surprising that they are generally better than those registered in photon radiotherapy series. The 5-year local control ranged between 53% (Hug et al., 1995) and 72% (Park et al., 2006) in the studies employing protons and between 55% (Schoenthaler et al., 1993) and 88% (Imai et al., 2011) in those using ions. The corresponding 5-year overall survival rates varied between 50% (Hug et al., 1995) and 82% (Park et al., 2006) in the proton series and between 85% (Schoenthaler et al., 1993) and 88% (Imai, 2011) in studies using ions. Disappointing results were reported only in one study (Nowakowsky et al., 1992). Almost all these series have not enough long follow-up. Hence, it is fair wondering whether such results are long lasting. With this regard data are not consistent and actuarial 10-year local control varied between 23% (Schoenthaler et al., 1993) and 57% (Park et al., 2006). Concerning overall survival, the actuarial rate at 10 years ranged between 22% (Schoenthaler et al., 1993) and 62% (Park et al., 2006). However, if the best results will be confirmed at adequate follow-up they will be consistent with data reported in patients treated by wide radical resection. This scenario could offer a new standard of care: a function-preserving surgery followed by high-dose radiotherapy (particle or mixed particle/photon). It is worth of note that results pointed out by Imai et al. (Imai et al., 2011) concern only inoperable patients even suggesting the possibility to avoid surgery. Finally, three authors (Nowakowsky, 1992, Schoenthaler, 1993, Park, 2006) pointed out the improvement of local control delivering the irradiation at the time of initial diagnosis.

In summary, radical tumor resection with adequate margins can achieve optimal results in terms of local control and overall survival even though at the expense of relevant peri-operative morbidity suggesting that such surgical procedures could be reserved for patients with a high cure possibility. The functional consequences should be clearly discussed preoperatively with the patient as well as the nature of the disease.

Radiation therapy, when positive surgical margin or residual tumor is present can improve the local control. Long lasting results in terms of local control can be achieved only delivering doses higher than 70 Gy. Such a strategy could also translate in high long-term overall survival rates. In order to optimize the outcome, adjuvant radiotherapy should be applied preferably at the time of initial diagnosis rather than at relapse.

Similar to skull base chordomas management, a function-preserving surgery followed by high-dose radiotherapy could represent a new standard of care.

4. Conclusion

Chordomas are rare primary bone tumors with a high risk for local recurrence and modest propensity for distant metastasis. Optimal therapy of chordoma is a combined approach of maximal safe surgical resection followed by proton beam irradiation for residual disease.

In our review, radiation therapy demonstrated to be a valuable modality for the achievement of durable local control in the postoperative setting, particularly with the advent of charged particle radiotherapy. The use of protons has shown better results in comparison to the use of conventional photon irradiation, with favourable long-term outcome and relatively few significant complications considering the high doses delivered.

5. References

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Clinical Application of Image-Guided Iodine-125 Seed Implantation Therapy in Patients with Advanced Pancreatic Cancer

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1. Introduction

Carcinoma of the pancreas is a devastating disease. The incidence of pancreatic carcinoma has increased in recent decades, yet the treatment outcome for this disease remains unsatisfactory. Despite the introduction of new therapeutic techniques and adoption of aggressive combined treatment modalities, such as external beam radiotherapy (EBRT) and chemotherapy, the prognosis of pancreatic carcinoma remained to be extremely poor, with a mortality rate of more than 90% (Geer & Brennan, 1993). Pancreatic cancer responds poorly to surgical resection or chemotherapy, moreover only 10–20% of patients are candidates for curative surgical resection, and even with resection, long term survival still remains poor (Levin et al., 1978; Crile, 1970), therefore, the remaining patients have to seek alternative therapeutic options (Joyce et al., 1990). Biliary and gastric bypass have been used for palliation in unresectable pancreatic carcinomas and median survival in these patients was often 5–6 months (Thompson & Nagorney, 1986; Schwarz & Beger, 2000; Khan et al., 2010). However most of pancreatic carcinoma was diagnosed in the locally advanced or metastatic stage, both local control and management of distant metastases are the major factors that affect the prognosis of such patients. More recently, EBRT and chemotherapy have been standard adjuvants for locally advanced pancreatic carcinoma. External beam radiotherapy therapy, one of the options, is usually regarded as insensitive to pancreatic cancer and associated with more systemic side effects, although it can relieve pain in up to 50–85% of patients (Minsky et al., 1988). Also EBRT alone has failed to control disease progression and yields a median survival of 5.5–7 months (Bodner et al., 2000; Nag et al., 2006). The introduction of intraoperative electron beam radiotherapy, combined with EBRT and chemotherapy, has also failed to significantly improve long-term results, with recent studies reporting median survival rates of 7–16 months (Blasko et al., 2002; Wang et al., 2010; Cengiz et al., 2008; Monk et al., 2002). Despite the availability of many treatments, there was currently no consensus regarding the optimal therapeutic modality for unresectable pancreatic carcinomas. Therefore, it is necessary to investigate new techniques that may improve the prognosis. It has been reported that compared with external irradiation therapy, 5-Fu chemotherapy combined with radiotherapy prolonged the median survival time of patients with local advanced pancreatic cancer from 23 weeks to 42–44 weeks

(Mattiucci et al., Furuse et al., 2010; Kouloulis et al., 2002). However, there are few reports on interstitial implantation of radioactive seeds in the treatment of pancreatic cancer (Sun et al., 2005; Mohiuddin et al., 1992). Interstitial brachytherapy has been reported to be a useful method for local control of malignant pancreatic tumors (Takacs et al., 2002; Enomoto et al., 2006). After placement of the radioactive seeds, the target tissue is exposed to a steady emission of gamma rays, which leads to localized ablation. Interstitial brachytherapy has been applied to unresectable pancreatic cancers in an attempt to maximize local tumor dose and minimize radiation dose to the surrounding normal structures. Therefore, radioactive iodine-125 seed implantation is another choice for treatment of malignant tumors, which is widely applied for its curative effect, minimal surgical trauma, and few complications (Ebara et al., 2008; Siegel et al., 1988; Holm et al., 1981).

2. Advantages of biological effects of radioactive iodine-125 seed

Advantages of ^{125}I seeds over other forms of radiotherapy are as follows:

1. Radiation from seeds is characterized by attenuation over short distance outside the target area and low grade of depth dose, which can keep a higher accumulative dose (up to 160 Gy) within the tumor. Unfortunately, serious complications such as radiation hepatitis can occur when the absorbed dose is less than 35 Gy as in traditional radiotherapy.
2. External radiotherapy in fractionation is only effective in cells in some phases of cell cycle. During the interval of irradiation, effect of radiotherapy is decreased because cells in the stationary phase enter into the mitotic stage. The ^{125}I seeds can kill tumor cells continually by keeping cells in the resting period and causing tumor stem cell apoptosis (Wang et al., 2010).
3. Deficiency of oxygen is a bottleneck of conventional external radiotherapy. Sensitive phase cells will be killed by the accumulated damaging effect of irradiation. However, cells deficient in oxygen avoid apoptosis by entering the sensitive phase.
4. Inhomogeneous radiation absorption occurs in the traditional method due to respiratory movements, which decrease the therapeutic volume. By contrast, the therapy using radioactive seeds is not affected by respiratory movements; the probability of therapeutic volume loss is obviously tiny.
5. Implantation of low energy, radioactive seeds is able to decrease the metastasis of tumor by changing the immunophenotype of tumor cells. Some research indicated that the radiobiological effect of radioactive seeds was superior to that of three dimensional conformal radiation (Mazeron et al., 2003).

3. Indications

1. Neutrophil leukocyte $3 \times 10^9/\text{L}$ or higher, platelets $70 \times 10^9/\text{L}$ or higher, and hemoglobin 90 g/L or higher in peripheral blood.
2. Prothrombin index (PI) greater than 50% and partial thromboplastin time (PTT) less than 50 s; kidney function within normal range.
3. Karnofsky physical scores (KPS) greater than or equal to 60.
4. With pathologically confirmed an advanced stage of pancreatic cancer.
5. Advanced pancreatic cancer was unable to undergo open surgery due to clinical or personal reasons.

6. Tumor maximum diameter of less than 6cm.
7. Previous course of chemotherapy were eligible.

4. Contraindications

1. Pregnant women and patients with distant metastasis (e. g. liver, lung).
2. Tumor maximum diameter greater than 6cm.
3. Expected survival time of less than 3 months.
4. Systemic failure.
5. Bleeding tendency.
6. Prothrombin time 3 seconds longer than the control were excluded.
7. Any previous irradiation or external radiotherapy were excluded.

5. Preoperative preparation

5.1 I seed sources

The ^{125}I sealed seed sources were supplied by XinKe Pharmaceutical Ltd, Shanghai. For the seed implantation we used 18-G implantation needles and turntable implantation gun (XinKe Pharmaceutical Ltd, Shanghai, China). The ^{125}I seeds were manufactured from silver rods, which absorbed ^{125}I , and were enclosed in a titanium capsule welded by laser. The diameter of each seed was 0.8 mm, the length was 4.5 mm, and thickness of the wall of the titanium capsule was 0.05 mm (Fig.1,2). The ^{125}I produces gamma rays (5% of 35 keV, 95% of 28 keV) with a half-life of 59.6 days, half-value thickness of 0.025 mm of lead, penetration of 17 mm, incipient rate of 7 cGy/h, a mean radioactivity of 0.694 ± 0.021 mCi (25.6 MBq), and activities of 0.5–0.9 mCi.



Fig. 1. Radioactive ^{125}I seeds



Fig. 2. Radioactive ^{125}I seed profile chart

5.2 Diagnosis of pancreatic cancer

Imaging methods were adopted for the clinical diagnosis of pancreatic cancer. Patients were first diagnosed by conventional computed tomography (CT) or magnetic resonance imaging (MRI), and by thin slice helix CT 10 days before implantation of seeds. Histological confirmation of the diagnosis was achieved by CT-guided, EUS-guided fine needle aspiration (FNA) 1 week before implantation. FNA has been accepted as a gold standard (Volmar et al., 2005; Gupta et al., 2002; Dickey et al., 1986; Mueller, 1993; Brandt et al., 1993) in the diagnosis of pancreatic cancer. Furthermore, patients had an abnormal serum CA19-9 level (higher than 37 U/ mL, 519±439 U/mL). All enrolled patients were diagnosed with pancreatic carcinoma before seed implantation.

5.3 Treatment planning

Dose distribution was calculated using a Fudan TPS2.00 brachytherapy planning system (Fudan University, Shanghai, China) based on the American Association of Physicists in Medicine TG43 brachytherapy formalism (Chen et al., 2008). The total volume of each tumor was calculated according to the CT image with the treatment planning system (TPS) before implantation (Cengiz et al., 2008). Patients underwent a detailed tumor volume study using CT scans 1-2 weeks before seed implantation. Images of each pancreatic carcinoma were obtained at 5 mm intervals. The radiation oncologist and surgeons together outlined the gross tumor volume (GTV) on each image and planning target volume (PTV) included GTV plus 0.5 - 1.0 cm peripheral tissue. These tracings were digitized and scanned to define the tumor volume, from which the D90 of 60-140 Gy for ¹²⁵I seed irradiation, with the median of 120 Gy and the number of ¹²⁵I seeds to be implanted could be calculated. The D90 was prescribed in a way that at least 90% of the tumor volume received the reference dose. In brief, the information from CT or MRI images was reconstructed into a three-dimensional form, and the precise margin of the tumor was outlined to facilitate the calculation of tumor matched peripheral dose (MPD). The expected number of implanted seeds was calculated according to the modified level formula (Monk et al., 2002). The ¹²⁵I with a nominal activity of 0.5-0.8 mCi/seed and a diameter of less than 1 mm was used as a radiation source and implanted into pancreatic tumor under image guidance, at a spacing of 1 cm.

5.4 Calculation of the number of seeds needed for implantation

The total volume of each tumor was calculated according to the CT image with the treatment planning system (TPS) before implantation (Bodner et al., 2000). In brief, the information from CT or MRI images was reconstructed into a three-dimensional form, and the precise margin of the tumor was outlined. An isodose curve and dose-volume histogram were drawn to concentrate the radioactivity in the target area. The expected number of implanted seeds was then calculated according to the modified Cevic formula (Monk et al., 2002) as follows:

Number of seeds needed

$$\frac{[\frac{1}{2} \text{Tumor length} + \text{width} + \text{height in cm}] \times 5}{\text{the mean activity per seed in mCi}} \quad (1)$$

In practice, to reach the maximum radiation effect, the number of seeds implanted was 15% more than needed. The seeds were sterilized by immersing in 2% glutaraldehyde solution for 20-30 minutes, then washed and placed into the specially designed releasing device.

During the course of implantation, and the seeds were released and deposited in the target position.

5.5 Pretreatment evaluation

In most cases, the initial request for a radiotherapy consultation occurred at the time of exploratory laparotomy, so a detailed preimplant evaluation by the radiation oncologist was not possible; however, in all cases, the patients were carefully studied by the surgical team preoperatively. All patients were evaluated by comprehensive medical history, physical examination, and standard presurgical studies including complete blood count, serum chemistries, liver function tests, urine analysis, and chest X-ray, as well as the following special imaging studies: abdominal ultrasound, computerized tomography, and MRI.

5.6 Patient preparation

All patients signed written informed consent before the study and were informed of potential benefits and risks. The whole study protocol was approved by the Ethics Committee.

Patients fasted for 24 h prior to the operation, and oral laxatives were given 12 h before the procedure. Pancreatic secretion was inhibited by medication 24 h before the operation to reduce the rates of complications. For patients with jaundice, percutaneous transhepatic cholangiodrainage (PTCD) was scheduled first in order to relieve symptoms, improve liver function, and reduce the surrounding edema, after which seeds implantation was performed.

6. Image-guided interstitial brachytherapy protocol

6.1 CT-guided interstitial brachytherapy protocol

The total volume of each tumor was calculated according to the CT image with the treatment planning system (TPS) before implantation (Cengiz et al., 2008). In brief, the information from CT or MRI images was reconstructed into a three-dimensional form, and the precise margin of the tumor was outlined to facilitate the calculation of tumor matched peripheral dose (MPD). The expected number of implanted seeds was calculated according to the modified level formula (Monk et al., 2002). In practice, to reach the maximum radiation effect, the number of seeds implanted was 15% more than needed. Implantation was guided by CT according to our TPS. The ^{125}I with a nominal activity of 0.5–0.9 mCi/seed and a diameter of less than 1 mm was used as a radiation source and implanted into pancreatic tumor under fluoroscopy CT guidance, at a spacing of 1 cm, avoiding puncturing vessels, pancreatic duct, and other nearby organs. Patients fasted for 24 h prior to the operation, and oral laxatives were given 12 h before the procedure. Pancreatic secretion was inhibited by medication 24 h before the operation to reduce the rates of complications. Sufficient breath training was given to ensure steady breath movement during the procedure. All the brachytherapy implants were performed in a standard CT room under local anesthesia. CT imaging was taken at intervals of 5 mm. The distance between the adjacent implantation needles was approximately 1 cm each. Transgression of the bowel during the puncture did not result in substantial complications in our study. However, a safer approach is achieved by transversing the stomach. Intestine and colon should be avoided especially when using large-bore needles. Repeated CT with the implantation needles in place permitted adjustment of depth and angle of needle direction.

Two to five seeds per needle were loaded, and seeds were released every 5–10 mm apart upon withdrawing the needles. For patients with jaundice, percutaneous transhepatic cholangiodrainage (PTCD) was scheduled first in order to relieve symptoms, improve liver function, and reduce the surrounding edema, after which seeds implantation was performed. Afterwards the implantation puncture site was bandaged and compressed to achieve hemostasis. Patients were kept in radiooncology/interventional ward for 4 full days.

6.2 EUS-guided interstitial brachytherapy protocol

All eligible patients underwent implantation of iodine-125 seeds. The operator wore a lead apron. A linear-array therapeutic echo endoscope (EG3830UT; Pentax Precision Instruments, Orangeburg, New York, USA) was inserted into the proximal stomach. The maximal diameter of the tumor was measured by real-time sector ultrasound (Olympus China Co. Ltd, Shanghai, China), with a frequency of 5-7.5 MHz. EUS was performed to show the conformation of the pancreatic tumor and EUS images were captured by computer. The tumor volumes were calculated using EUS and CT images and 3D diameters of the tumors and treatment plan system software (Zhiye Medical Software Co., Shenyang, China). The minimum peripheral dose was then set to 140 Gy and the dose of every seed was entered into the software. The number of implants required was calculated by the software and the distribution plan maps were drawn: the experienced operator would then know the distance and direction of every target site from the center of the tumor. Iodine-125 radioactive seeds could be inserted easily through the channel of a 19-gauge therapeutic needle (Wilson-Cook Medical Inc., Winston-Salem, North Carolina, USA). When the needle was inserted into the target site under EUS guidance, the stylet was removed and a seed was inserted into the needle; the stylet of the needle was then advanced to push the seed forward, and the seed was released from the needle and implanted into the tissue. This implantation procedure was repeated until all the seeds were implanted into target sites according to the treatment plan. The lesion was observed by multi-slice scanning, and the relationship between the surrounding vasculature and the tumor was then identified. The puncture points and method of puncturing were determined by color Doppler technology to prevent injuring the pancreatic duct or the vasculature of the pancreas.

In principle, the seeds should be in a line and parallel to each other. The distance between each seed should be the same (1.0~1.5 cm). The distribution of seeds should be denser in the peripheral area so as to avoid high-dose-induced complications.

6.3 Intraoperative ultrasound-guided interstitial brachytherapy protocol

After the diagnosis of pancreatic cancer had been established by biopsy intraoperation, tumor volume was measured during laparotomy by intraoperative ultrasonography utilizing a megahertz linear probe. Guided by ultrasound, 18-gauge needles were implanted into mass and spaced in a parallel array at intervals of 1.0 cm, extending at least 0.5~1 cm beyond the margins of the pancreatic lesions. During the placement of the needles, care was taken to avoid the needles from the pancreatic duct, small blood vessels, and the adjacent transverse colon at least 1 cm. After needles were implanted, ¹²⁵I seeds were implanted using a Mick-applicator and the spacing was maintained at 1.0 cm intervals. The number of ¹²⁵I seeds implanted ranged from 10 to 75, with the median number implanted of 38. The specific activity of ¹²⁵I ranged from 0.40 to 0.60 mCi per seed, and the total isotope

radioactivity implanted ranged from 4 to 37.5 mCi. An omental fat pad was placed over the implanted volume to protect the gastric and transverse colon mucosa from irradiation.

7. Post-implant adjuvant therapy

7.1 Chemotherapy

Patients who gave consent to chemotherapy received combined treatment with gemcitabine 1.0 g/m² (body surface area) and 5-fluorouracil (5-Fu) 300 mg/m² 1 week after the implantation. The chemotherapy was a 5-day schedule which contained gemcitabine on the first day followed by 4 days of 5-Fu. The chemotherapy was repeated every 4 weeks for up to six cycles if tolerated.

7.2 External beam radiotherapy (EBRT)

EBRT was generally recommended to all patients for an adjuvant aim. The patient received EBRT at 4–6 weeks after ¹²⁵I seed implantation. The total doses of EBRT ranged from 35 to 50 Gy at 1.8–2.0 Gy per fraction if tolerated.

8. Clinical benefit response (CBR)

The clinical benefit response assessment in these patients with locally advanced pancreatic cancer was derived from the measurement of pain levels, functional impairment (assessed by the Karnofsky performance status score), and weight loss (Burris et al., 1997). For patients to achieve an overall rating of positive CBR, they had to be positive for at least one parameter (pain, performance, status, or weight) without being negative for any of the others (Hwang et al., 2004). This improvement had to last for at least 4 weeks. Patient survival, tumor responses, and the clinical benefit responses were recorded. Visual analog scale (VAS) pain score was recorded as level 0 to 10, in which 0 indicated no pain, 1 to 3 indicated mild pain, 4 to 7 meant moderate pain, and 8 to 10 severe pain. Scoring began after ¹²⁵I seeds were implanted.

9. Evaluation of curative effect

Patients were monitored for adverse events and for abnormalities in laboratory indices, including hematological parameters, lipase, amylase, carcinoembryonic antigen (CEA), CA19-9, and liver function tests. They were assessed by physical examination (including weight, Karnofsky performance status, and visual analog scale pain score), and the tumor size was monitored by CT scan or EUS. Patients were examined by CT 2 month after the operation. The short-term efficacy was determined according to the tumor response standards suggested by the World Health Organization (Miller et al., 1981). Briefly, complete response (CR) was defined as the complete disappearance of the lesion lasting for more than 4 weeks. Partial response (PR) referred to the situation where the size (i.e., the longest dimension multiplied by maximal upright dimension) of the lesion decreased by more than 50% and then remained unchanged for 4 weeks. Stable disease (SD) was defined as the situation where the size of the tumor decreased by less than 50% or increased by less than 25%. Response rate was defined as the sum of CR and PR. Local tumor control after brachytherapy was defined as the absence of tumor progression in CT (SD+PR+CR). The long-term efficacy included the median survival time, tumor-free survival, and 1-year

survival rate. Serum CA19-9 level was checked every month post-implantation as an indicator of prognosis.

10. Follow-up

The tumor diameter, general condition, and pain score of patients were monitored and recorded during follow-up. KPS and visual analog scale (VAS) pain score were used as the main indicators of quality of life (Burriss et al., 1997; Hwang et al., 2004). VAS scoring began when chemotherapy started (1 week after brachytherapy). One month after seed implantation, patients were evaluated by radiation oncologists and surgeons by physical examination, complete blood panel, chest X-ray, abdominal CT and ultrasound. One month later, a clinical consultation was provided. After that, evaluation was given every 2-3 months or sooner if a new clinical sign or symptom appeared. Time of survival was calculated from the date of diagnosis to the date of death or last follow-up. A local recurrence was defined as tumor progression (PD) within the implanted area or surrounding regions as seen on CT. Local recurrence and distant metastasis were scored until patient death and censored thereafter. The short term efficacy was determined according to the tumor response standards suggested by the World Health Organization. The long term efficacy included the median survival time, tumor-free survival, and survival rate.

11. Complications

The significant causes of high morbidity of ¹²⁵I seed intraoperative implantation were due to the needles penetrated into pancreatic duct, small blood vessels in the pancreas and/or organ at risk resulting in fistula and abscess formation. The major long-term complication from the combined effects of multimodality treatments has been gastrointestinal bleeding and obstruction (Shipley et al., 1980). Clinical evaluation, ultrasound, and CT scans determined that the majority of patients developed metastases to the liver and peritoneal surface.

12. Clinical status and prospects

The survival for patients with pancreatic cancer remains poor despite standard surgical approaches, new adjuvant therapeutic techniques, and combined modality treatment. The treatment of unresectable pancreatic cancer continues to be a major challenge. More than half of patients have a locally or regionally confined tumor requiring local treatment. Resection of primary pancreatic malignancies with a curative intention is only feasible in less than 15% of all patients (Barkin & Goldstein, 2000). Most patients will have unresectable disease even before the diagnosis is made. The median survival time for untreated patients is 4 months, and they will suffer in varying degrees from pain, anorexia, weight loss, jaundice, and intestinal obstruction (Korinthenberg et al., 2011; Jiang et al., 2010). The management of these patients is still controversial. Combined modality treatment may have a positive effect on survival and quality of life in this group of patients.

Traditional treatment for local control of advanced or metastatic pancreatic cancer involves intravenous chemotherapy with 5-fluorouracil (5-Fu) or gemcitabine; however, local recurrence and progression in the pancreas and peripancreatic lymph nodes under this treatment has been reported to be as high as 58% (Xie et al., 2006). Stereotactic radiotherapy

(SRT) allows an escalation of radiation doses to be applied to a small target volume within a small margin. SRT is administered in one or a few fractions with the goal of sparing the surrounding normal tissue by using multiple non-coplanar field arrangements for the administration. In a phase II study on the use of SRT in the treatment of locally advanced pancreatic carcinoma by Huyer et al, the median survival time was only 5.7 months, and the one-year survival rate was 5% (Gudjonsson, 1987). These data associate SRT with a poor outcome, unacceptable toxicity, and questionable palliative effects, making SRT inadvisable for patients with advanced pancreatic carcinoma.

In the context of multimodal oncologic therapy concepts a minimally invasive approach is often desired. Percutaneous image-guided seed implantation which can be performed without surgery or general anesthesia has attracted increasing attention because of its ability to increase radiation dose to pancreatic tumors without damaging neighboring organs (Peretz et al., 1989). With this technique, highly effective radiation doses are applied as a single fraction, ensuring protracted cell killing over a period of up to several weeks or months. Compared with other interventional procedures, advantages exist regarding interference-free and accurately predictable energy distribution, treatable size of a target lesion, and lower rate of acute adverse effects possible by maintaining tissue continuity. Extensive experiences with this technique had been collected during several preceding studies targeting liver malignancies as well as one study targeting lung malignancies (Korinthenberg et al., 2011; Jiang et al., 2010; Xie et al., 2006) ¹²⁵I seed placement has become a routine treatment for malignant tumors at various sites.

In contrast, interstitial permanent implantation of radioactive seeds into the tumor site provides the advantage of delivering a high dose of irradiation to the tumor (range 140–160 Gy) which drops off sharply outside the local implanted field. ¹²⁵I seeds with a half-life of approximately 59.6 days were selected as the radioactive source for permanent implantation in this study, allowing approximately 95% of the needed dose to be delivered within a year (Hoyer et al., 2005). Implantation of radioactive isotopes for the treatment of pancreatic carcinoma has been used for the past several decades. For example, Handly et al. reported the use of radium needle implantation in 7 patients for the treatment of pancreatic carcinoma in 1934 (Hilaris, 1975). Of those, one patient survived up to two years. Hilaris, who was a pioneer in the development of ¹²⁵I seeds for implantation for the treatment of pancreatic carcinoma, published a study of 98 patients receiving seed implants that responded with a median survival of 7 months (Handley, 1934), with 1 patient surviving for five years. Pain control was achieved in 65% of patients and lasted between 5 and 47 months (with a median of 6 months). In a review study by Morrow et al., no difference in survival between patients treated with interstitial brachytherapy and patients treated by surgical resection at the same institution were observed (Hilaris, 1975). The median survival time was 7 months, and at least one patient survived up to five years. Pain control was achieved in 65% of the patients (Morrow et al., 1984). Syed et al. reported 18 patients treated with biliary bypass surgery, ¹²⁵I interstitial brachytherapy, and EBRT (Syed et al., 1983). Ten patients with the interstitial brachytherapy were "sandwiched" between two courses of EBRT. Typically, patients received 30 Gy EBRT following biopsy and bypass surgery, then 2 weeks later an additional interstitial brachytherapy of 100–150 Gy, and then an additional 15–20 Gy EBRT was administered 3–4 weeks after interstitial implantation. The results showed a 13 month median survival time in 12 patients with head and body pancreatic carcinoma. ¹²⁵I seed implantation has been attempted in patients with locally advanced pancreatic carcinoma, and no difference in overall survival was found compared with the

use of other techniques (Morrow et al., 1984). Wang et al. reported 14 patients treated with ¹²⁵I seed implantation guided by intraoperative ultrasound (Wang et al., 2009). The interstitial needle position and distribution were determined using ultrasound supervision and with the intent to spare at least 1 cm from nearby or normal tissues including the internal pancreatic duct and small blood vessels. The placement of an omental fat pad over the implanted volume was also used to protect the gastric and transverse colon mucosa from irradiation. The result indicates that the local control of disease was achieved in 78.6% of all patients. 87.5% (7/8) of all patients experienced complete and partial pain relief and shown satisfactory palliative effect. The overall 1-, 2- and 3-year survival rates were 33.9%, 16.9% and 7.8%, respectively with the median survival of 10 months. The survival rate and survival times were found to be the most advantageous for some selected stage II/III patients.

However, there are few reports on CT-guided implantation of radioactive seeds in the treatment of pancreatic cancer. At present, the most commonly used isotope is ¹²⁵I, and ¹²⁵I placement has become a routine treatment for recurrent tumors at various sites. Wang et al. reported in this group of pancreatic cancer patients (Wang et al., 2010). 31 patients implanted ¹²⁵I seeds under CT guidance and yielded good local control of the disease. The results showed even distribution of the radioactive seeds with overall response rate of 61.3%, local control rate of 90.3%, and pain relief rate of 92%.

Permanent interstitial administration of radioactive seeds appears to offer consistent and improved local control, although a major drawback is the high rate of perioperative morbidity and mortality. The significant causes of high morbidity of ¹²⁵I seed intraoperative implantation were due to the needles penetrated into pancreatic duct, small blood vessels in the pancreas and/or organ at risk resulting in fistula and abscess formation. The major long-term complication from the combined effects of multimodality treatments has been gastrointestinal bleeding and obstruction (Schwarz & Beger, 2000). The high incidence of complications maybe related to that the seeds were implanted nearby normal tissues such as gastric, colon and jejunum. The second reason may be the activity of seeds was high. The third reason maybe the doses of seeds beyond the tolerance of normal pancreas tissue. In earlier studies, perioperative mortality was 16% – 25% from acute pancreatitis, fistulization, and abscess formation (Peretz et al., 1989). Side effects reported in the Hilaris et al., study included 1 patient developing a post-operative mortality, another patient suffered from a pancreatic fistula, 4 patients developed biliary fistula, 4 developed abscesses, 4 developed gastrointestinal bleeding, 6 developed obstruction of the gastrointestinal tract, 5 patients developed sepsis, and 4 patients developed deep venous thrombophlebitis (Handley, 1934). In comparison, the study by Syed et al. included 8 patients with a poorer prognosis, 2 patients with prolonged wound drainage, 3 patients developed insulin-dependent diabetes, and 2 patients developed other interstitial complications (Peretz et al., 1989). Also Wang et al. reported [48], one patient suffered from chylous fistula, one patient suffered from pancreatitis and one suffered from gastritis, seven patients suffered from low fever, there were no grade III and grade IV toxicity and complications, and less than most series of surgically-treated pancreatic cancer patients published in the literature (Morrow et al., 1984; Wang et al., 2009).

Local complications of advanced pancreatic carcinoma result in significant morbidity and mortality. Although systemic therapy is ultimately needed for cure, an effective locoregional therapy for the treatment of the pancreatic primary and/or regional metastases in the liver would be beneficial in patients who do not have extensive extrahepatic disease at the time of

presentation. Current therapies, however, are of limited benefit in most patients. The high incidence of complications associated with resection of advanced pancreatic cancer and the significant gastrointestinal toxicity of external-beam radiation limit their usefulness (Yao et al., 2002). Other series of intraoperative iodine-125 implantation have been associated with mortality ranging from 0% to 16% and major morbidity of 18% (Order et al., 1996). Sun S et al. reported (Sun et al., 2006), there was no significant immediate complications, such as significant bleeding or infection. The incidence of complications was 3/15 (20%), and the adverse events were mild and not life-threatening. Although the objective response rate in patients with locally advanced pancreatic cancer was moderate, five patients experienced clinical benefit and four patients showed a partial tumor response. Pancreatic fistula is the most common complication after implantation of seeds, especially in surgery cases. In contrast, the needle used in EUS is thinner and the procedure is real-time monitored by EUS. Therefore, no pancreatic fistulae were observed in the present study.

Nevertheless there were fewer complications compared with other interventional ablation procedures. From these data it appeared that ^{125}I implantation of unresectable pancreatic tumors offered high control of the primary tumor and significant palliation of symptoms. Wang et al. reported (Wang et al., 2010), their data suggest that local control rates can be enhanced by the addition of chemotherapy. Despite lacking definitive proof, positive results allow us to continue the use of drug-seeds combination therapy. Cron et al. (Cron et al., 2005) suggested that the best time for chemotherapy is within 3–4 days after implantation of ^{125}I seeds, because the permeability of the surrounding vasculature is promoted by the radiation effects of the seeds at that time. Wang et al. reported ten out of 31 patients in this group underwent additional chemotherapy 1 week post-treatment and tolerated it well (Wang et al., 2010). The median survival time for pure seeds implantation and drug-seeds combined therapy was 7 months and 11 months, respectively; it reached statistically significant and therefore encouraged our further evaluation. In the present study, implantation of seeds combined with chemotherapy in the treatment of pancreatic carcinoma showed preliminary effects. Although no complete remission cases were observed, the tumor progression was effectively controlled (stable disease or partial remission) in more than half of the patients (59.1%) (Jin et al., 2008).

After promising results, we will further evaluate interventional brachytherapy as an additional tool in multimodal oncologic therapy concepts (Chen et al., 1999; Trombetta et al., 2008). This study suggested that image-guided brachytherapy using ^{125}I seeds implantation appeared to be safe, effective, uncomplicated, and could produce adequate pain relief for treating unresectable pancreatic cancer. The present study is limited: a multimodality approach, with image guided-interstitial brachytherapy in combination with chemotherapy or external radiation, may be indicated and should be tested in further studies. Therefore, future studies should be focused on how to design a mature and feasible integrated protocol based on radioactive seeds.

13. In conclusion

This study suggested that image-guided brachytherapy using ^{125}I seeds implantation appeared to be safe, effective, uncomplicated, and could produce adequate pain relief for treating unresectable pancreatic cancer. ^{125}I seed implantation with image-guided provides a satisfactory distribution of seeds in tumor mass, minimizes radiation to surrounding organs due to the sharp dose fall-off outside the implanted volume, and generates no damage (Armstrong et al., 1994). We hypothesize that a further improvement in median survival of

patients with unresectable pancreatic carcinoma may be obtained with the combined aggressive use of EBRT, systemic chemotherapy.

14. Examples of clinical application

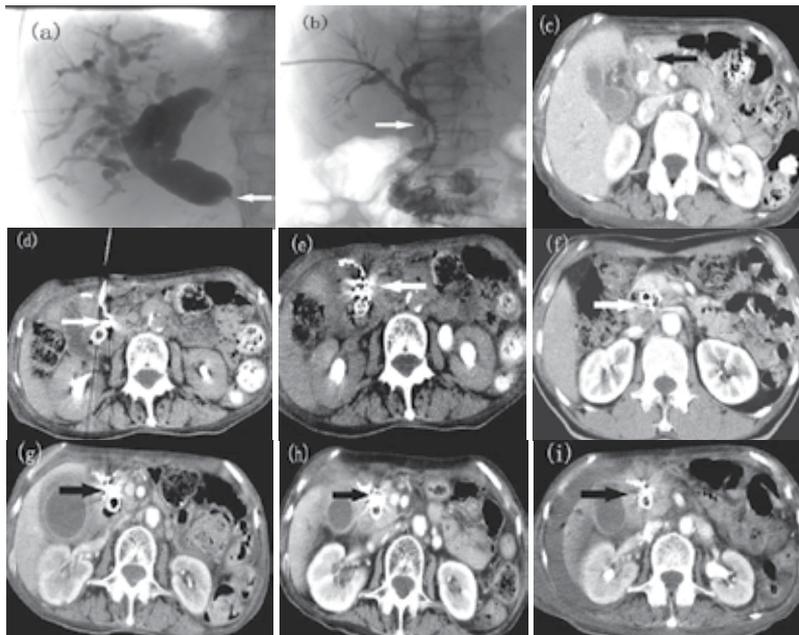


Fig. 3. 80-year-old female patient. Pancreatic carcinoma patient with ^{125}I seeds implant under CT guidance. (a)PTCD shows the lower common bile duct stenosis (*arrow*), while the upper common bile duct and intrahepatic bile ducts shows expansion. (b) Common bile duct stent is implanted (*arrow*). (c) Contrast CT done prior to ^{125}I seeds implantation revealed mass measured around 1.7×1.8cm at head of pancreas (*arrow*), adjacent vessels and important organs were showed clearly on the film. (d-f) Puncture needle was inserted precisely to the tumor through subcutaneous tissue under CT guidance, and ^{125}I seeds were implanted (*arrows*). (g) 2 months follow-up. Repeated contrast CT showed reduced size of mass and aggregation of ^{125}I seeds (*arrow*). (h) 4 months follow-up. Pancreatic mass showed stabilization with repeated contrast CT (*arrow*). (i) 6 months follow-up. Repeated contrast CT showed increased size of mass (*arrow*) and ascites.

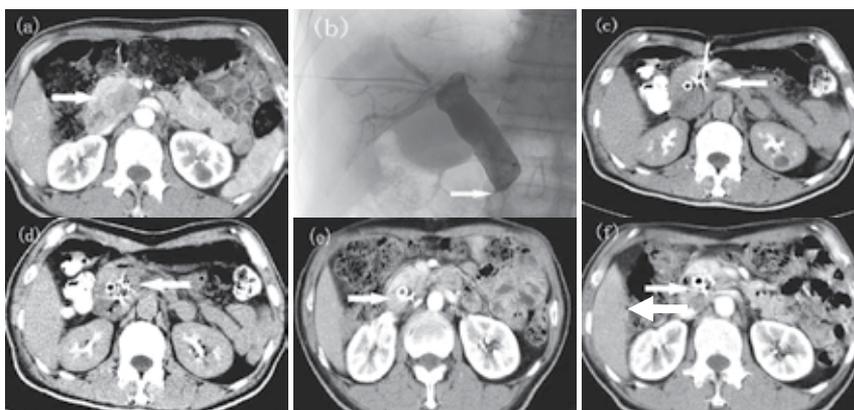


Fig. 4. 69-year-old male patient. Pancreatic carcinoma patient with ^{125}I seeds implant under CT guidance. (a) Contrast CT done prior to ^{125}I seeds implantation revealed mass measured around $2.1 \times 2.6\text{cm}$ at the head of pancreas (*arrow*), adjacent vessels and important organs were showed clearly on the film. (b) PTCD shows the lower common bile duct stenosis (*arrow*), while the upper common bile duct and intrahepatic bile ducts show expansion. (c-d) Puncture needle was inserted precisely to the tumor through subcutaneous tissue under CT guidance, and ^{125}I seeds were implanted (*arrows*). (e) 6 months follow-up. Repeated contrast CT showed reduced size of mass and aggregation of ^{125}I seeds (*arrow*). (f) 12 months post ^{125}I seed implantation, repeated contrast CT showed reduced size of mass and aggregation of ^{125}I seeds, pancreatic mass showed stabilization (*arrow*).

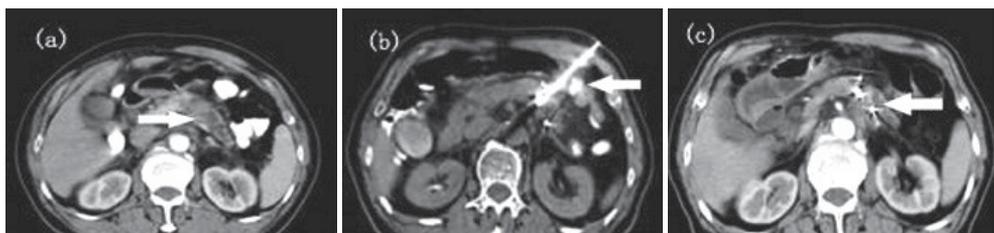


Fig. 5. 76-year-old male patient. Pancreatic carcinoma patient with ^{125}I seeds implant under CT guidance. (a) Contrast CT done prior to ^{125}I seeds implantation revealed mass measured around $3.5 \times 5.5\text{cm}$ at the body and tail of pancreas (*arrow*), adjacent vessels and important organs were showed clearly on the film. (b) Puncture needle was inserted precisely to the tumor through subcutaneous tissue under CT guidance, and ^{125}I seeds was implanted then (*arrow*). (c) 2 months follow-up. Repeated contrast CT showed reduced size of mass and aggregation of ^{125}I seeds (*arrow*).



Fig. 6. 57-year-old female patient. Pancreatic carcinoma patient with ^{125}I seeds implant under CT guidance. (a) Contrast CT done prior to ^{125}I seeds implantation revealed mass measured around $4.5 \times 5.5\text{cm}$ at the tail of pancreas (*arrow*), adjacent vessels and important organs were showed clearly on the film. (b) 12 months follow-up. Repeated contrast CT showed reduced size of mass and aggregation of ^{125}I seeds (*arrow*). (c) 24 months follow-up. Repeated contrast CT showed reduced size of mass and aggregation of ^{125}I seeds (*arrow*). Pancreatic mass showed stabilization.

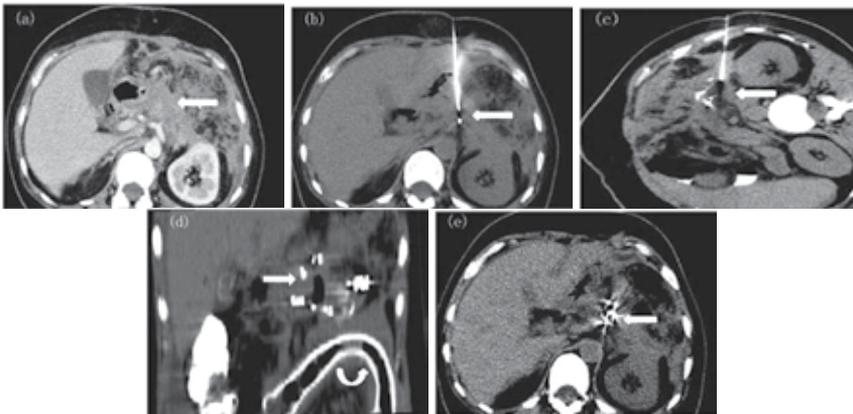


Fig. 7. 56-year-old female patient. Pancreatic carcinoma patient with ^{125}I seeds implant under CT guidance. (a) Contrast CT done prior to ^{125}I seeds implantation revealed mass measured around $4.5 \times 6.5\text{cm}$ at the body and tail of pancreas (*arrow*), adjacent vessels and important organs were showed clearly on the film. (b) Puncture needle was inserted precisely to the tumor through subcutaneous tissue under CT guidance, and ^{125}I seeds was implanted then (*arrow*). (c) Puncture needle reached precisely from prerenal space to the body and tail of pancreas cancer, which as far as possible to avoid the intestinal lumen (*arrow*). (d) CT 2Dimensional reconstruction after ^{125}I seeds implantation was done to determine the distribution of ^{125}I seeds (*arrow*). At the same picture the duodenal stent can be seen (curved arrow). (e) 2 months follow-up. Repeated contrast CT showed reduced size of mass and aggregation of ^{125}I seeds (*arrow*).

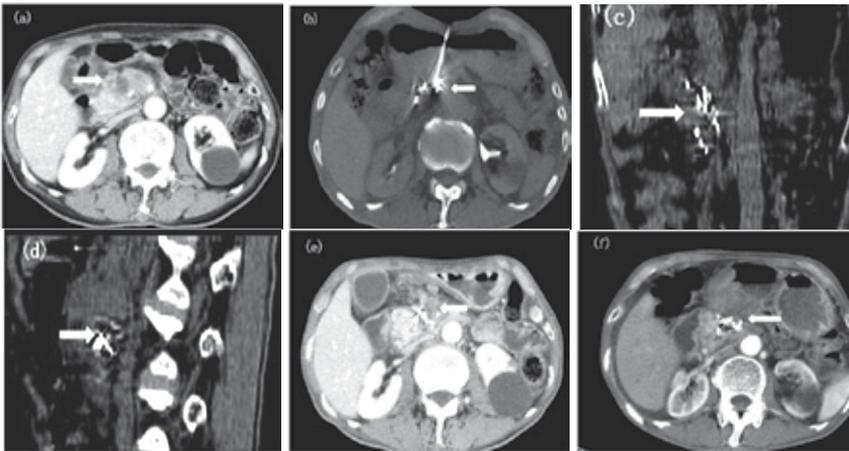


Fig. 8. 78-year-old male patient. Pancreatic carcinoma patient with ^{125}I seeds implant under CT guidance. (a) Contrast CT done prior to ^{125}I seeds implantation revealed mass measured around $3.5 \times 4.5\text{cm}$ at the head of pancreas (*arrow*), adjacent vessels and important organs were showed clearly on the film. (b) Puncture needle was inserted precisely to the tumor through subcutaneous tissue and stomach under CT guidance, and ^{125}I seeds was implanted then (*arrow*). (c-d) CT 2Dimensional reconstruction after ^{125}I implantation was done to determine the distribution of ^{125}I seeds (*arrows*). (e) 2 months follow-up. Repeated contrast CT showed reduced size of mass and aggregation of ^{125}I seeds (*arrow*). (f) 12 months follow-up. Repeated contrast CT showed reduced size of mass and aggregation of ^{125}I seeds, pancreatic mass showed stabilization (*arrow*.)

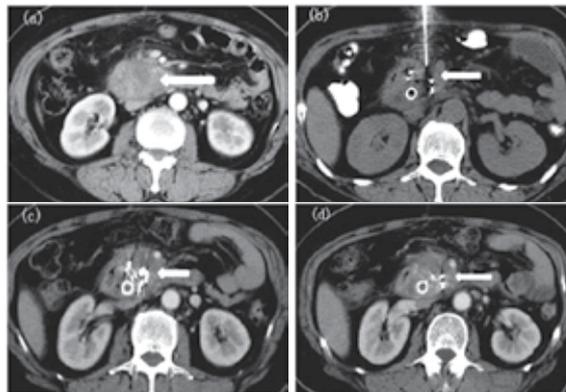


Fig. 9. 56-year-old female patient. Pancreatic carcinoma patient with ^{125}I seeds implant under CT guidance. (a) Preoperative contrast-enhanced CT scan shows a $3.5\text{cm} \times 4.5\text{cm}$ tumor at the head of pancreatic carcinoma (*arrow*). (b) CT scan shows that ^{125}I seeds are implanted into the tumor via 18G implantation needles (*arrow*). (c) CT scan shows the distribution of ^{125}I seeds post implantation (*arrow*). (d) 2 months follow-up. CT scan shows pancreatic tumor partially decreased and ^{125}I seeds gathered together (*arrow*).

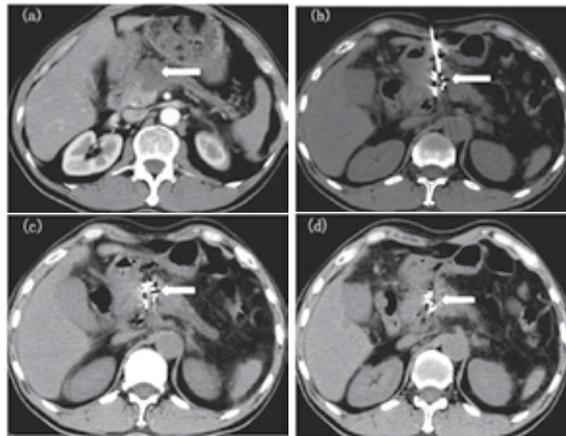


Fig. 10. 70-year-old male patient. Pancreatic carcinoma patient with ^{125}I seeds implant under CT guidance. (a) Preoperative contrast-enhanced CT scan shows a 3.0cm x 3.0cm tumor at the body of pancreatic carcinoma (arrow). (b) CT scan shows that ^{125}I seeds are implanted into the tumor via 18G implantation needles (arrow). (c) CT scan shows the distribution of ^{125}I seeds post implantation (arrow). (d) 2 months follow-up. CT scan shows pancreatic tumor partially decreased and ^{125}I seeds gathered together (arrow).

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V-ATPase Inhibitors in Cancer Treatment and Their Implication in Multidrug Resistance in Oral Squamous Cell Carcinoma

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1. Introduction

ATPases are enzyme systems that originated in a common ancestor and are distributed universally among all organisms. There are three types of ATPases: those found in archaea (A-ATPases), synthases (F-ATPases), and vacuole or vacuolar ATPases (V-ATPases) (Nelson, Nelson 1989). They are essential for life and have in common the fact that they create an electrochemical ion gradient across the membrane to hydrolyze or synthesize ATP. Structurally, they are enzymatic complexes that work as molecular rotary motors. ATPases are formed by two domains, a hydrophobic domain (A0, V0, and F0) and a hydrophilic domain (A1, V1, and F1) connected by a central axis and either one or two lateral axes. In this chapter, we are going to discuss V-ATPases.

1.1 Biological functions

Unlike F-ATPases, whose primary function in eukaryotic cells is to generate ATP from proton motive force, V-ATPases function exclusively as ATP-dependent proton pumps, performing diverse biological functions within cells (Nelson 1992; Kane 1999; Saroussi & Nelson 2008, Stevens & Forgac 1997).

Regarding to the membrane transport, V-ATPases play an important role in receptor-mediated endocytosis (Forgac 1998), intracellular transport, and the acidification of late endosomes (Kane 1999; Stevens & Forgac 1997; Nishi & Forgac 2002; Kawasaki-Nishi & Forgac 2003; Finbow, Harrison 1997). Vacuolar acidification has also been reported to be involved in the transport of lysosomal enzymes from the Golgi apparatus to the lysosomes (Stevens, Forgac 1997; Moriyama, Nelson 1989). V-ATPases appear to play an important role in the creation of the microenvironment needed for correct protein transport, exchange, and secretion (Schoonderwoert et al. 2000).

Although V-ATPases were initially identified in intracellular compartments, knowledge on the roles they play in the plasma membrane has increased enormously. V-ATPases located at the apical membrane of type A intercalated cells are involved in the secretion of protons in renal fluid (Smith et al. 2005; van Hille et al. 1993). Type B intercalated cells, whose function is to secrete bicarbonate, also contain V-ATPases, but they are located between the apical and basolateral membranes (Nishi & Forgac 2002, van Hille et al. 1993). In macrophages and neutrophils, plasma membrane V-ATPases (pmV-ATPases) are involved

in the homeostasis of cytoplasmic pH (Stevens & Forgac 1997, Nishi & Forgac 2002, Nanda et al. 1996). These ATPases also play an important role in bone reabsorption (Marshansky, Futai, Stevens, Forgac 1997, Nishi, Forgac 2002, Smith et al. 2005, van Hille et al. 1993). Another of their functions is to regulate sperm motility and maturation on the apical membrane of epididymal cells and vas deferens by stabilizing the sperm medium (Nishi, Forgac 2002). The role of V-ATPases in cancer cells will be discussed in a specific place. Other additional functions of V-ATPases involves the low pH maintained by them in lysosomes and phagosomes, which is necessary for the activity of the degradative enzymes in these compartments (Sun-Wada, Wada & Futai 2003, Sun-Wada, Wada & Futai 2004, Kurashima et al. 1996) and the transport of small molecules and ions (Nishi, Forgac 2002, Kurashima et al. 1996). The driving force necessary for the accumulation of neurotransmitters in synaptic vesicles is proton motive force, which is generated by V-ATPases (Nelson, Harvey 1999). The fusion-fission balance of the vacuolar system of eukaryotic cells is also controlled by V-ATPases, i.e. via the interaction between vacuolar SNARE proteins and GTPase Vps1p (Baars et al. 2007, Muller et al. 2003). Exocytosis in eosinophils and binding to actin cytoskeleton is also regulated by V-ATPases (Kurashima et al. 1996). The association between V-ATPase subunits and other cellular proteins, for example, that which occurs between the C subunit of the V₀ domain and the E5 oncoprotein, or between platelet-derived growth factor (PDGF) and b1 integrin, indicate that these subunits play a role in cell growth and transformation. V-ATPases also allow the entry of certain viruses (e.g. influenza) and toxins (e.g. diphtheria) into the intracellular space via the binding of these pathogens to the endosomal membrane (Stevens, Forgac 1997). In the case of the human immunodeficiency virus (HIV), the association between the V-ATPase H subunit and the HIV-1 Nef protein, which controls the expression of CD4 (the main HIV receptor), facilitates endocytosis of Nef and/or alterations in the acidification of the endosomal pathway by this protein (Nishi, Forgac 2002)(Marshansky, Futai). The most recent function attributed to V-ATPases is their involvement in the regulation of cell-cell fusion to form larger cells, as is the case with osteoclasts and macrophages (Wada et al. 2008).

1.2 V-atpase structure

The V-ATPase proton pump has multiple subunits, each with multiple isoforms, hence the need for a clear, standardized nomenclature system. Initially, the HUGO Gene Nomenclature Committee agreed to use the ATP as the stem, or root, symbol. ATP6, for example, indicated ATPase, H⁺ transport, lysosomal (vacuolar proton pump). In 2003, the nomenclature system for genes encoding V-ATPase subunits was revised and it was decided to maintain the root ATP6 and add the domain to which a particular subunit belonged, followed by the letter of the subunit, and finally the number of the isoform, where relevant, (e.g. *ATP6V1C1*, *ATP6V1E*, etc.) (Smith A.N. et al. 2003).

V-ATPase structure, function, biogenesis, and regulation was widely revised by Stevens and Forgac (Stevens & Forgac 1997). We will use the nomenclature system proposed by these authors to explain the structural subunits of V-ATPase together with relevant modifications based on recent research using transmission electron microscopy (Wilkens, Zhang & Zheng 2005).

V-ATPases have been found to be practically identical in terms of the composition of their subunits in all eukaryotic cells. They have two distinct structures: a peripheral catalytic

sector (V1) and a hydrophobic membrane sector (V0) responsible for driving protons (Gruber 2005). The catalytic sector is composed of five polypeptides known as subunits A, B, C, D, and E, with a molecular weight, in decreasing order, ranging from 72 to 33 kDa. Recent advances in knowledge of the mechanism of action of F-ATPases have clarified the relationship between function and structure for each of the subunits of these enzymes (Qi, Wang & Forgac 2007, Inoue et al. 2005) (Figure 1).

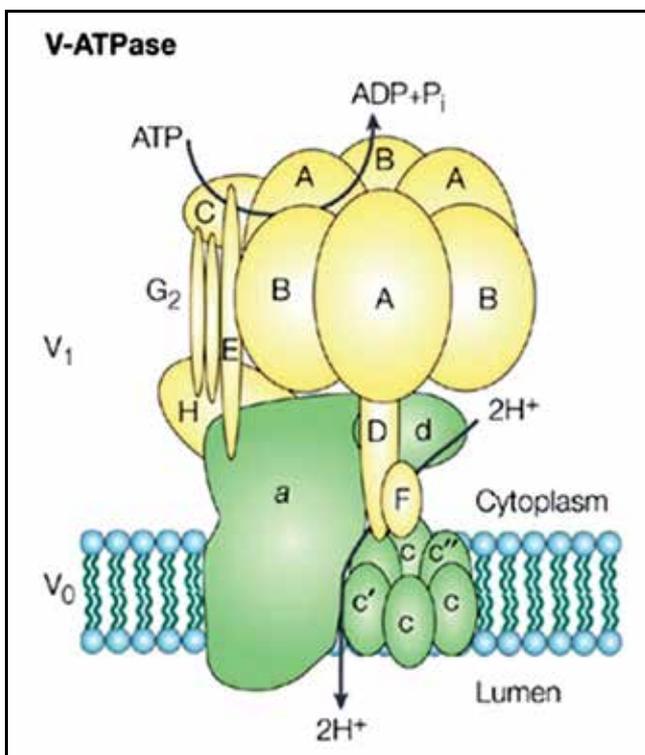


Fig. 1. Diagram of V-ATPase. The cytosolic domain (in yellow) is formed by three A subunits, three B subunits, three G subunits, and one C, D, E, F, and H subunit. The V0 transmembrane domain is formed by five subunits: a, c, c', c'' and d. The V1 domain contains the catalytic unit (Nishi & Forgac 2002).

1.3 V-ATPase regulation

Three major regulatory mechanisms have been described for V-ATPase: 1) the regulation of pump density, which allows different cells to maintain their cytoplasmic and vacuolar pH stable; 2) the regulation of V1 and V0 domain association/dissociation, for example, a decrease in glucose levels can cause a 70% dissociation of the V1 domains of the membrane; and 3) the regulation of secretory activity, via the maintenance of balance in the formation of bisulfite and binding efficiency between H⁺ and the pump. Other mechanisms include the necessary modifications in the membrane potential for the generation of electrogenic force (Forgac 1998; Peng, Stone & Xie 1993) and alterations in the vacuolar transporter chaperone (Vtc) complex, which affect the conformation of the V0 domain and its function in vacuole fusion of the membrane (Muller et al. 2003).

2. V-ATPase inhibitors

Scientific evidence suggests that the acidic tumor microenvironment is key to managing cancer progression and metastasis. In particular, V-ATPases play a major role in metastasis tumor development because many tumor cells secrete lysosomal enzymes that participate in the extracellular matrix degradation necessary for metastatic invasion. These enzymes are most active at low optimal pH; moreover, V-ATPases are responsible for microenvironment acidification (Nishi, Forgac 2002, Martinez-Zaguilan et al. 1993). Among the many mechanisms that regulate the tumor microenvironment, V-ATPases are especially significant because they can be inhibited by proton pump inhibitors. (Fais et al. 2007).

2.1 Classes of V-ATPase inhibitors

Initial attempts to block V-ATPases were made after bafilomycin and concanamycin were discovered in 1988 (Bowman, Siebers & Altendorf 1988). New molecules capable of inhibiting V-ATPase to a greater or lesser extent via different mechanisms of action were later discovered. Such molecules include benzolactone enamides salicylilalamide (Erickson et al. 1997), lobatamide A and B (Galinis et al. 1997), apicularen (Kunze B., Janse R., Sasse F., Höfle G. and Reichenbach H. 1998), indolyls (Gagliardi et al. 1998, Nadler et al. 1998), oximidine (Kim et al. 1999), macrolactone archazolid (Sasse et al. 2003), lobatamide C (Shen et al. 2003), and cruentaren (Kunze et al. 2006). The latest generation of inhibitors include NiK12192 (Saroussi, Nelson 2008, Petrangolini et al. 2006), FR202126 (Niikura 2007), and PPI SB 242784 (Hesslink et al. 2008). We can see the differences and similarities of V-ATPase inhibitors in Table 1:

The V-ATPase inhibitors studied most thoroughly and used most often are macrolide antibiotics with 18-membered lactone rings, namely, bafilomycins and concanamycins. Bafilomycin and concanamycin are commercially available, and various laboratories have developed in vitro synthesis processes for experimental purposes (Scheidt et al. 2002). The remaining V-ATPase inhibitors are still in experimental phase, due to possible side effects that may occur in humans. However, PPIs are the treatment of choice for peptic diseases such as gastroesophageal reflux (Larsson et al. 1985). While these pumps block the secretion of gastric acid, they also directly inhibit V-ATPase activity. Examples of PPIs include omeprazole, esomeprazole, lansoprazole, pantoprazole, and rabeprazole (Horn 2000), all of which accumulate in acidic compartments (De Milito, Fais 2005a). PPI treatment has been associated with V-ATPase activity inhibition and an increase in both extracellular pH and pH in lysosomal organelles. In vivo experiments using mice/human xenografts have shown that pretreatment with PPIs can sensitize solid human tumors to chemotherapy drugs (De Milito, Fais 2005a).

Treatment with PPIs has also been found to sensitize tumor cells to cisplatin, 5-fluoracil, and vinblastine through changes in cellular pH gradients, with retention of the drugs in the cytoplasm, and in the nucleus in the case of doxorubicin (De Milito, Fais 2005a, Luciani et al. 2004, Luciani et al. 2004, Cianfriglia et al. 1990).

It is also known that low pH levels are suitable for the complete activation of PPIs (De Milito et al. 2007), suggesting that tumor alkalization may be an extremely interesting target for future anticancer treatments (De Milito, Fais 2005a, Luciani et al. 2004, De Milito, Fais 2005b). Specific V-ATPase inhibitors such as concanamycin and bafilomycins are other candidates for investigation, not only to treat cancer but also to reduce MDR in tumors (Perez-Sayans et al. 2009, Sasazawa et al. 2009).

CLASSES OF V-ATPase INHIBITORS					
		Chemistry	Provenience	Binding site	Action
Plecomacrolide	Concanamycin & Bafilomycin	Macrolide antibiotics with 18-membered lactone rings	<i>Streptomyces</i>	Unknown	V-ATPases inhibition Ionophoric properties
	Salicylihamide A	Macrocyclic salicylate	Sponge <i>Haliclona</i> sp.	VO complex	Animal V-ATPases inhibition Cytotoxin
Benzolactone enamides	Apicularens	Lactone ring	<i>Chondromyces</i>	VO complex	Highly toxic for human and animal cell
	Lobatamides	Substitution of enamide NH, salicylate, and phenyl salicylate	<i>Tunicate Aplidium lobatum</i>	VO complex	Animal and mammalian V-ATPases inhibition
	Oximidines	Lactone ring	<i>Pseudomonas</i> sp.	VO complex	Animal and mammalian V-ATPases inhibition
	Cruentaren	Lactone ring	<i>Byssovorax cruenta</i>	VO complex	Cytotoxicity on mammalian and fungal cells at mitochondrial F-ATPases
	Archazolid	Macrocyclic lactone ring with a thiazole side	<i>Archangium gephyra</i> <i>Cystobacter violaceus</i>	VO subunit c	Cytotoxicity on mammalian cell line
	Indolyls	Bafilomycin-based	Synthesis	VO subunit c	V-ATPase inhibitor
Late-generation V-ATPase inhibitors	NiK12192, SB 242784, FR202126, 3-bromopyruvate (3-Br PA), Tributyltin chloride (TBTCI), FR177995, FR167356				

Table 1. Classes of V-ATPase inhibitors

3. Role of v-ATPases inhibitors in cancer

3.1 Tumor metastasis

The development and maintenance of the proton gradient present in tumors is due directly to the ability of tumor cells to secrete protons (H^+) (Martinez-Zaguilan et al. 1993, McLean et al. 2000), acidify the extracellular medium (Cardone, Casavola & Reshkin 2005, Perona, Serrano 1988), and keep the cytosolic pH alkaline (Sennoune, Martinez-Zaguilan 2007). This ability also

increases with tumor aggressiveness (Montcourrier et al. 1997, Parkins et al. 1997). In addition, low pH may cause extracellular matrix (ECM) degradation and remodeling through activation of proteolytic enzymes which contribute to invasion and cancer metastasis (Martinez-Zaguilan et al. 1996, Rofstad et al. 2006). Proteases need low extracellular pH to optimize their activation, including metalloproteinases (MMP), morphogenetic bone metalloproteinases (protein type 1), tissue serine proteases, and adamalysin-related proteinases. Among them, MMPs are the proteases basically involved in degradation and remodeling of all extracellular matrix (ECM) structural components (Montcourrier et al. 1994, Rozhin et al. 1994, Johnson et al. 2000, Kato et al. 2005, Gocheva, Joyce 2007).

Sennoune et al. assessed the effect of bafilomycin A1 in breast tumor cells and found that cytoplasmic pH recovery was inhibited in response to acid load, in both highly and lowly metastatic cells, although to a greater extent in highly metastatic cells (Sennoune et al. 2004). This suggests that V-ATPases in the plasma membrane are involved in the acquisition of a more metastatic phenotype and that the use of V-ATPase inhibitors allows distant metastasis to be minimized (Figure 2).

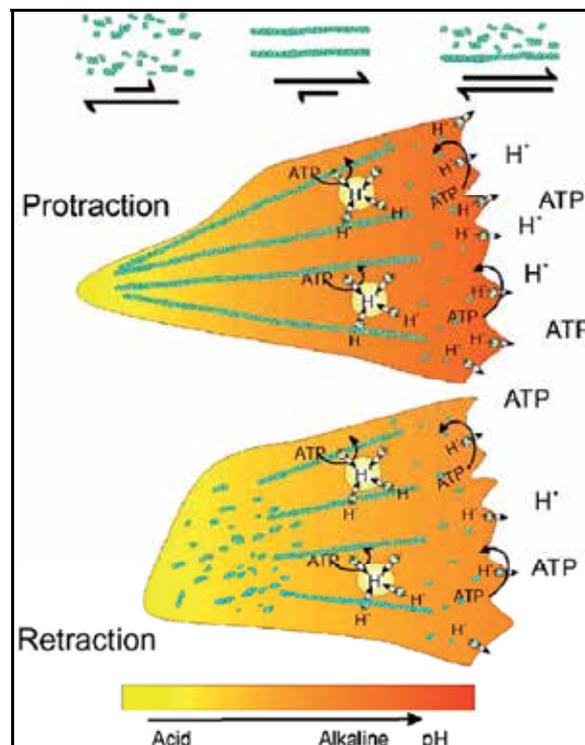


Fig. 2. Proposed mechanism by which overexpression of pmVATPase at the leading edge of the cell modulates cell migration/invasion. The proposed model should be viewed as a framework to explain how pmV-ATPases determine the acquisition of an invasive phenotype needed for angiogenesis and metastasis. Changes in pH_{cyt} are critical for establishing cell polarity needed for cell movement. A critical step in directed motility and migration is the asymmetric actin polymerization at the leading edge (Sennoune, Martinez-Zaguilan 2007).

Using RNA interference techniques, Lu et al. found that distant metastasis could be delayed and suppressed in human hepatocellular carcinoma *in vitro* by reducing proton extrusion and gelatinase activity through the inhibition of V-ATPase subunit c (ATP6L) (Lu et al. 2005). This fact is consistent with subunit c block by bafilomycin and concanamycin, as this is their main binding site to V-ATPase (Bowman et al. 2004). In tyrosinase-positive amelanotic melanoma cells, inactive tyrosinase accumulates in the endoplasmic reticulum because the presence of aberrant V-ATPases blocks trafficking through secretory pathways. The use of V-ATPase inhibitors, such as bafilomycin A1 or concanamycin A, improves transport, demonstrating the involvement of this enzyme and preventing conditions that favor metastatic dissemination (Halaban et al. 2002).

Hence, both *in vitro* or *in vivo*, V-ATPases are a target for anticancer therapeutic agents, either directly by regulating the pH gradient in the tumor environment or indirectly by preventing ECM protease activation (Fais et al. 2007).

3.2 Tumor cell growth and survival

V-ATPases may also play a significant role in tumor cell survival by regulating pH and preventing apoptosis. As previously reported, plasma membrane V-ATPases help regulate cytosolic pH in macrophages and neutrophils (Nanda et al. 1996). This mechanism may also be used by tumor cells, which produce more H⁺ due to high glycolytic activity (Gatenby, Gillies 2004). Treatment with V-ATPase inhibitors lowers H⁺ extrusion, both *in vitro* and *in vivo* (Volk, Albert & Kempinski 1998, McSheehy et al. 2003).

Bafilomycin A1 was assessed as a potential anticancer agent because it inhibits cell proliferation and tumor growth. Although this effect has been attributed to the inhibition of intracellular acidosis by blocking V-ATPases, the precise mechanism remains unknown (Bowman et al. 2004). A study conducted by Lim et al., hypothesized that bafilomycin A1 and its analogue, concanamycin A, stimulate a tumor growth factor, hypoxia-inducible 1 α (HIF-1 α) (Zhong et al. 1999). The interaction of bafilomycin with HIF-1 α increases with hypoxia, causing strong induction of the p21 gene which, in turn, leads to cell cycle detection in cancer cells (Lim et al. 2006).

V-ATPase inhibition has also been shown to trigger apoptosis through caspase-dependent and caspase-independent mechanisms (De Milito et al. 2007, Aiko et al. 2002), and bafilomycin and concanamycin induce apoptosis in other types of cells, including neutrophils (Gottlieb et al. 1995) and osteoclasts (Xu et al. 2003).

Morimura et al. described the growth-inhibiting effect of apoptosis stimulation in human hepatoblastomas using bafilomycin A1. In particular, electron microscopy, morphological observations, and flow cytometry showed higher apoptotic cell ratios and diminished cell reproduction. Cell growth inhibition in normal liver cells was insignificant (Morimura et al. 2008).

In the case of human gastric cancer cells, Nakashima et al. investigated the mechanism of apoptosis induced by bafilomycin A1. Bafilomycin inhibits the growth of MKN-1 cancer cells through apoptosis. Flow cytometry was used to measure alterations in lysosomal pH, which increased in the presence of bafilomycin. Caspase-3 activity was also increased by bafilomycin; such findings suggest that bafilomycin A1 induces apoptosis in MKN-1 cells mediated by proteases released after lysosomal dysfunction, followed by caspase-3 activation of the cytochrome c-independent manner (Nakashima et al. 2003, Hishita et al. 2001).

A study conducted by Wu et al. has shown that bafilomycin A1 suppresses macroautophagy by preventing lysosome acidification (Wu et al. 2009). Macroautophagy is a protein

degradation pathway that allows increased cell survival under stress and in cancer (Meijer, Codogno 2004, Mortimore, Hutson & Surmacz 1983). Macroautophagic inhibition in HT-29, HCT-116, and SW1116 colon cancer cells is accompanied by down-regulation of cyclin D and E and up-regulation of p21^{Cip1} and various caspases, causing an antiproliferative effect (Wu et al. 2009).

ancer cells are more likely to express V-ATPase than normal cells, causing abnormalities in the acidic microenvironment and affecting cancer cell growth and infiltration significantly (Saroussi, Nelson 2008, Cardone, Casavola & Reshkin 2005, Perona, Serrano 1988). Moreover, neoplastic cells are more sensitive to bafilomycin A1 than normal cells, a fact that may be used in anticancer therapy (Ohta et al. 1996).

3.3 Chemoresistance

Resistance to chemotherapy agents is the main reason for treatment failure in patients with cancer, and multidrug resistance (MDR) occurs in many types of tumors. The main mechanism that gives rise to the MDR phenotype is the overexpression of drug efflux transporters such as the P glycoprotein (Pgp) in the plasma membrane (Nielsen, Skovsgaard 1992) (Figure 3).

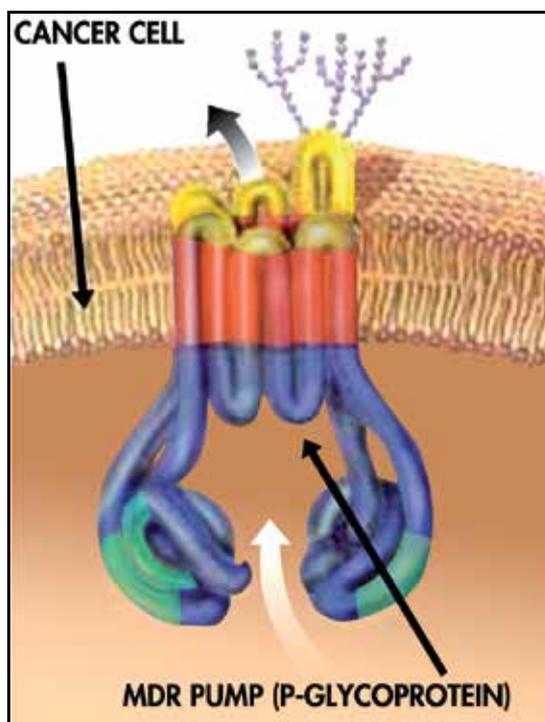


Fig. 3. Pgp resides in the cell membrane, where it may act to pump toxins out of the cell. The painting shows a model of the protein's structure that is based on its known sequence of amino acids. The protein chain is thought to snake back and forth 12 times across the lipid bilayer of the membrane forming a 12-sided pore. The pan of the protein outside the cell bears sugar chains (purple); two large and nearly identical domains protrude into the cell. They include regions (green) that bind the cellular energy-carrying compound ATP, which probably provides the energy that drives the efflux (arrows) (Kartner, Ling 1989).

Extracellular pH is considerably more acidic in oral squamous cell carcinoma (OSCC), a solid tumor, than in normal tissue. This increased acidity interferes with the absorption of standard chemotherapy drugs, reducing their effect on tumors (Griffiths 1991, Negendank 1992). Vacuolar ATPases (V-ATPases) have been reported to be largely responsible for this acidic environment (Newell et al. 1993, Yamagata et al. 1998). While a clear association has been established between MDR and Pgp expression in some tumors, the mechanism by which drug resistance occurs within the multistep process of OSCC has not yet been fully elucidated (Tanigawara 2000). OSCC is highly resistant to a wide range of structurally different drugs with diverse cytotoxic mechanisms of action (McLeod, Evans 1999). This suggests that OSCC may be intrinsically chemoresistant and it is possible that V-ATPases play a key role in this resistance (Perez-Sayans et al. 2009).

3.3.1 Multidrug resistance in OSCC

As already mentioned, while a clear association has been established between MDR and Pgp expression in certain tumors, the mechanism by which drug resistance occurs within the multistep process of OSCC has not yet been fully elucidated (Ling 1997). Several genes have been implicated in MDR, including *MDR1*, *MRP* (multidrug resistance-associated protein), *GST-π*, and *DNA* topoisomerase II.

Pgp is encoded by *MDR1* and flow cytometry studies have shown increased Pgp levels in recurrent OSCCs compared to normal mucosa with oral lesions at different stages of tumorigenesis (Jain et al. 1997). These findings were confirmed by immunohistochemical staining in a study that compared recurrent tumors with untreated primary oral tumors (Chomczynski, Sacchi 2006, Xie et al. 2000). The best known *MDR1* gene product is Pgp/P-170, which has been implicated in resistance to chemotherapy agents such as taxanes, anthracyclines, vinca alkaloids, podophyllotoxins, and camptothecins (Juliano, Ling 1976).

The mechanism by which Pgp-mediated MDR is acquired in head and neck tumors, however, is different. Immunohistochemical studies, for example, have revealed high levels of Pgp in salivary gland adenocarcinoma (SGA) cell lines but insignificant levels in OSCC cell lines (Naramoto et al. 2007). Reverse transcription quantitative polymerase chain reaction analysis of *MDR1* expression has also revealed increased Pgp levels in different cell lines treated with vincristine (alkaloid cancer drug). These results suggest that Pgp-induced MDR in OSCC is essentially an acquired phenotype caused by the genetic induction of Pgp (Uematsu et al. 2001).

MRP has been linked to MDR in multidrug-resistant Pgp-negative cells lines in small cell lung cancer, cancer of the stomach, bladder, cervix, and prostate, and leukemia (Kim et al. 1996, Kim et al. 1995, Endo et al. 1996b, Endo et al. 1996a). In head and neck tumors, overexpression of MRP1 mRNA has been found in human and murine OSCC and SGA cell lines mice treated with vincristine. suggesting the theory of Pgp- and MRP-independent MDR in OSCC.

Overexpression of the isozyme *GST-π* is often associated with malignant transformation and/or MDR (Ruzza et al. 2009). *GST-π* is responsible for detoxifying xenobiotics such as carboplatin (used in chemotherapy) and elevated levels of this enzyme cause treatment resistance (Engel et al. 2005, Koshiyama et al. 2001). Whether or not this is also the case in OSCC, however, is a matter of debate. In a study by Chen et al (Chen, Lin 1997), *GST-π* levels increased with increased severity of oral epithelial dysplasia in line with the development of OSCC. The immunohistochemical expression of placental *GST-π* has been

studied in the oral epithelium of premalignant and malignant oral lesions, and has indeed been proposed as a good marker for premalignant lesions and tumors (Zhang, Xiao & Priddy 1994). Another study, however, that analyzed GST- π levels using enzyme-linked immunoassay failed to find a significant relationship between GST- π and TNM stage (Oude Ophuis et al. 1998). Finally, in a study that analyzed GST- π expression using Northern blot analysis and gene amplification with Southern blot analysis, Wang et al (Wang et al. 1997) concluded that the amplification of the *GST- π* gene was not critically related to the overexpression of GST- π mRNA. Furthermore, they found no relationship between GST- π mRNA overexpression and tumor size, neck nodal status, or patient survival.

The downregulation of topoisomerase II—an enzyme that breaks and rejoins double-stranded DNA in the interconversion of different topological forms of DNA—has also been associated with MDR (Deffie, Batra & Goldenberg 1989, Shi et al. 2008).

The mechanisms underlying MDR response are less clear in OSCC than in other types of tumors (Yajima et al. 2009). MDR holds challenges for both researchers and the pharmaceutical industry. Accordingly, efforts are being made on all sides to find anticancer compounds characterized not only by high tolerability and oral bioavailability but also by the ability to overcome the problem of drug resistance. OSCC is highly resistant to a wide range of structurally different drugs with different cytotoxic mechanisms of action (McLeod, Evans 1999). This suggests that OSCC may be intrinsically chemoresistant, with other molecules, including V-ATPases, playing an important role (Perez-Sayans et al. 2009).

3.3.2 The role of v-ATPases in multidrug resistance

It has been demonstrated that hypoxia and acidity contribute to the transition from benign to malignant growth via the selection of tumor cells capable of surviving in an acidic, oxygen-deprived environment. Acidity, for example, has been associated with chemotherapy resistance (Raghunand et al. 2001), proliferation (Morita et al. 1992), and metastatic behavior (Martinez-Zaguilan et al. 1996). Indeed, alteration of the pH gradient between the extracellular environment and the cell cytoplasm has been suggested as a possible mechanism of resistance to cytotoxic drugs (De Milito, Fais 2005a) (Figure 4).

The alteration of cytosolic pH also plays an important role in drug resistance in chemotherapy. Extracellular pH in solid tumors is significantly more acidic than in normal tissue. This increased acidity interferes with the absorption of basic chemotherapy drugs, reducing their effect on tumors (Griffiths 1991, Negendank 1992). Martínez-Zaguilán et al., found that unlike chemoresistant cells, chemosensitive cells did not recover from acid load (Martínez-Zaguilán et al. 1999). Becelli et al (Becelli et al. 2007) found that reversed pH gradient was directly related to drug resistance.

Anaerobic metabolism is an important determinant of tumor acidity that allows the selection of cells capable of surviving in a hypoxic-anaerobic environment via the synthesis of lactate. A complex system appears to regulate pH homeostasis in mammal cells, and it seems as if malignant tumor cells are capable of exploiting some of these mechanisms to protect themselves from the acidic environment, while maintaining levels of acidity that are poorly tolerated by normal or more differentiated cells (De Milito, Fais 2005b).

Recent studies have suggested that V-ATPases, which secrete protons through the plasma membrane, may play a key role in the acidification of the tumor environment (Perez-Sayans et al. 2009). Several human tumor cells are characterized by increased V-ATPase expression and activity (Perez-Sayans et al. 2010), and pretreatment with proton pump inhibitors (PPIs)

has been found to sensitize tumor cell lines to the effect of different chemotherapy drugs (De Milito, Fais 2005a, Luciani et al. 2004, De Milito, Fais 2005b, Sennoune, Luo & Martinez-Zaguilan 2004). The effectiveness of the drug transport mechanism appears to be comparable to that of drug efflux pumps such as Pgp, although vesicle acid exchange (above all in vesicles that have an active H⁺/cation exchange system) may be an important factor in drug resistance, and particularly in cells that do not overexpress Pgp-type efflux pumps in the plasma membrane (Raghunand et al. 1999).

Murakami et al (Murakami et al. 2001) found cisplatin-resistant tumors to contain elevated levels of all V-ATPase subunits but levels of ATP6C were particularly. They also found significantly higher levels of cellular pH in cisplatin-resistant tumor cells than in cells sensitive to vincristine and etoposide. In a later study, however, Zhang et al (Zhang et al. 2006) identified 38 overexpressed genes and 25 underexpressed genes in cisplatin-resistant OSCC. Torigoe et al (Torigoe et al. 2002) showed that treatment with anticancer agents increased ATP6L (c subunit). Interfering RNA targeting the c subunit has also been found to improve drug resistance in breast cancer cells (You et al. 2009).

4. Conclusions

The above findings suggest that the induced expression of V-ATPases in MDR is an anti-apoptotic defence and that the combined use of PPIs or V-ATPase inhibitors and low chemotherapy doses might be a possible treatment target (Torigoe et al. 2002). We believe that the future of these molecules in cancer treatment involves measuring the overexpression of specific V-ATPase subunits in tumors to be treated and then using inhibitors specific for the subunits being expressed (Perez-Sayans et al. 2009, Perez-Sayans et al. 2010). This will allow clinicians to provide more specific treatment, while also minimizing adverse effects.

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Farnesyltransferase Inhibitor in Cancer Treatment

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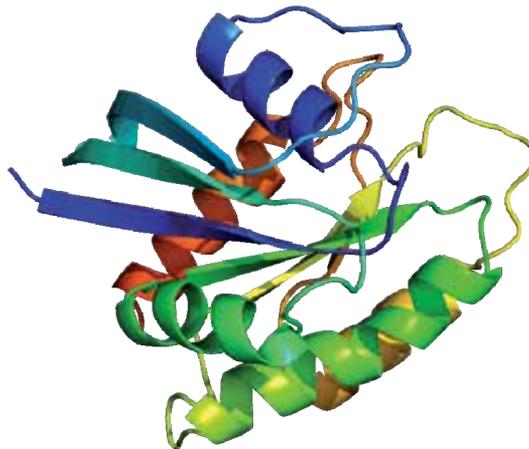
1. Introduction

Cancer is a class of diseases characterized by uncontrolled growth of abnormal cells anywhere in the body and the ability of these cells to invade other locations in the body, either by direct growth into adjacent tissue or by migration of cells to distant sites. This unregulated growth is caused by damage to DNA, resulting in mutations to genes that encode proteins controlling cell division. To prevent this unregulated growth various anticancer drug (Chen et al., 2011; Kim & Dass 2011) have been developed. But these drugs have severe toxicity and are not well tolerated in the patient. Therefore, the major goal in anticancer drug discovery process is to discover and develop innovative therapies that exhibit a real improvement in effectiveness and/or tolerability. In cancer therapy, continuous effort has been made to explore the new targets. Cancer research is largely focused on prospective targets identified by basic science such as the oncogenic signal transduction pathway, oncogenes, tumor suppressor genes, and genes involved in the regulation of the cell cycle and apoptosis or programmed cell death (Gridelli et al., 2003; Hochhaus et al., 2004; Lau et al., 2011; Minna et al., 2004). Proteins mediating their effects are obvious targets for cancer therapy because, by definition, these proteins are involved in the primary transformation of normal cells. Proteins that transmit abnormal growth signals offer enticing points of intervention for the treatment of cancer. One potential target is the Ras family of proteins, which are mutationally activated in a wide range of human tumor types and are important contributors to the neoplastic phenotype (Barbacid et al., 1987; Biagi et al., 2010; Bollag et al., 1991; Bos et al., 1989).

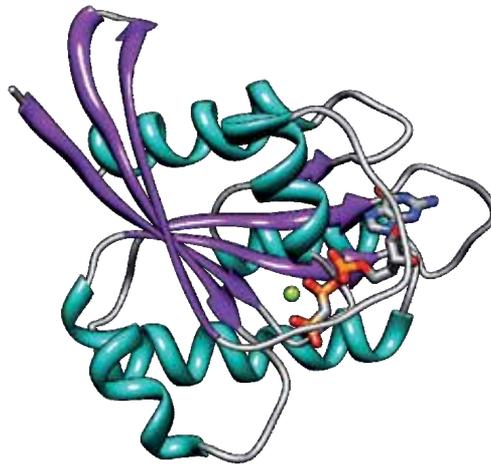
2. Ras protein

Ras proteins have been the subject of intense research investigation by the biomedical research community since 1982 (James et al., 1996). Ras is the name of a protein, the gene that encodes it, and the family and superfamily of proteins to which it belongs. Ras proteins are guanine nucleotide-binding proteins that play pivotal roles in the control of normal and transformed cell growth. The Ras superfamily includes the Ras, Rho, and Rab families. There are three Ras proto-oncogenes: the *H-ras* gene (Harvey murine sarcoma viral

oncogene homolog, Fig. 1), the *K-ras* gene (Kirsten murine sarcoma viral oncogene homolog), and the *N-ras* gene (neuroblastoma oncogene homolog) (Boguski et al., 1993; Ellis et al., 1981; Marcos et al., 2003; Ruta et al., 1986; Shimizu et al., 1983). The *ras* oncogenes encode four low molecular weight (21 kDa) proteins, Ras (H-Ras, N-Ras, and K-Ras4A and K-Ras4B, resulting from two alternatively spliced *K-ras* gene products) (Morgillo et al., 2007), that, in normal untransformed cells, cycle between an inactive guanosine 5'-diphosphate (GDP)-bound state and active guanosine 5'-triphosphate (GTP)-bound state at the inner surface of the plasma membrane in mammalian cells.



(a)



(b)

Fig. 1. a. Structure of the HRAS protein (Elaine 2009), b. Ribbon diagram of H-ras (Elaine 2010).

The highly conserved nature of the variable region across mammalian species indicates that Ras proteins serve specific functions. They are very important molecular switches for a wide

variety of signal pathways that control such processes as cytoskeletal integrity, proliferation, cell adhesion, apoptosis, and cell migration (Zhao et al., 2011). The final four amino acids play an important role in specifying subcellular localization of the Ras protein. All Ras proteins have a specific amino acid sequence motif at the carboxyl (C) terminus, commonly referred to as the CAAX sequence (C, cysteine; A, aliphatic amino acid; X, any amino acid usually methionine or serine) which signals for posttranslational modifications (Cadinanos et al., 2003; Epifano et al., 2007; Roberts et al., 2008; Rowinsky et al., 2006).

Ras is a G protein and functions as a molecular switch cycling between GTP-bound "on" and GDP-bound "off" states (Seki et al., 1996). It is activated by guanine exchange factors which are themselves activated by mitogenic signals and through feedback from Ras itself. It is inactivated by GTPase-activating protein, which increases the rate of GTP hydrolysis, returning Ras to its GDP-bound form, simultaneously releasing an inorganic phosphate. Ras is synthesized in the cytoplasm as a biologically inactive cytosolic propeptide (Pro-Ras) and undergoes a series of closely linked posttranslational modifications by the covalent addition of a non-polar farnesyl group to the COOH-terminal, thereby increasing its hydrophobicity (Kyathanahalli & Kowluru, 2011). The C-termini triplet of amino acids is cleaved off, leaving a farnesylated, methylated cysteine residue at the carboxyterminus. Ras is then localized to the inner surface of the plasma membranes (Gibbs et al., 1993; Hancock et al., 1989, 1990; Jackson et al., 1990; Salaun et al., 1999), in which Ras cycles from an inactive GDP-bound state to an active GTP-bound state. Once in its GTP-bound form, Ras activates several downstream effector pathways that mediate increased gene transcription and rapid cell proliferation (Fig. 2). The most critical step, farnesylation, adds a 15-carbon farnesyl isoprenoid group to H-, K-, and N-Ras through a thioether bond and is catalyzed by Farnesyl transferase (FTase) (Kho et al., 2004; Ljuca et al., 2011).

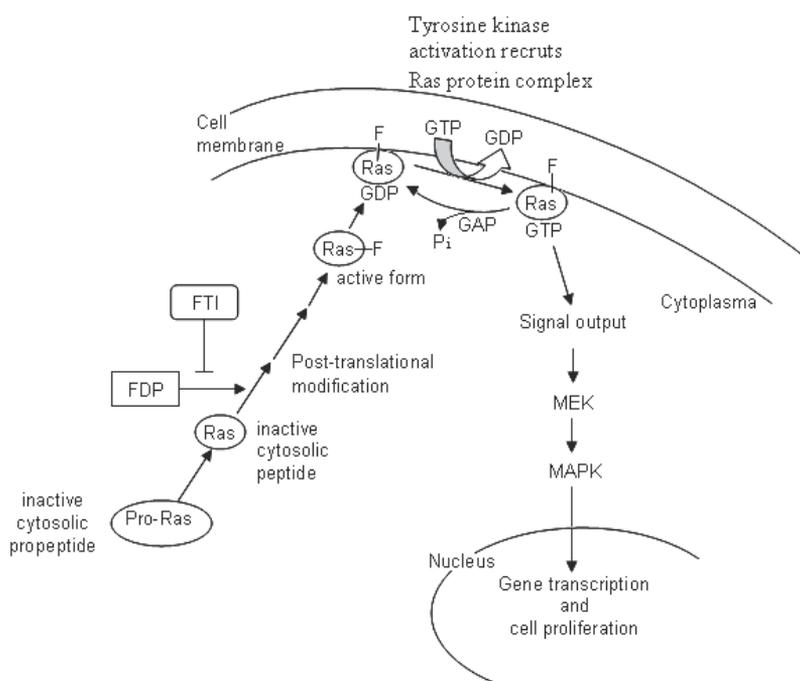


Fig. 2. Ras-dependent signal transduction with Farnesyltransferase inhibitor (FTI) target.

3. Mutations of Ras in human cancers

Ras is mutated to an oncogenic form in cancer, so the Ras and Ras-related proteins are often deregulated, leading to increased invasion and metastasis, and decreased apoptosis. In part of the human tumors, one of the three *ras* genes harbored a point mutation, they result in a permanently active GTP-bound form of Ras (Le Moulec et al., 2009; Lowry & Willumsen et al., 1993).

Mutant Ras proteins transform cells because they continuously activate the downstream effector pathways, including those involved in cell proliferation, in the absence of any upstream growth factor stimulation. Mutations of *ras* occur in approximately 30% of all human cancers, including a significant proportion of pancreatic and colorectal carcinomas (Clark et al., 1995; Khosravi-Far et al., 1994; Shimoyama, 2011; Widemann et al. 2006). With regard to the three *ras* genes, mutation of K-*ras* is most commonly found in human tumors, whereas N-*ras* mutations are encountered less often and H-*ras* mutations rarely. The type of *ras* mutation seems to correlate with tumor type. Although activating *ras* mutations are mainly involved with myeloid malignancies and carcinomas of the breast, colon, pancreas, lung, and thyroid, they have also been detected in many other types of cancer (Beaupre et al., 1999; Zheng et al., 2010).

4. Post-translational modification of Ras

Ras proteins are tethered to the inner face of the membrane by posttranslational modifications that make them more hydrophobic (Ageberg et al., 2011), which involve prenylation (addition of a lipid moiety) of the protein. After its synthesis as cytoplasmic Pro-Ras, Ras is sequentially modified by farnesylation of the cysteine residue, proteolytic cleavage of the AAX peptide by proteases, and carboxymethylation of the new C-terminal carboxylate by carboxymethyl transferase. As the first step in this sequence, farnesylation is the most critical part of the process (Casey et al., 1989; Cox & Der, 1997; Gibbs & Oliff, 1997; Gelb et al., 1997; Kato et al., 1992; McCormick et al., 1993; Omer et al., 1997; Schafer et al., 1989; Yamane et al., 1990), in which a 15-carbon farnesyl isoprenoid group is transferred from farnesyl diphosphate (FDP) to form a thioether bond with the cysteine moiety in the C terminal tetrapeptide sequence of the Ras protein (Fig. 3).

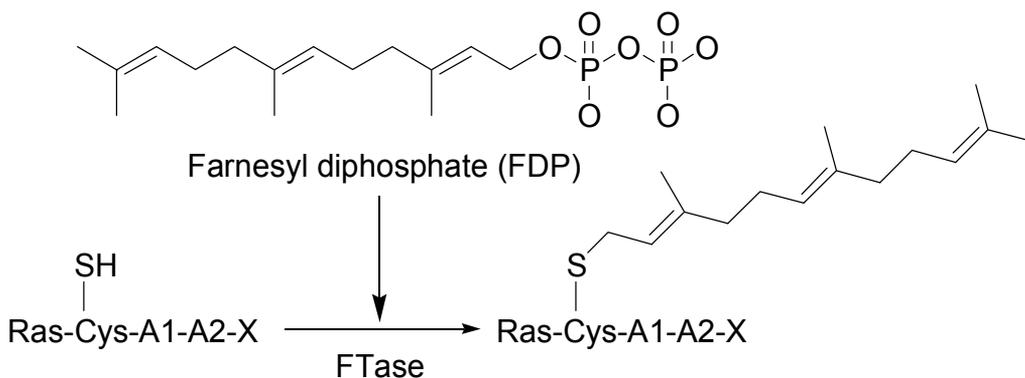


Fig. 3. The first step in Ras posttranslational modification is mediated by FTase, which transfers a farnesyl moiety from FDP to the cysteine moiety in the CAAX motif at the carboxyl terminus of Ras.

In addition, there are other prenyltransferase enzymes, including geranylgeranyl transferases which transfer one or two 20-carbon geranylgeranyl isoprenoid lipid moieties to proteins, again facilitating membrane incorporation. Both farnesylation and geranylgeranylation result in more hydrophobic proteins. The potential for cross-prenylation of proteins such as Ras suggests that geranylgeranyltransferase could restore the function of these proteins if FTase was inhibited (Kim et al., 2010; Marks et al., 2007). However, not all Ras proteins are prenylated by geranylgeranyltransferase, and it is not clear that the function of geranylgeranylated Ras is the same as that of farnesylated Ras, as suggested by the fact that geranylgeranylated normal Ras may be inhibitory. Strategies that are capable of blocking FTase and preventing farnesylation may be expected to inhibit the maturation of Ras into a biologically active molecule, thus turning off signal transduction (Appels et al., 2011; Geryk-Hall et al., 2010).

5. Farnesyl transferase

Farnesyl transferase is located in cell cytosol. FTase is one of the three enzymes in the prenyltransferase group that catalyzes most prenylation reactions and differs in their isoprenoid substrates and protein targets (Fig. 4). FTase adds a 15 carbon (Subramanian et al., 2008) isoprenoid lipid called a farnesyl group to proteins bearing a CAAX motif and its targets include members of the Ras superfamily of small GTP binding proteins critical to cell cycle progression.

FTase is a zinc metalloenzyme that exists as a heterodimer. This heterodimer has two distinct subunits denoted as α and β , having molecular weights of 48 kDa and 46 kDa respectively (Machida et al., 2011; Zhang & Casey, 1996). The X-ray crystal structure of FTase reveals that it has binding sites for both the CAAX peptide and the FDP (Kauh et al., 2011; Park et al., 1997; Wei et al., 2011). It has been shown that geranylgeranyltransferase can prenylate some of the substrates of FTase and vice versa.

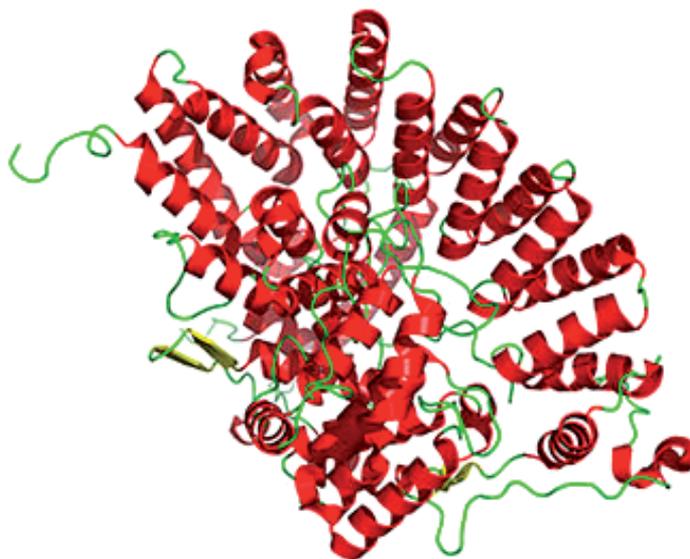


Fig. 4. Structure of Farnesyltransferase (Berman et al., 2000)

6. Farnesyltransferase inhibitors

The introduction of the first 'anti-Ras' agents, the farnesyl transferase inhibitor (FTI), which were proposed to interrupt the crucial post-translational modification of Ras, led to much anticipation of their potential therapeutic benefits (Niessner et al., 2011). The detailed kinetic information about the FTase reaction and the physicochemical nature of FTase substrates has led to the rational design of FTI (Heimbrook & Oliff, 1998; Sebt & Hamilton, 1998). FTI comprise a novel class of antineoplastic agents recently developed to inhibit FTase with the downstream effect of preventing the proper functioning of the Ras protein, which is commonly abnormally active in cancer (Babcock & Quilliam, 2011; Hourigan & Karp, 2010; Kohl et al., 1999). FTIs interfere with bipolar spindle formation during transition from prophase to metaphase in mitosis (Ashar et al., 2000; Crespo et al., 2001).

Currently known FTIs can be divided into three categories based on their mechanism of action: FDP competitive inhibitors, CAAX competitive inhibitors and compounds that inhibit both CAAX and FDP (so-called "bisubstrate analogues") (Crul et al., 2001; Wasko et al., 2011). The second class of compounds in particular has shown promising results. This group can be divided into two subclasses comprising peptidomimetic and nonpeptidomimetic agents, respectively. The high-throughput screening of natural products or compound libraries also led to the discovery of some FTIs which possess good activity.

A number of specific inhibitors have been developed in each of these categories, and subjected to rigorous testing in pre-clinical studies. In the laboratory setting, FTIs revealed the ability to inhibit growth of a wide range of human tumour cell lines, as well as in xenograft and transgenic models (Appels et al., 2005). The anti-tumour outcome has been linked with pleiotropic effects on apoptosis, angiogenesis and the cell cycle.

6.1 FDP analogs

FDP analogs were the first reported active inhibitors of FTase and were designed based on the farnesyl moiety of the FDP substrate. FDP based inhibitors of FTase offer several advantages over bisubstrate analogs or CAAX peptidomimetics in that they are small and non-peptides. Although the compounds that competed with FDP and inhibited Ras processing showed no antitumour activity in animal models (Rowinsky et al., 1999). However, the use of FDP inhibitors in chemotherapy raises several concerns about toxic side effects, since FDP is involved in several biological pathways including cholesterol biosynthesis (Patel et al., 1995). Therefore clinically useful compounds need to be much more selective for FTase than other FDP using enzymes in the cell.

6.2 Peptidomimetics

Development of peptidomimetic inhibitors was initiated upon discovering that FTase activity can be inhibited by a tetrapeptide having the CAAX motif. This was followed by the finding that introduction of an aromatic residue such as phenylalanine at the second "A" position of the CAAX tetrapeptide destroys the ability of the peptide to serve as a substrate while maintaining its ability to inhibit FTase reaction (Goldstein et al., 1991).

When this modification contains an aromatic residue at the terminal A position, the tetrapeptide is a non-substrate inhibitor, and this aroused interest in developing low-molecular-weight CAAX peptidomimetics as a principal strategy for FTase inhibition (Brown et al., 1992; Duque et al., 2011; Symons, 1995). Some chemical structures of peptide CAAX peptidomimetics is given in Fig. 5.

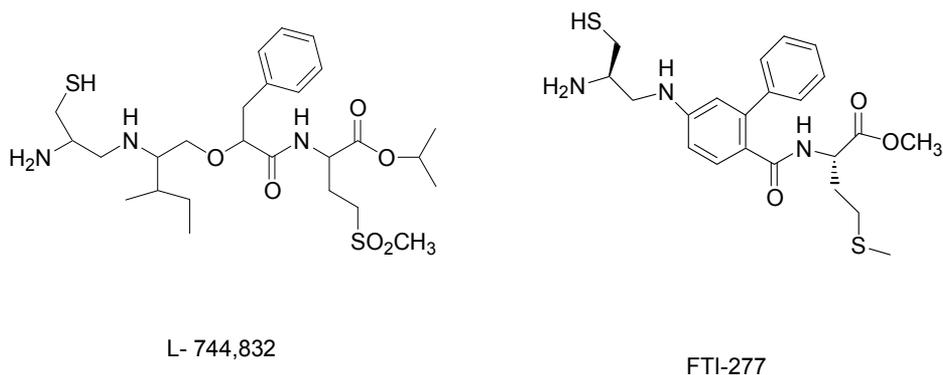


Fig. 5. Peptide CAAX peptidomimetics.

6.3 Nonpeptidomimetic

The molecules of this class are potentially able to inhibit almost selectively the farnesylation of different target proteins involved in malignant cell signalling processes. These class of inhibitors constitute a heterogeneous group of FTIs with different action profiles for each target cell type (Manne et al., 1995). R115777 and SCH66336 (Fig. 6), both of which are orally active nonpeptidomimetic, have now entered clinical development (Castaneda et al., 2011). R115777 is an imidazole-containing heterocyclic compound (Epling-Burnette & Loughran 2010; Skrzat et al., 1998), initially developed as antifungals and possess high enzyme specificity and interesting levels of growth inhibition (End et al., 1998; Smets et al., 1999). *In vitro* tests of human tumor cell lines showed 80% overall sensitivity to R115777. SCH66336 is a tricyclic halogenated compound, which inhibits the growth of several tumour cell lines as well as *K-ras*-transformed xenografts *in vivo* (Bishop et al., 1995). BMS-214662 is an example of a new class of nonpeptide imidazol FTIs, showing high affinity for FTase over geranylgeranyltransferase and it exhibits complete tumour regressions in various tumor xenograft models after both oral and intraperitoneal administration. This compound has recently entered clinical studies.

6.4 Bisubstrate analogs

Bisubstrate analog inhibitors of FTase combine the features of FDP analogues and non-peptide CAAX peptidomimetics and are highly potent *in vitro*. The bisubstrate analog BMS-186511 (Fig. 6), which is 3-log-fold more selective for FTase than for geranylgeranyltransferase, inhibits Ras signalling and transformed growth with a minimal effect on normal cells. Cytotoxic effects were not seen (Manne et al., 1995; Yan et al., 1995).

6.5 Natural products

A variety of compounds with inhibitory activities against FTase have been identified by screening of natural products isolated from microorganisms (Hara et al., 1993), plants (Khan et al., 2010) and soils. This led to the identification of manumycin, chaetomelic acids, actinoplanic acid A, pepticinnamins, fusidienol, cylindrol A, preussomerin, gliotoxin, 10'-desmethoxystreptonigrin and related analogues as inhibitors of FTase (Singh et al., 1993, 1994, 1995a, 1995b; Tamanoi & Mitsuzawa 1995). Natural compounds, such as Manumycin, which is isolated from *Streptomyces* sp., act on the FDP-CAAX complex (Leonard et al.,

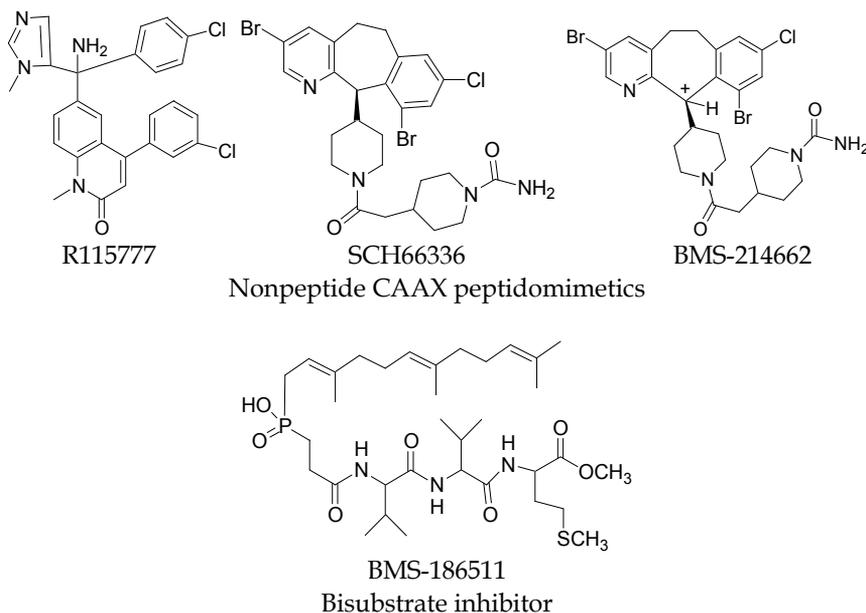


Fig. 6. Nonpeptide CAAX peptidomimetics and Bisubstrate inhibitor.

1997). Some natural products, including the chaetomelic acids, actinoplanic acid A, and manumycin analogs, compete with FDP, whereas other inhibitors, such as the pepticinnamins, compete with the Ras CAAX tetrapeptide (Kainuma et al., 1997). Other natural products, such as fusidienol, preussomerin, gliotoxin, 10'-desmethoxystreptonigrin, and cylindrol A, inhibit FTase noncompetitively.

7. Clinical development of FTIs

The FTIs entered in clinical development, so far, are R115777 (Zarnestra) (Tomillero & Moral 2010), SCH-66336 (Sarasar), L-778, 123 and BMS-214662 (Eskens et al., 2000; Yasui et al., 2002). Among these, R115777 is the most advanced in the clinical development (Fig. 9) since some phase III studies have been already completed (Tsimberidou et al., 2010). BMS-214662 and L-778, 123 are administrated intravenously, whereas the two other agents, R115777 and SCH66336, are given orally with different schedules (Widemann et al., 2011). Dose-limiting toxicities have included myelosuppression, gastrointestinal disorders, peripheral neuropathy and fatigue. Because of cardiac conduction abnormalities, the clinical development of L-778, 123 has been discontinued. The results from Phase I studies are encouraging. R115777 has given evidence of clinical activity in a minority of patients including those with non small cell lung cancer (NSCLC), colorectal cancer and pancreatic cancer (Zujewski et al., 2000). Phase I studies showed that myelosuppression and neurotoxicity were dose-limiting toxicities. Gastrointestinal toxicities and fatigue were also observed (Crul et al., 2002; Punt et al., 2001; Schellens et al., 2000). A phase II trial in breast cancer with R115777 showed a modest activity with a low toxicity profile and achieving a response rate of 11% and disease stabilization in 35% of patients (Johnston et al., 2003). Other trials are conducted in patients with malignant glioma and haematological malignancies and interesting results are documented (Kurzrock et al., 2003). A phase III

study was conducted in patients with advanced refractory colorectal cancer who had failed two prior chemotherapy regimens. R115777 is currently under study in acute myeloid leukemia (Baer & Gojo, 2011; Robak et al., 2011). Because of its relatively low toxicity profile, R115777 provides an important alternative to traditional cytotoxic approaches for elderly patients who are not likely to tolerate or even benefit from aggressive chemotherapy. SCH66336 is orally active (Field et al., 2008) and its first phase I trial was started in 1997. SCH66336 has shown to inhibit the in vitro anchorage-independent growth of many human tumour cell lines and the growth of a number of human xenografts in a dose-dependent manner (Castaneda et al., 2011). In the first phase I study with SCH66336, 5% NSCLC patient experienced a partial response, disease stabilization in 40% were also described for 5-10 cycles (Adjei et al., 1999). Phase II study of SCH66336 in patients with chemorefractory, advanced squamous cell carcinoma of the head and neck was well-tolerated at a dose of 200 mg twice daily (Hanrahan et al., 2009, Raza et al., 2011). In the phase II study in transitional cell carcinomas, myelosuppression was dose limiting with patients experiencing additional toxicities. Despite significant toxicities, no responses were observed (Winquist et al., 2005). Also, in a second phase II study investigating the effect of SCH66336 in patients with metastatic colorectal cancer, no responses were observed. Phase III studies with SCH66336 have just been started.

Drugs	Trial Stage
R115777 (Zarnestra)	Phase III (leukemia, refractory colorectal) Phase II (bladder, brain, breast, malignant glioma, colorectal, leukemia, lymphoma, melanoma, myeloma, pancreatic, sarcoma, haematological malignancies)
SCH-66336 (Sarasar)	Phase II (brain, breast, genitourinary, head and neck)
BMS-214662	Phase II (leukemia)
L778, 123 ^a	Phase I

^a Denotes agents which have been withdrawn because of concerns over demonstrated or potential toxicity

Table 1. FTIs in clinical development

BMS-214662 is administered intravenously and has shown significant activity against several tumour lines in preclinical models as well as potent cytotoxic effects in vitro and in human tumour xenografts (Rose et al, 2001). The oral formulation exhibits dose-dependent gastrointestinal toxicity, which limits its oral dosing (Camacho et al., 2001). BMS-214662 is unique in inducing apoptosis in hematopoietic stem cells. BMS-214662 significantly and selectively induced apoptosis in chronic myeloid leukemia stem cells compared with normal cells [Pellicano, et al 2009]. Phase I clinical trial of the BMS-214662 has shown promising suggestions of single agent activity in patients with advanced solid tumors. There are currently no published phase II trials with this agent. [Eder et al., 2006]

8. Combination with other anticancer drug

As multiple pathways are important for the proliferation, invasion, and metastases of malignant cells, and because combination therapies are often far more effective than are

single-agent regimens, the FTase inhibitors may complement other anticancer agents that may or may not affect Ras-mediated pathways. FTIs target different downstream effectors according to host-tumor interactions, histological tumor type and stage of the tumor and their anti-tumor effects are quite heterogeneous from a prominent anti-angiogenic to an anti-proliferative and an apoptotic effect in different tumors (End et al., 2001). Moreover, resistance to FTIs is reported probably by overexpression of antiapoptotic proteins. Thus, as a single agent, FTIs appear to have modest clinical effects that are not sufficient to induce a long-term tumor inhibition. Additionally, although FTIs demonstrated the capacity to rapidly reduce and nearly ablate large tumors in preclinical studies (rather than simply prevent tumor growth), residual tumors proliferated after withdrawal of the agents. Therefore, combination with other well-chosen targeted therapy might synergize with FTIs and may reduce the need for protracted therapy (David et al., 2010). The overlapping antitumor spectra and nonoverlapping toxicity profiles of FTIs and cytotoxic agents provide a rationale for assessing the efficacy and feasibility of combination regimens. Pre-clinical studies confirm that FTIs can be useful in combination therapy and have showed that combination with cisplatin, taxanes or gemcitabine can improve response (Adjei et al., 2006; Sun et al., 1999; Weber et al., 2011). Although the choice of chemotherapeutic agents to be evaluated in combination with FTIs will ultimately be dependent on the logistics and appropriateness of the agents for the particular clinical setting, the selection may also be based on a unique mechanistic rationale (Table 2). For example, the combination of FTI L-744,832 and taxanes is sustained by the fact that FTIs sensitize tumor cells to paclitaxel-induced mitotic arrest (Moasser et al., 1998).

Therapy	Trial Stage	
Cytotoxic chemotherapy		
Alkylating agents	I/II	Glioblastoma
Antimetabolites	I/II	Breast
Taxanes	I/II	Breast
Topoisomerase Inhibitors	I	AML advanced solid tumours
Endocrine therapy		
Aromatase inhibitors	II	Breast
Anti-oestrogen	II	Breast
Targeted therapy		
Trastuzumab	I	Breast
Sorafenib	I	Advanced solid tumours
Bortezomib	I/II	Myeloma
Imatinib	I	CML
Ionizing radiation		
External beam radiotherapy	I	Pancreas/lung/ glioblastoma

Table 2. Current combination studies employing FTIs (R115777 or SCH66336)

SCH66336 potentiate the activity of temozolomide and radiation for orthotopic malignant gliomas (Chaponis et al., 2011). Combination of SCH66336 with paclitaxel has been reported, which demonstrated either synergistic or additive activity against a broad panel of human tumor cell lines, except for one breast cancer cell line against which the combination demonstrated antagonism (Khuri et al., 2004; Sharma et al., 2000). Promising preliminary

evidence of efficacy was documented with 38% patients demonstrating partial response (Khuri et al., 2000). The study revealed that the inhibitor SCH66336 did not sensitise cells to all anticancer drugs; whereas the combination with cisplatin was synergistic, for melphalan was additive and no potentiation was observed with 5-FU. Moreover this study reported that the synergism between cisplatin and SCH66336 was cell lines specific and did not appear to correlate with the status of Ras. In addition, in many models the effect of SCH66336 was additive to the effect of cytotoxic agents such as vincristine and cytoxan (Shi et al., 1999). Docetaxel- SCH66336 combination therapy in refractory solid tumors was tolerated in all cohorts with the exception of a 28% incidence of diarrhea (Kauh et al., 2011). Coadministration of continuous and intermittent SCH66336 enhanced the antitumor activity of docetaxel in a panel of prostate cancer models (Liu et al., 2009). In phase II when SCH66336 was given with imatinib, 33% patients had a clinical response or improvement with combination therapy (Druker et al., 2003). Responses were encouraging also in another study of SCH66336 combined with gemcitabine in patients with advanced urothelial tract cancer (Theodore et al., 2005).

The combination of R115777 with cytotoxic agents such as cisplatin and paclitaxel induced additional antiproliferative activity against human breast, pancreatic, and melanoma cells growing in tissue culture and as well-established tumor xenografts. The interaction between R115777 and paclitaxel was additive irrespective of the order of drug administration, and the duration of the response to R115777 was not enhanced by paclitaxel. The addition of R115777 to irinotecan failed to enhance the antitumor effect of this topoisomerase inhibitor (Skrzat et al., 1999). The R115777 was combined with 5-fluorouracil and leucovorin in patients with advanced colorectal and pancreatic cancers (Peeters et al., 1999; Verslype et al., 2001). Phase I study of R115777 with imatinib mesylate combination is well tolerated and demonstrates antileukemia activity (Verslype et al., 2001). Phase II trial of R115777 and radiation in children with newly diagnosed diffuse intrinsic pontine gliomas offered no clinical advantage over historical controls (Haas-Kogan et al., 2011; Poussaint et al., 2011; Zukotynski et al., 2011). The combination of R115777 with bortezomib, a proteasome inhibitor, in patients with advanced leukemias was well-tolerated, demonstrated relevant target inhibition, promoted synergistic death, overcomes de novo drug resistance and was associated with signals of clinical activity in patients with advanced and refractory acute leukemias (Lancet et al., 2011; Yanamandra et al., 2011). Sorafenib, a vascular endothelial growth factor receptor kinase inhibitor, combined with R115777 is well tolerated and active against thyroid cancer (Hong et al., 2011). A phase I-II study of R115777 combined with idarubicin and cytarabine for patients with newly diagnosed acute myeloid leukemia and high-risk myelodysplastic syndrome showed a better complete remission (Jabbour et al., 2011). R115777 was well tolerated when given with radiation therapy and temozolomide in patients with newly diagnosed glioblastoma (Nghiemphu et al., 2010).

BMS-214662 in combination with imatinib mesylate or dasatinib, potently induced apoptosis of both proliferating and quiescent chronic myeloid leukemia stem/progenitor cells (Copland et al., 2008). Also combination with PD184352, a MEK inhibitor, improves the ability of BMS-214662 to selectively target chronic myeloid leukemia cells (Pellicano et al., 2011). BMS-214662 and taxol combination have shown 33% response in larynx and prostate cancer, with neutropenia, nausea as dose limiting toxicity (Bailey et al., 2001). One phase I combination study has been reported for the BMS-214662 (Dy et al., 2005; Bailey et al., 2007), in combination with paclitaxel and carboplatin, in patients with advanced solid tumors. This

combination was well tolerated, with broad activity in solid tumors. In parallel, combination of FTI with radiotherapy is under investigation. *ras* oncogenes have been reported to confer resistance to ionizing radiation (Cengel et al., 2005; Kim et al., 2004; McKenna et al., 1990). Presently, many other combinations in phase I/II trials are ongoing, the results of which will hopefully soon be reported. FTIs are a promising class of novel antineoplastic agents. As single agents have significant activity in myeloid leukemias, but in solid tumors their activity seems to be modest and these drugs probably need to be studied in combination with cytotoxic agents, ionizing radiation and other novel targeted drugs, such as antiangiogenic agents.

9. Conclusion

FTIs are a new class of agents and have been developed rapidly as potential cancer therapeutic drugs. They can be quoted as the rolling stones to some of the current generation of cancer research. They have shown promise in early preclinical and clinical studies as a novel anticancer agent. Combinations with other signal transduction inhibitors may be an additional strategy that merits further research. However, FTIs represent one of the first small molecule signal transduction inhibitors to enter the clinic and show promise for the future.

10. List of abbreviations

GDP	=	Guanosine 5'-diphosphate
GTP	=	Guanosine 5'-triphosphate
CAAX	=	"C" cysteine, "A" any aliphatic amino acid, "X" any amino acid
FTase	=	Farnesyl transferase
FTI	=	Farnesyltransferase inhibitor
FDP	=	Farnesyl diphosphate
NSCLC	=	Non small cell lung cancer

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Part 2

New Version of Conventional Modalities in Cancer Therapy

Laser Photo Chemotherapy: An Alternative Treatment for Cancer

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1. Introduction

The application of quantum electronic devices like lasers and high speed MRI provide the potential for less invasive detection techniques and drastically improved treatment of cancer (Upile et al., 2011). Lasers employ the principle of stimulated photon emission to produce light that markedly differs in quality, depending on the wavelength of the radiation in ultraviolet (UV), infrared (IR) or visible regions of the spectrum (Joffe et al., 1989). In this context, laser fiber optics have been used for minimally invasive access and treatment of inoperable cerebral and head and neck neoplasms as well as tumors in other organs and systems (Feyh et al., 1996; Jager et al., 1996; Vogl et al., 2002a; Vogl et al., 2002b).

For the past 50 years, several laser technologies have been employed in surgical oncology, including CO₂ laser for cutting and coagulating and laser-induced thermal therapy (LITT) for thermal ablation of cancer. Photodynamic therapy (PDT) is an approach which has a wide range of application from acne to wrinkles, bactericidal cleansing, cancer treatment, etc (D'Cruz et al., 2004). In PDT, the absorption of photons by organic compounds can excite orbital electrons and increase kinetic energy levels leading to chemical reactions, but also may include light emission as fluorescence or transfer energy directly to oxygen molecules forming singlet and free radical species (Carmichael et al., 1983; Paiva et al., 1996).

Anthracycline derivatives, such as adriamycin and daunomycin, are the most common anticancer agents that interact with light to elicit fluorescence, membrane photo labeling, laser activation and killing of tumor cells (Andreoni et al., 1991; Nahabedian et al., 1988; Saxton et al., 1996). Studies with these anticancer agents include reports of excited states of these drugs and further generation of radical oxygen species which appear to be wavelength dependent, from 313 to 498nm (Paiva et al., 1996). Some reports have shown that yeast cells were sensitized and killed by adriamycin after irradiation at 365nm, a wavelength that presented no effect in the absence of the drug. Also, cytotoxicity of several anthracycline derivatives are significantly enhanced by continuous wave green light of argon (514nm) or KTP (532nm) laser illumination of different types of cancer, both *in vitro* (Li and Chignell, 1987; Paiva et al., 1996) and *in vivo*. (Nahabedian et al., 1988; Peavy et al., 1992; Soudant et al., 1992; Paiva et al., 1995).

Minton and Ketcham (1965) first described enhanced potentiation of the oncolytic capability of a pulsed ruby laser when combined with cyclophosphamide in a melanoma tumor model in 1965. Although lasers were initially very cumbersome in the operating room, these authors visualized the potential benefits of combined drug and laser tumor ablation. Carmichael et al. (1983) and Li and Chignelli (1987) raised the possibility of using chemotherapeutic drugs to photosensitize tumors since many are chromophoric and absorb light at specific wavelengths of the visible spectrum leading to energy transfer and oxygen species which cause photo-oxidation. Based on such concept, several clinical studies combining chemotherapy with laser therapy were developed in the mid-1990s (Lueder and Goyal, 1996; Murphree et al., 1996).

Previous studies with anthracyclines such as adriamycin and daunomycin have shown that tumor cell peroxidation and membrane photo labeling occur immediately after drug uptake (Myers). Both drugs cause oxidative damage to cardiac tissue even in the absence of light. However, spin label experiments have been reported that show light activation of these drugs induces chemical bonding to many different cell membrane proteins (Li and Chignell, 1987). Anthracyclines initially bind to tumor cell membranes before transport in the nucleus and are considered to be poor type-II photosensitizers, which do not generate significant amounts of singlet oxygen (Andreoni et al., 1993). Nevertheless, these chemotherapy agents are the most tested in hopes of enhancing three distinct anti-cancer effects when combined with visible light: photo toxicity, thermal-toxicity and chemotherapy per se (Saxton et al., 1995).

Recent attempts to reduce anthracycline toxicity led to the development of a variety of anthrapyrazole derivatives including DUP-941 (CI-941), which possess reduced side effects but has been shown to retain clinical efficacy in breast cancer patients (Diwu and Lown, 1994). An unusual property of DUP-941 is that it has an over 100-fold increased photo-oxidation potential compared to anthracyclines as reported by Reszka et al. (1992). Ongoing clinical studies are being conducted to determine the impact that this alternative approach of combining laser and anti-cancer agents may have on the control of disease, survival and quality of life in cancer patients (Paiva et al., 2005b; Paiva et al., 2005c).

In addition to the advantage of photo-activation, several of these light sensitive chemotherapeutic agents have been reported to exhibit enhanced toxicity in tumor cells after photo-thermal-activation (Chung et al., 2003; Graeber et al., 1999; Graeber et al., 1998; Paiva et al., 1995; Saxton et al., 1996). As a result, a potentially useful and less invasive approach for treatment of malignancies is to combine imaging guided interstitial laser surgery with conventional chemotherapy (Paiva et al., 2005b; Saxton et al., 1995). Several FDA approved anti-cancer drugs are highly photosensitive or heat-responsive, including anthracycline derivatives and cisplatin (Paiva et al., 1997; Graeber et al., 1999). This experimental technique broadens the concept of tumor ablation based on thermal denaturation of malignant cells to include anti-cancer drug activation by laser energy (Chung et al., 2000; Diwu and Lown, 1994; Nahabedian et al., 1988).

A major development is already taking place in photo chemotherapy as computer-modeled nanoparticles have proven to be "intelligently-guided" by heat sensitization. Nanoshells are optically tunable core/shell nanoparticles that can be fabricated to strongly absorb in the near-infrared (NIR) region where light transmits deeply into tissue (Saxton et al., 1995). Injecting nanoshells systemically will allow enhanced photo thermal ablation of tumor irradiated with the Nd:YAG laser (Feyh et al., 1996; Joffee et al., 1989).

Tumor specificity can be increased by functionalizing the nanoshell surface with tumor-targeting moieties. Moreover, nanoshells can also be made to strongly scatter light and therefore can be used in various imaging modalities such as dark-field microscopy and optical coherence tomography (OCT). After adjustments to the NIR absorbing nanoshells have been finalized, the next step will be to explore image-guided systems in combination with LITT, which will be the ultimate goal of photochemotherapy as a minimally invasive alternative therapy for cancer.

In this article, the authors review the literature that supports combining anti-cancer drugs and laser for cancer therapy. These studies suggest that photo chemotherapy with currently approved drugs and lasers may soon become an attractive alternative for cancer treatment. PDT studies were not in the scope of this work, as photodynamic application in clinical oncology is already a well-established subspecialty in laser technology.

2. Laser photo-thermal therapy for cancer

The pathway towards the realization of optical solid-state lasers was gradual and slow. After Einstein's paper on absorption and stimulated emission of light in 1917 it took until 1960 for the first solid state laser device to see the light. Laser systems are widely spread in the field of medicine (Upile et al., 2011). The applications are divided into therapeutical and diagnostic applications. The main field however, is therapeutical procedures. Depending on the indication lasers were used for removing and cutting of smooth and hard tissue or for coagulation. A relative new procedure is image guided laser thermal ablation of solid tumors. As early as 1964, Goldman and Wilson reported clinical results on the treatment of basal cell epithelioma using laser radiation.

In 1964 experimental investigations on the potential of lasers to destroy malignant tumors began with studies by using high energy pulsed laser radiation for treatment of multiple intra-abdominal tumor implants in experimental animals (Minton and Ketcham, 1964). Richey and Dixon (1981) investigated a similar treatment of gastric polyps by endoscopic application of lasers, a similar technique used by Sasako et al. (1982) for treating gastric cancers with the Nd:YAG laser. Hofstetter and Frank (1979) previously reported the successful use of laser endoscopy for clinical treatment of bladder tumors. Shapshay and Strong (1982), Brunetaud et al. (1981), and Carpenter et al. (1977) presented in separated publications, that successful management of tracheal lesions could be obtained using the Nd:YAG laser. Fleischer et al. (1982) reported tumor palliation in a series of 14 patients with end stage carcinoma of the esophagus treated with the Nd: Y AG laser.

The Nd:YAG laser emits luminous energy in the near infrared portion of the visible spectrum at 1064nm and is poorly absorbed by hemoglobin, melanin and water, which leads to scattering of the laser beam with deep penetration into tissues (Feyh et al., 1996; Joffee et al., 1989; Mueller-Lisse et al., 1998; Sturesson and Andersson-Engels, 1996). Transmitting laser energy directly through a fiberoptic cables allows the surgeon to deliver more effective localized treatment, minimizing undesired tissue damage to adjacent structures, which is the main advantage of this adjuvant therapy for malignancies (Castro et al., 1994; Feyh et al., 1996; Joffee et al., 1989; Paiva et al., 1998a). The Nd:YAG laser is widely used in endoscopic and open surgery due to its photo thermal ability to coagulate, cut, vaporize and ablate tissue (Castro et al., 1994; Joffee et al., 1989; Paiva et al., 1998a). Thus, the Nd:YAG is a unique versatile tool for localized thermal ablation of tumors.

The minimally invasive methods for tumor ablation currently available are using thermal probes, infrared thermography, and histological studies, which are reliable but have limited clinical usefulness (Stafford et al., 2010). Since the mid-1970s, interstitial placement of laser fiber optics has been applied successfully in large subcutaneous metastasis. In 1985, Svaasand developed some preliminary optical dosimetry for interstitial phototherapy of malignant tumors. About the same time Matthewson et al. (1987) investigated interstitial use of the Nd:YAG laser (1-2W) for hepatic hyperthermia to produce areas of thermal necrosis up to 16mm in the normal rat liver, and to eradicate induced rat colon tumors and implanted fibrosarcomas. Hashimoto et al. (1986) applied 5-15 W of Nd: YAG laser power with a modified diffuser fiber tip to treat liver tumors with evidence of reduction of tumor size. Godlewski et al. (1988) used high Nd: YAG powers (100 W), with one second duration to produce areas of vaporization and necrosis of 16-22 mm in porcine liver at great speed. However, the high power density at the distal end of the fiber optics resulted in frequent tip damage, burning, non-uniform distribution of laser energy, and poorly reproducible tissue effects. In 1985, Daikuzono and Joffe introduced synthetic sapphire probes that have high melting points (2020-2050°C), greater tensile strength, and a uniform pattern of laser beam delivery from the lateral surface of the probe. These probes allowed testing of Nd:YAG laser-induced hyperthermia in a dog model, and further developed a computer-controlled Nd:YAG system for interstitial local hyperthermia.

To increase the area of necrosis that could be produced and still use one laser as the energy source, Steger et al. (1989) used fiber optic coupling systems to increase the number of fibers that could be inserted. This concept, while attractive, has proved difficult to achieve in practice. Nevertheless, the same author introduced the term interstitial laser hyperthermia in cancer treatment, which later on established itself as laser-induced thermal therapy, or LITT. Gatenby et al (1987) used computerized tomography (CT)-guided laser therapy for treatment of resistant human tumors in ten patients with favorable results. Steger et al. (1992) used ultrasound imaging to monitor the percutaneous interstitial Nd:YAG laser treatment of ten patients with liver tumors and found ultrasound imaging to be a sensitive monitoring technique of thermal necrosis.

Computed tomography scanning demonstrates poor sensitivity to early tissue changes after laser exposure because of low soft-tissue contrast resolution. Using image-guided systems as CT, and ultrasound. Castro et al. (1994), at the University of California, in Los Angeles (UCLA) , made significant contributions to define Nd:YAG laser doses that could be safely translated into effective clinical application. Blackwell et al. (1993) first reported results of a pilot study testing interstitial laser ablation of neck tumors guided by ultrasound. The method later evolved into minimally invasive MRI-guided treatments as described by Pushek et al. (1995). Sinha et al. (1997) established temperatura monitoring system for thermal-ablative procedures using a software system that correlated tissue changes with MRI imaging.

MRI is a non-invasive and extremely sensitive diagnostic imaging system, used to locate and guide biopsies of deep and difficult tumors and to access target tissues using needles with extremely low magnetic susceptibilities (Kahn et al., 2008; Pushek et al., 1995). MRI has the potential to become a three-dimensional, real-time, video imaging technique that can provide accurate and detailed anatomic information during laser surgery (Kahn et al., 2008; Vogl et al., 2002b). Important advances in clinical applications of imaging-guided laser ablative procedures were developed in the mid 1990s by different groups in Germany.

Particularly in MR-guided LITT liver tumors, Vogl et al. (1997) showed local tumor control between 96.3% and 98.8% after 3 months and between 95.6% and 98.8% after 6 months. No local recurrence was observed later than 6 months after LITT. Although the intention for LITT was originally palliative, its favorable survival rates compared with those obtained with surgical resection of liver metastases on the basis of analyses of large surgical series with lower complication rates, are encouraging (Vogl et al., 2002a; Vogl et al., 2002b; Vogl et al., 2009).

The recent development of software programs for mathematical models of imaging-guided LITT should lead to more effective tumor palliation with laser therapy as an alternative to surgery (Mohammed and Verhey, 2005). In sum, image-guided ablation of tumors is assuming an increasingly important role in many oncology services as a minimally invasive alternative to conventional surgical interventions for patients who are not good candidates for surgery. LITT is now a multi-specialty procedure used for treatment of inoperable tumors of the brain, head and neck, breast, liver, prostate and colon, as a minimally invasive and low cost cancer therapy (Castro et al., 1994; Chapman, 1998; Mueller-Lisse et al., 1998; Paiva et al., 1998a; Schulze et al. 2002; Vogl et al., 1997; Vogl et al., 2002a; Vogl et al., 2002b; Mack et al., 2004; Marga et al., 2011). Further improvement will be possible by combining LITT with novel systemic therapy such as targeting agents, gene therapy, or nano-designed thermo-sensitive drugs (Kanekeal et al., 2009).

3. Photo-chemical and photo-thermal activation of anti-cancer drugs

Tissue interactions with laser irradiation are typically classified as photochemical, photomechanical, and photo thermal (Feyh et al., 1996; Joffee et al., 1989). Photochemical effects depend on the absorption of light to initiate chemical species in photodynamic therapy (PDT). It is associated with low fluency rates that do not produce a significant temperature increase in the treated tissue, but do not interact with a natural or exogenous photosensitizer to produce the desired reaction. Although we will discuss photochemical effects of laser at length, PDT is not in the scope of this chapter for simplicity. Photomechanical responses occur during applications of extremely high fluency rates (greater than 10^8 W/cm² and short laser pulses 10^{-6} sec. or less), which produces shockwaves and plasmas. Such effects occur, for example, when a laser operates in a Q-switch mode.

Photo chemotherapy with lasers is an alternative therapy which consists of using a monochromatic light delivered via external irradiation or via interstitial fiber optics to enhance the "killing" threshold in tumors containing light and/or heat -sensitive anti-cancer agents (Saxton et al., 1995). The development of photoactivatable pro-drugs of platinum-based antitumor agents is aimed at increasing the selectivity and thereby lowering toxicity of this important class of antitumor drugs (Bednarski, 2007; Farrer et al., 2010). Hence, laser chemotherapy explores two distinct mechanism of antitumor action: (1 -) direct toxic effect, and (2 -) additional photochemical and/or photo thermal toxicity (Bednarski, 2007; Crescenzi et al., 2004; Eshraghi et al., 1997; Mackay et al., 2007).

These drugs may be injected intravenously at concentrations lower than normal chemotherapeutic levels, or at higher intratumor doses reducing systemic toxicity while enhancing local tumoricidal effects by laser photoactivation in situ (Nahabedian et al., 1988; Paiva et al., 1998c). Other anthracyclines have also been identified that have greater photosensitization potential than daunomyucin (Diwu and Lown, 1994). With all the supporting evidence of translational studies, photochemotherapy has been established as an

alternative treatment for retinoblastoma (Peterson et al., 2011). Most of these studies were conducted in children where there has been a few standardized clinical protocols, in particular for unilateral retinoblastoma (Leng et al., 2010; Mallipatna et al., 2009; Gallie et al., 1996; Lueder and Goyal, 1996; Murphree et al., 1996; Saxton et al., 1996

The first reports of DUP-941 as an intratumor sensitizing drug for phototherapy in a subcutaneous squamous cell carcinoma (SCCA) transplant model in nude mice were published in 1995 (Paiva et al., 1995). This experimental treatment showed intralesional injections at 60µg DUP-941 per gram of tumor led to stasis but no regression. LITT administered via fiber optics at low energy levels (continuous green light, 532nm, power density = 13.4 J/cm²) also resulted in tumor recurrence in over 80% of the animals. However, combined intratumor DUP-941 and LITT at the same dose levels of drug and laser energy produced tumor eradication in over 90% of treated animals with no recurrence detected during a 12-week follow-up period. This was the first effort in the literature to explore the concept of local injection of anti-cancer drug combined with laser interstitial therapy for cancer treatment. Additional testing in a larger group of animals in Germany by Graeber et al. (1998) confirmed these results, which provide strong preclinical evidence in support of the hypothesis that photo chemotherapy may be an alternative treatment for human tumors. Similar LITT studies have followed with a translational experimental model combining intratumor cisplatin with Nd:YAG laser for thermal ablation of recurrent and advanced head and neck cancer (Paiva et al., 1998b; Graeber et al., 1999).

Despite important advances in current therapy with surgery, radiation, and chemotherapy, nearly one half of all head and neck cancer patients will develop persistent or recurrent disease (Adelstein, 1998; Argiris et al., 2010; Bonner et al., 2006; Correa and Burkey, 1999; Weisman and Robbins, 1998). Head and neck tumors are an excellent disease model for testing intratumor chemotherapy and laser because of their accessibility for surgery and their loco-regional biological behavior (Paiva et al., 1997). The laser energy can be delivered via interstitial fiber optics using drugs activated by photo-chemical and photo-thermal energy as a potential less invasive treatment alternative for cancer (Paiva et al., 2005c; Paiva et al., 1997; Graeber et al., 1999). Moreover, there has been no generally accepted standard of care for recurrent head and neck cancer (Goodwin et al., 2000; D'Cruz et al., 2004; Arnold et al., 2004; Fury et al., 2011). At UCLA, Phase I and II clinical trials have been conducted using LITT in a stepwise fashion to palliate patients with advanced and recurrent head and neck tumors as an alternative to more radical and at times disabling surgery (Castro et al., 1994; Paiva et al., 1998a; Paiva et al., 2002a).

4. The rationale for cisplatin and laser thermal-therapy for cancer

Platinum-based compounds were first synthesized in the nineteenth century but their clinical use against cancer did not start until the 1970s (Higby et al., 1974). Cisplatin [(*SP*-4-2)-diamminedichloroplatinum] was the first of the platinum drugs to be approved for the treatment of both ovarian and testicular cancer in 1978 and is also administered for many other types of solid tumors. Second-generation platinum derivative carboplatin [*cis*-diammine (cyclobutane-1,1-dicarboxylate-*O,O*)platinum(II)] differs from cisplatin in the substitution of two chlorides by a 1,1-cyclobutane dicarboxylate group. Its efficacy in the treatment of the above malignancies is equal to that of cisplatin, and its toxicity profile is more favorable and was approved in March 1989 for treatment of ovarian cancer (Ozols et al., 2003). Thus, carboplatin has often been used in place of cisplatin (Syrigos et al., 2010;

Gkiozos et al., 2007). Oxaliplatin [(1*R*,2*R*)-cyclohexane-1,2-diamine](ethanedioate-*O*,*O*)-platinum(II)] is a third generation platinum compound approved in 2002 (Charalabopoulos et al., 2002). Oxaliplatin is widely used for the treatment of metastatic colorectal cancer and a variety of other malignancies, such as breast cancer, melanoma, non-Hodgkin lymphoma, and head and neck cancer (Makrilia et al., 2010). The other names for cisplatin are DDP, cisplatinum, and cis-diamminedichloridoplatinum(II), or CDDP. Common adverse events are myelotoxicity, nausea, vomiting, diarrhea, paresthesia, and dysesthesias (Makrilia et al., 2010).

The mechanism of anticancer activity of cisplatin is by forming a platinum complex inside of a cell which binds to DNA and cross-links DNA. Consequently, cross-linked DNA causes the cell to undergo apoptosis, or systematic cell death. Cross-linking ensues apoptosis by damaging the DNA so that the repair mechanisms for DNA are activated, and once the repair mechanisms are activated and the cells are found to not be salvageable, the death of those cells is triggered instead. Clearly, the use of platinum derivatives is essential in the fight against cancer, and currently it is the most commonly used chemotherapy drug in the United States (Seiwart et al., 2007). However, the toxic side effects must be attenuated to reap the maximum benefits, which is why other uses are devised exploring distinct synergistic effects when combining cisplatin with a regimen of other anti-cancer drugs, radiotherapy, whole body hyperthermia, and more recently biologic therapy (Amichetti et al., 1993; Goodwin, 2000; Bonner et al., 2006; Seiwart et al., 2007; Fury et al. 2010).

Whole body hyperthermia has been studied clinically for nearly two decades with respect to cancer treatment and has been shown to enhance tumoricidal effects of irradiation as well as chemotherapy with adriamycin (doxorubicin) and cisplatin (Amichetti et al., 1979; Hahn, 1979; Baba et al., 1989; Storm et al. 1989; Ohno et al., 1994; Engin, 1996; Cezamar et al., 2001; Arancia et al., 2001; Hahn and Shiu, 1983; Noel et al., 2003). In these studies, near maximum effects were observed when both modalities (i.e., hyperthermia and chemotherapeutic agents) were administered simultaneously (Hahn, 1979; Teicher and Herman, 1992). At temperature levels above 43°C, heat also appears to reverse acquired resistance to cisplatin (Hahn, 1979; Cezamar et al., 2001). In particular, hyperthermia enhances the activity of cisplatin by potentiating tumoricidal effects progressively as intra-tumor temperature is elevated to 43°C resulting in synergistic lethal effects for cancer cells (Ohno et al., 1994; Engin, 1996). Thermo-chemotherapy using systemic fever-range (40 C°) temperature for long durations (4-6 h) has better or equal anti-cancer efficacy compared to maximally-tolerated systemic temperatures (41.5 C°-42 C°), and generally results in less toxicity (Bull et al., 2008a).

Bull et al. (2008a) determined the maximally tolerated dose (MTD) of cisplatin administered within a regimen of fever-range whole body thermal therapy to be 60mg/m². Since hyperthermia has the potential to interfere with many mechanisms that cause cisplatin resistance, it may also be a suitable modality to interfere with acquired resistance (Oldenburg et al., 1994; Bull et al., 2008a). Apparently, the mechanisms responsible for hyperthermic cell killing and hyperthermic drug sensitization must, in part, be the same (Hettinga et al., 1995). Heat has been shown to cause denaturation of cellular proteins. This leads to an increase in protein mass of nuclei isolated from heated cells. Under several conditions, including thermo tolerance, a good correlation has been found between the extent and duration of this "nuclear protein aggregation" and thermal cell killing (Hettinga et al., 1995). This nuclear protein aggregation may also be one of the major mechanisms

responsible for thermal potentiation of killing by a number of drugs, which is by hampering repair of drug-induced DNA damage (Hettinga et al. 1995).

Timing of chemotherapy, with respect to potentiation by heat, and of two drugs relative to each other, is critical in determining antitumor efficacy, toxicity, and survival (Bull et al., 2008b; Teischer and Herman, 1992). Significant sequence-mediated differences in antitumor response demonstrated that when cisplatin is administered with gemcitabine an optimization of the administration schedule of multidrug chemotherapy regimens may also prove to be important. Therefore, preclinical optimization of the timing of chemotherapy drugs relative to each other, and drugs relative to heat, in multi-agent thermo chemotherapy regimens could significantly increase tumor response while minimizing toxicity.

Refaely et al. (2001) studied the results of thermo chemotherapy with cisplatin in stage IVa patients diagnosed with thymic malignancies. They found a relatively high 3-year and 5-year survival rates of 70% and 55% for the entire study population, and 90% and 70% for the thymoma patients. These results are comparable, and apparently higher than that reported in other series (Yellin et al., 2001). While it is important to recognize that whole body thermal therapy can enhance some of the toxicities associated with other treatments, the synergy of hyperthermia with several chemotherapy agents means that lower doses can be used, resulting in less toxicity. Among the collateral effects that cisplatin can cause, we find nausea, both acute, and chronic renal toxicity as well as long-lasting neuropathy. These toxicities are acute and treatable (McWhinney et al., 2009).

There are three proposed explanations for the synergism between hyperthermia and cisplatin: (1) an increase level of drugs within cells; (2) significant enhancement of the DNA cross-linking effect of the drugs; and (3) heat-induced inhibition of DNA repair (Refaely et al, 2001). Evidence indicates that heat induction leads to ultrastructural changes in cell membranes resulting in altered cellular metabolism and increased susceptibility to drug toxicity (Arancia et al., 1989; Hahn and Shiu, 1983). Higher intracellular uptake of CDDP induced by heat also enhances cytotoxicity in proliferating cancer cells and stimulates protein kinase-mediated pathway to tumor apoptosis (Noel et al., 2003).

Drug resistant human carcinoma cells also exhibit increased CDDP uptake and cytotoxicity in the setting of hyperthermia (Flood et al., 1999). In addition, combined CDDP and hyperthermia is effective in the eradication of hypoxic tumor cells which may be resistant to single modality treatment with either radiation or chemotherapy alone (Storm, 1989). The oncogenic potential of CDDP is markedly enhanced by hyperthermia compared to drug treatment alone (Zanke et al., 1996; Beketic-Oreskovic et al., 1997). Studies of CDDP at the molecular level have shown that hyperthermia induces CDDP to bind to DNA forming more DNA adducts, contributes to delayed tumor growth, and amplifies inter-strand crosslinking (Storm et al., 1989; Hettinga et al. 1996; Los et al., 1994; Los et al., 1993). Altogether, the exact biological mechanism of the synergistic effect of combined cisplatin and heat remains unclear (Bednarski et al, 2007; Los et al., 1993).

An initial report by Nahabedian et al. (1988) on photochemotherapy using cisplatin and laser (wavelength 688nm) showed that laser treatment was enhancement by systemic chemotherapy in murine tumors, and was the first *in vivo* evidence of cisplatin-photo activation by visible light. More recently, the development of photoactivatable prodrugs of platinum-based antitumor agents is aimed at increasing the selectivity and thereby lowering toxicity of this important class of antitumor drugs (Bednarski, 2007; Crescenzi et al., 2004; Mackay et al., 2007; Cubo et al, 2010). Cisplatin forms mainly intrastrand cis-diguanine

cross-links on DNA between neighboring nucleotides, whereas photoactivated complex-1 rapidly forms unusual trans azido/guanine, and then trans diguanine Pt(II) adducts, which are probably mainly intrastrand cross-links between two guanines separated by a third base. DNA interstrand and DNA-protein cross-links were also detected. Importantly, DNA repair synthesis on plasmid DNA platinated by photoactivated-1 was markedly lower than for cisplatin or its isomer transplatin (an inactive complex). Single-cell electrophoresis experiments also demonstrated that the DNA damage is different from that induced by cisplatin or transplatin. Cell death is not solely dependent on activation of the caspase 3 pathway and, in contrast to cisplatin, p53 protein did not accumulate in cells after photosensitization of photoactivated complex-1. The trans diazido Pt(IV) complex-1 therefore has remarkable properties and is a candidate for use in photoactivated cancer chemotherapy (Cubo et al., 2010). Even though there was a lack of basic science evidence supporting photochemotherapy with cisplatin in the beginning of the eighties, a wealth of clinical investigations followed testing this experimental treatment for cancer.

5. Clinical relevance of laser and cisplatin in photo chemotherapy

Photochemotherapy (PCT) uses Federal Drug Administration (FDA) approved chemotherapeutic drugs which are first injected into tumors, thus reducing systemic effects. The sensitized tumor is then activated either by photo oxidation and/or by photo thermal levels of energies using a monochromatic laser light which is delivered via external and/or interstitial fiber optics to enhance the "killing" threshold (Saxton et al., 1995). Head and neck cancers are accessible for surgery and have a well documented loco-regional biological behavior. These tumors can therefore serve as an ideal model to test photochemotherapy, as this approach is a possible alternative for less invasive treatment for cancer.

Local recurrence in head and neck cancer is thought to be caused, in many cases, by residual disease and/or contamination of the surgical field during resection. Using an animal tumor model to investigate local recurrence, Maker et al. (1995) observed that Nd:YAG contact laser ablation provided about 50% improvement in the control of local disease in vivo ($p < 0.05$) compared to animals treated with a scalpel. Pilot studies have demonstrated that Nd:YAG (1064 nm) laser induced thermal therapy may also play a role in pain control and allow continued nutritional support for patients with advanced or recurrent head and neck tumors (Castro et al., 1995a; Castro et al., 1994; Jager et al., 1996) Through repeated laser endoscopy, the need for a nasogastric or gastrostomy tube has been avoided as well (Jager et al., 1996; Mitty et al., 1996). Several studies report successful relief of malignant dysphagia and improvement in the quality of life with endoscopic Nd:YAG laser treatment (Karlín et al., 1987; Mitty et al., 1996; Sawant and Moghissi, 1994).

Although LITT procedures are safe, feasible, and can extend survival, margin recurrence is seen in many cases, particularly in advanced obstructing cancers of the gastrointestinal tract or bronchial tree (Castro et al., 1995a; Feyh et al., 1996; Joffe et al., 1989; Mueller-Lisse et al., 1998). In order to improve local tumor recurrence after laser treatment, Firusian (1988) was the first to propose combining Nd:YAG therapy with systemic chemotherapy to improve the final outcome in the palliation of esophageal cancer. The author reported that 13 patients with stenotic upper gastrointestinal cancers treated by endoscopic recanalization with laser ablation and systemic cisplatin in combination with other anticancer drugs responded more favorably compared to laser alone as a palliative approach for advanced malignant disease.

In most of the 13 patients, combined therapy led to immediate patency of the upper alimentary tract and 8 months median survival in the cohort studied. In this study it was also found that local laser thermal effects were enhanced by systemic chemotherapy leading to additional ablation of malignant cells down to 7-8mm depth compared to 4mm for laser treatment alone. Thus, it was concluded that combined drug and laser therapy was more effective in preventing rapid cell proliferation at the tumor margins (Firusian, 1988). The author also compared different anti-cancer agents combined with LITT ablation for inoperable esophageal cancer and reported that cisplatin (CDDP) was more effective than anthracyclines or cyclophosphamide when combined with Nd:YAG laser. However, other studies done in children with retinoblastoma found that Adriamycin derivatives were more cytotoxic in tumors after laser photo-thermal activation (Andreoni et al., 1991; Gallie et al., 1996; Lippert et al., 2004; Lueder and Goyal, 1996; Murphree et al., 1996). From a historical perspective, initial work on chemotherapy and lasers was reported in clinical models investigating combined palliative therapy for inoperable esophageal cancers. Such reports were produced in Germany (Semler et al., 1985), who noticed significant improvement in quality of life in 21 of 24 patients with advanced gastrointestinal tumors treated by systemic chemotherapy administered before intraluminal endoscopic Nd:YAG laser thermal ablation. Mache et al. (1986) also reported on the survival benefits of this alternative combined treatment for palliation of advanced upper gastrointestinal tumors. In a randomized clinical trial, Mason et al. (1996) confirmed that patients with esophageal cancer presented a significant reduction in need for additional laser therapy to maintain swallowing when adjunctive chemotherapy was given before laser treatments. Expanding the same concept, Vogl et al. (2009) have recently proposed a combination of chemoembolization and LITT for liver tumors with promising results. An additional boost for laser thermal therapy has also been reported, when associating systemic cycles using IL-2 human cytokines for palliation of metastatic renal cell carcinoma to the head and neck (Paiva et al., 2007).

Clinical experience with this form of combined therapy for head and neck SCCA was first reported on eight patients with recurrent tumors who enrolled in the first study conducted in the United States testing systemic chemotherapy (CDDP at 80mg/M²) followed 24 hours later by palliative Nd:YAG laser thermal ablation (Paiva et al., 2000). Four of the 8 patients treated in this manner remained alive after a median follow up of 12 months. A total of twelve tumor sites were treated, and complete responses were seen in the following anatomic locations: oral cavity (n=3), oropharynx (n=1), hypopharynx (n=1), and maxillary sinus (n=1). The median survival for these patients was 9.5 months. The adverse effects of treatment included mild alopecia in a 82 year-old female, and a bout of gastrointestinal infection in another patient (Paiva et al., 2000). A total of 21 patients were treated on this study that showed minimal toxicity of the combined treatment. However, the therapeutic benefit could not be demonstrated because of the variability of the tumor sites in the head and neck that were analyzed (Paiva et al., 2005a). One of the patients treated in this series presented with a recurrent SCCA of the neck after having previously undergone a reconstructive free flap transfer (Joo et al., 2009). The patient underwent 6 concurrent treatment sessions using the protocol mentioned above and demonstrated an unusually long period of survival (i.e., over 5 years). The remarkable survival of this patient suggests that the combination of LITT and chemotherapy warrants further investigation as an alternative treatment for patients with recurrent head and neck cancer. Studies have shown that even more effective eradication of head and neck cancer is possible by combining LITT

with local, intratumor injections of CDDP (Clayman et al., 1999). Currently, cisplatin or cis-diamminedichloroplatinum (CDDP) are the most commonly used chemotherapeutic agents for head and neck cancer (Argiris et al., 2010; Bonner et al., 2006; Forastiere et al., 2006). technology.

6. Intratumor injections of chemotherapy and laser for effective local tumor control

Initial experimental studies combining intratumor injections of cisplatin followed by local hyperthermia were carried out by Kitamura et al. (1992) in a melanoma model. The authors demonstrated that the combined treatment led to 6-fold decreased tumor growth rate of melanoma and improved prognosis without nephrotoxicity or the promotion of hematogenic metastasis. Also, intratumor administration of cisplatin led to animal weight gain in this study, while mice treated with systemic drug had a significant weight loss or absence of weight gain (Kitamura et al., 1992).

In the mid-1990's several studies explored local adjunct chemotherapy to eliminate marginal tumor regrowth and improve final outcomes after surgery and radiation. These studies had a strong rationale based on two reports by authors Theon et al. (1994) and Begg et al. (1994), who proposed adjunct local chemotherapy combined with tumor resection as a more effective approach for cancer treatment. The local chemotherapy proposed was based on a therapeutic implant of cisplatin in a gel vehicle (CDDP/gel) which consisted of purified bovine collagen (a protein carrier), cisplatin and a vaso-constrictor, epinephrine (Deurloo et al., 1991). The therapeutic implant provides sustained release of drug in tumors and greatly reduces systemic toxicity (Krag et al., 1990). Recent studies with human SCCA transplant models combining intratumor chemotherapy with LITT encourages further development of this novel combined therapy to successfully eradicate marginal disease for treatment of recurrent head and neck cancer (Chung et al., 2003).

The rationale for combined treatment is that during LITT, high photo thermal laser energy levels are delivered to the area of maximum obstruction in the tumor core, inducing irreversible coagulative changes and lower levels at the tumor margin. Less energy is delivered to the margins because of higher risk of organ perforation or damage to neighboring tissues (Paiva et al., 1997). LITT debulking procedures causes boiling of tissue water and subsequent irreversible thermal damage, which will ultimately lead to photo-evaporation at the tumor core (≥ 100 °C) (Chapman, 1998; Halldorsson and Langerholm, 1978; Marchesini et al., 1985; Morrison et al., 1998). Local recurrence after LITT is related to residual disease due to reversible cellular thermal damage on the margins treated with subtherapeutic energy levels (40-60°C) in head and neck and prostate tumor ablation (Anzai et al., 1991; Marchesini et al., 1985). Toxicity enhancement of cisplatin at these temperatures (40-60°C) would promote optimal eradication of cancer cells at the tumor margins. Under such rationale, enhanced laser therapy by adjuvant local chemotherapy using CDDP/gel in the region of subtherapeutic energy levels was first demonstrated in an experimental model by Paiva et al. (1997).

Using authographic imaging method, Kanekal et al. (1995) tested cisplatin radiotracer in suspension (^{195m}Pt -CDDP, 4mg/ml) and gel implant form (^{195m}Pt -CDDP/gel, 4mg/ml) to compare tumor retention in 600mm³ subcutaneous tumors in experimental murine model. Intratumor CDDP/gel provided a uniform drug concentration at the margins of the tumor

at 4 hours, and 80% drug retention in the tumor as opposed to rapid systemic washout of ^{195m}Pt -CDDP in suspension after intratumor administration. Higher intratumor CDDP levels can allow enhanced direct toxicity as well as a synergistic interaction with laser hyperthermia to further promote cisplatin cytotoxicity (Begg et al., 1994; Graeber et al., 1999; Theon et al., 1994). This hypothesis was tested in multiple studies in which intra-tumor CDDP/gel injections were combined with LITT for enhanced therapy in the tumor margins and further improvement in cancer treatment. This body of translational work provided the groundwork for clinical application of CDDP/gel and LITT for recurrent head and neck cancer (Paiva et al., 2005b). The CDDP/gel dose was based in phase II-III studies where 2.0 mg CDDP/cm³ tumor was determined to be tolerable, contributed to tumor responses, and provided volume dosing to adequately infiltrate tumor masses. Using this vehicle, 1 mL of CDDP/gel delivers 2 mg of CDDP (Burris et al., 1998; Castro et al., 2003). In one study, eight patients were treated using the CDDP/gel implant and laser combination (oral $n=1$; neck $n=3$; skin $n=2$; maxillary sinus $n=1$; breast $n=1$) where the end point was to study drug toxicity (Bublik et al., 2010). None of the patients experienced toxicity at the levels of laser (2,200J/cm²) and drug (2mg/CDDP per cm³ of tumor) tested. However, additional studies are needed to guarantee the safety of combining both treatment modalities. Other studies have confirmed that intratumor injection of cisplatin at 2mg/ml is safe in well-established clinical protocols exploring electro-chemo-poration treatment for skin cancer (Sersa et al., 2000a; Sersa et al., 2000b).

A more recent study by Kanekal et al. (2009) showed that intratumor injections of CDDP in solution washed out after 5 minutes, which shows that combined treatment with LITT could be performed as a one-step procedure in the operating room. This would obviate the need for waiting between tumor injection and laser treatment (typically 4 to 48 hours), making the treatment protocol more efficient and streamlined for patients. Probably the most relevant clinical investigation testing intratumor injection of chemotherapy combined with laser thermal therapy was published by Wang et al. (2001) who reported 100% complete responses in 33 patients with T1-2 esophageal squamous cell carcinoma. In this work intratumor injection of bleomycin was combined with LITT for esophageal tumors and followed for an average 29 months. The combined minimally invasive procedure proposed has a strong translational potential as a cost effective alternative treatment not only for head and neck but for other accessible inoperable primary and/or recurrent tumors of the chest wall, breast, liver, prostate and colon (Castro et al., 1994; Chapman, 1998; Mueller-Lisse et al., 1998; Paiva et al., 1998a; Schulze et al., 2002; Vogl et al., 2002a; Vogl et al., 1997; Stafford et al., 2010; Sercarz et al., 2010).

7. Future development

The next phase of the experimental model for intratumor injections of CDDP followed by LITT will be to introduce the concept of “vascular collapse” potentiating high dose chemotherapy and localized hyperthermia. Peralta et al. (2010) et al. recently described massive thermal injury to arteries and veins resulting in vascular collapse due to high energy density using the Nd:YAG laser powered at 30-45W for treatment of severe twin-twin transfusion syndrome. This is the same energy level used by our group for head and neck thermal ablation (2,200-3,300 J/cm²) of cancer (Bublik et al., 2006; Sercarz et al., 2010). In theory, the peripheral vascular collapse of arteries and veins produced by LITT will isolate blood flow to and from the tumor, thereby impeding drug (CDDP) washout to the

rest of the body. Consequently, high cisplatin concentration in the tumor will potentiate the cytotoxic synergistic combination of drug and heat, and promote more effective treatment. Potentially, intratumor injections of cisplatin may be increased up to 40mg as recently reported by Celikoglu et al. (2006) in a clinical trial testing local chemotherapy (CDDP/sol) and radiation for inoperable bronchogenic tumor, where no adverse effects were observed. The vascular collapse will avoid systemic diffusion of cisplatin and allow us to attain a very high concentration of CPPD in the tumor margins as first suggested by Sakurai et al., in 1996. High doses of CDDP/sol will significantly improve the combined treatment proposed (Kanekal et al., 2009).

Further progress in laser chemotherapy may be an outgrowth of the current excitement surrounding nanoscience and the promise of new nanoscale applications in cancer diagnostics and therapy. Because of their strongly resonant light-absorbing and light-scattering properties that depend on shape, noble metal nanoparticles provide a new and powerful tool for innovative light-based approaches. Nanoshells are spherical, dielectric core, gold shell nanoparticles that have been central to the development of photo thermal cancer therapy and diagnostics for the past several years. By manipulating nanoparticle shape, researchers can tune the optical resonance of nanoshells to any wavelength of interest (Lal et al., 2008).

Nanoshells are optically tunable core/shell nanoparticles that can be fabricated to strongly absorb in the near-infrared (NIR) region where light transmits deeply into tissue. When injected systemically, these particles have been shown to accumulate within the tumor due to the enhanced permeability and retention effect and induce photo thermal ablation of the tumor when irradiated with an NIR laser. Tumor specificity can be increased via functionalizing the nanoshell surface with tumor-targeting moieties. Nanoshells can also be made to strongly scatter light and therefore can be used in various imaging modalities such as dark-field microscopy and optical coherence tomography - OCT (Morton et al., 2010).

An interesting application of nanoshell research was demonstrated by Hirsch et al. (2006) by incubating human breast carcinoma cells with nanoshells *in vitro*. The breast carcinoma cells found to have undergone photo-thermally induced morbidity on exposure to NIR light (820 nm) as determined by using a fluorescent viability stain. Cells without nanoshells displayed no loss in viability after the same periods and conditions of NIR illumination. Likewise, *in vivo* studies under magnetic resonance guidance revealed that exposure to low doses of NIR light in solid tumors treated with metal nanoshells reached average maximum temperatures capable of inducing irreversible tissue damage ($\Delta T = 37.4 \pm 6.6^\circ\text{C}$) within 4-6 min. Controls treated without nanoshells demonstrated significantly lower average temperatures on exposure to NIR light ($\Delta T < 10^\circ\text{C}$). These results demonstrate a good correlation with the histological findings: tissues heated above the thermal damage threshold displayed coagulation, cell shrinkage, and loss of nuclear staining, which are all indicators of irreversible thermal damage, whereas control tissues appeared undamaged. Now that a regime of nanoshell and laser dosage has been established for successful therapy of nanoshell-treated tumors, survival studies monitoring tumor growth/regression of entire tumors after treatment with the NIR nanoshell therapy under MR guidance are possible.

Future investigation of a targeted nanoshell therapy that is similar in many ways to the delivery of stealth liposomes is warranted. In this approach, nanoshells "stealthed" with PEG are systemically injected and preferentially accumulate at the tumor site due to the highly permeable, poorly organized vascular networks commonly found in tumors

(Papahadjopoulos et al., 1991; Wu et al., 1993). This preferential accumulation behavior is often referred to as the enhanced permeability and retention effect (Maeda, 2001). NIR treatment of the bulk tissue then selectively heats and destroys the nanoshell-laden tumor regions within the tissue, leaving surrounding tissue intact. As an additional adjuvant, nanoshells may also be conjugated with antibodies targeting surface oncoproteins overexpressed by tumor cells. These antibodies would enhance target specificity and cellular internalization resulting in more selective thermal damage to the tumor. Such therapies could have a large impact on the treatment of secondary metastases and other tumors considered to be otherwise inoperable (Lal et al., 2008).

8. Conclusions

Innovative approaches are being explored in many areas of medicine to reduce costs by increasing both efficiency and effectiveness of patient care. Less invasive surgical access has allowed more rapid recovery and decreased inpatient costs for procedures ranging from laparoscopic cholecystectomy to neurosurgery for cerebral neoplasms. Advanced electronic devices including color Doppler ultrasound, open magnetic resonance imaging, and low-cost semiconductor laser fiber optics can be coupled for accurate detection and improved tumor ablation without open surgical access in many anatomic sites. Imaging guided interstitial laser therapy is minimally invasive and can be repeated as an outpatient procedure for improved palliation of recurrent tumors. We and others have performed UTZ-guided or MRI-guided LITT during the last 18 years for unresectable and recurrent head and neck cancer.

Because head and neck cancers are accessible for surgery and have a well described locoregional biological behavior, they are an ideal model to test combined laser energy delivered via interstitial fiber optics and chemotherapeutic agents activated by photo-thermal energy as an alternative, less invasive treatment for cancer. Long term remission and tumor eradication may be possible by combining intratumor chemotherapy with photo-thermal energy delivery via laser fiber optics. In this model, cisplatin and hyperthermia have been shown to be an effective combined therapy in the laboratory and in recent clinical trials. In addition to therapeutic benefits and improved tumoricidal effects, combining intralesional anti-cancer drugs with interstitial photo-thermal laser treatment reduces systemic toxicity and is less invasive than conventional chemotherapy or surgical resection. This research will benefit from recent advances in development of low-cost imaging systems for real-time monitoring during minimally invasive procedures. Photo-chemotherapy promises to become a useful adjunctive modality for tumor palliation in advanced cancer patients and may represent one of many future biomedical applications of quantum microelectronics devices and nanotechnology in surgical oncology.

9. References

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Laser-Driven Radiation Therapy

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1. Introduction

A class of novel cancer treatment methods in radiation therapy using intense lasers has been investigated and developed in recent years. We review the latest status and future prospects in this field. Radiation therapy is playing an ever increasing role in the treatment of cancer and other illnesses, compared to other treatment options, it shows a good response with acceptable side effects for the patient and a good cost-benefit ratio.

Radiation therapy comes in different variants, mainly *external beam radiation therapy (EBRT)*, where the radiation sources are positioned outside the patient, and *endoradiation therapy*, where sealed radiation sources are positioned in the treatment zone, or *systemic radionuclide therapy*, where unsealed radionuclides are injected into the patient's body.

EBRT can be performed with X-rays, gamma-rays, electrons, neutrons or ion beams. Ion beams (such as proton and carbon beams Tajima (2009)) have the distinct advantage in reducing unwanted radiation dose on healthy tissues before and behind the tumor region due to the Bragg peak. On the other hand, the physical installation (accelerator, gantry, and radioprotection measures) for ion beams is larger and more costly. Laser-driven ion beams are small and lend themselves also for the treatment of smaller tumors. For this purpose, a new dose monitoring method is introduced, namely on-line monitoring of laser-driven ion beams via prompt gamma ray detection.

Endoradiation therapy too can profit from advanced laser technology and laser applications. Here laser-driven γ beams are produced by Compton backscattering of laser light from relativistic electron beams. They are nearly monoenergetic and have a low divergence and high brilliance. Compared to present day γ beams, new beams with much higher flux and much better monochromaticity become available, using new techniques of intensified interaction between laser and electron beam. Due to their much smaller opening angle they allow focusing with refractive γ lens optics for the first time, opening a new world of *nuclear photonics*. These γ beams can be employed to induce photonuclear reactions to selectively generate new radioisotopes or nuclear isomers with high specific activity Habs (2011). With conventional methods, such radionuclides cannot be produced in the required quality or quantity, but they will become available with γ beams. Thus, e.g., in many cases "matched" pairs of isotopes of the same element become available, one for therapy and one for diagnostics, allowing for an optimized therapy. Some new isotopes kill the cancer cells

due to the short-range interaction only in a small volume, making a very localized treatment possible. This will be outlined in detail in Section 3 of this chapter.

We present here one possible school of thought for these goals based on an oncological consideration. This idea is based on the following observation Abe (2007); Molls (2009). Dr. Molls argues: "In chemotherapy the tumor cell kill depends on the transport of the substance to the clonogenic cells and molecular targets, DNA repair capacity, repopulation, pO₂, pH, etc. In macroscopic tumors not all the subvolumes of the tumor, clonogenic cells and relevant molecular targets are reached by those doses of the medical substance which are needed for cell kill. In other words," he goes on, "the chemotherapy dose distribution is intrinsically inhomogeneous." On the other hand, Dr. Molls points out, "In radiation therapy the tumor cell kill depends on intrinsic radiation sensitivity, DNA repair capacity, repopulation, oxygenation status etc. However, the entire tumor can be irradiated matched with the dose, which is necessary to kill all clonogenic tumor cells, even the most resistant ones." In other words, "it delivers a matched dose distribution," citing the superior feature of radiation therapy.

The process in the detection of small and even micro tumors by such methods as phase contrast imaging Pfeiffer (2006) gives us hope for such a vision. Such techniques point us towards small (or even micro) tumor detection. For proton laser-driven (or ion) beam radiotherapy, the therapy of small tumors is well suited.

2. Laser-driven ion therapy

2.1 New trends in ion acceleration

It is important to improve the laser ion interaction in both its energy and the quality of the beam. The progress in recent years makes us hopeful. Energetic proton and ion beams with high beam quality, such as quasi-monoenergetic spectra, have been produced in the last few years from thick metallic foils (e. g. μm thick aluminum) irradiated by ultra intense short laser pulses with appropriate target preparations Hegelich (2006); Schwoerer (2006); Snavely (2000); Ter-Avetisyan (2006). Most previous experiments fall in the regime called TNSA (Target Normal Sheath Acceleration) using what we regard as relatively thick targets ($\sim \mu\text{m}$) Fig. 1). In this regime electrons are first accelerated by the impinging intense laser pulse and penetrate the target. Leaving the target at the rear side, the electrons set up an electrostatic field that is normal to the rear surface of the target. Ions accelerated from solids originate primarily from contaminant layers of water vapor and hydrocarbons on the target surface. As these targets are thick, the laser pulse is mostly reflected and the conversion efficiency of laser energy to ion energy is normally less than 1%, the maximum energy scales with less than a linear function of the laser intensity. The maximum proton energy based on the TNSA mechanism has not improved since 2000 (~ 60 MeV). As reviewed by Robson et al. Robson (2007) (in particular their Fig. 1a), their fit of the data experimentally obtained (what they collected are all TNSA) shows that ion energies are proportional to the square root of the laser intensity I_L , which is proportional to a_0^2 , where the dimensionless normalized vector potential a_0 is defined as: $a_0 = \frac{e E_L}{m_e c \omega_L} = \left(\frac{I_L [\text{W cm}^{-2}] \cdot \lambda_L^2 [\mu\text{m}^2]}{1.37 \cdot 10^{18}} \right)^{1/2}$, where m_e is the electron mass and ω_L the laser frequency. In TNSA, the laser energy is absorbed at the front surface of a solid target. Hot electrons are generated by a variety of mechanisms. Since electrons gain kinetic energy through interaction with the laser, typically up to the ponderomotive potential

$$\varepsilon_0 \approx e\Phi = m_e c^2 \left(\sqrt{1 + a_0^2} - 1 \right), \quad (1)$$

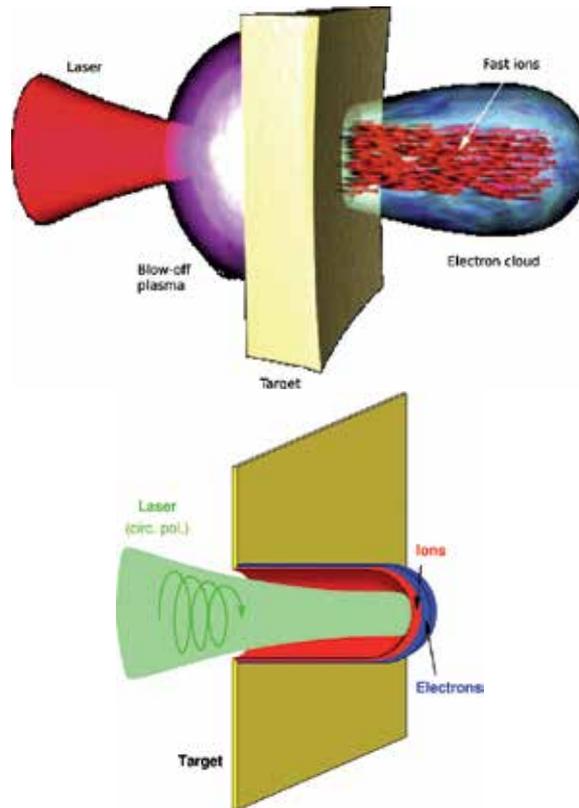


Fig. 1. Schematic picture of “Target Normal Sheath Acceleration” (TNSA, left)Wilks (2001) and Coherent Acceleration of Ions by Laser (CAIL, right), in particular Radiation Pressure Acceleration (RPA). The dynamics of electrons remains coherently slaving to the laser fields, in sharp contrast to TNSA Tajima (2009).

the electron energy gain is approximately proportional to a_0 , when a_0 is much greater than unity (which is the case for the data analyzed). Then through certain mechanisms the heated electrons transmit their energy to ions, which is again thus proportional to a_0 .

In the present review, however, we would like to report the progress that now emerges with a new class of experiments and theory that supports such experiments, in which a regime of much more efficient and possibly higher energy acceleration processes exists. We will scrutinize the process of this energy transfer between electrons and ions, both for TNSA and the new regime that we now describe as Coherent Acceleration of Ions by Laser (CAIL), i. e. a regime of interaction more direct than TNSA. However, it is worthwhile to look at one aspect of the laser interaction with electrons. At higher laser intensities above 10^{22} W/cm², numerical simulations seem to indicate that a laser could also accelerate protons to high energies Bulanov (2009). With a compact high-repetition laser system, however, the highest proton energy is still lower than 20 MeV in all experiments Fuchs (2006). In order to realize 200 MeV/u beams for proton/ion therapy, the laser intensity required should be as high as 10^{22} W/cm² Fuchs (2006).

Experiments, producing high-energy ions with sub-micrometer to nanometer thick targets that are much thinner than those used so far, have shown far superior acceleration

characteristics Henig (2009) and have recently attracted strong interest. A typical physical situation is depicted in the sketch in Fig. 1. With the emerging nanometer target of diamond-like carbon (DLC), the conversion efficiencies are one to two orders of magnitude higher ($> 10\%$) even with modest-energy lasers (less than 1 Joule per pulse, and highly repetitive lasers) compared with those in the regime of TNSA with the thicker targets, and so the laser pulse can accelerate the ions to higher energies. The experiments show that the proton energy increases as the target thickness decreases for a given laser intensity, and that there is an optimum thickness of the target (several nm) at which the maximum proton energy peaks and below which the proton energy now decreases.

This optimum thickness for the peak proton energy is consistent with the thickness dictated by the relation Esirkepov (2006); Liu (2008); Matsukado (2003); Rykovanov (2008)

$$a_0 \sim \sigma = \frac{n_0 d}{n_c \lambda_L}, \quad (2)$$

where σ is the (dimensionless) normalized electron areal density, n_0 the electron density of the target, and n_c is the critical density. Note that this optimum thickness for typically available laser intensities is much smaller than the previously attempted target thicknesses (for ion acceleration). Thus we attribute the observed singularly large value of the maximum proton energy in the recent experiments Steinke (2010; 2011) to the ability to identify and provide prepared thin targets of the order of nm to reach this optimum condition. In reality, at this target thickness the laser field comes to the point of partial penetration of the target, rendering the realization of optimum rather sensitive. The experiments show that transparency plays an important role in energy enhancement. As we shall show, in this new regime (CAIL) with σ/a_0 of the order of unity (as opposed to $\sigma/a_0 \gg 1$ in TNSA), the electron dynamics remains coherent, directly following the laser field. Thus we call this regime of acceleration Coherent Acceleration of Ions by Laser (CAIL).

2.2 How ions are accelerated with laser and electrons

The characteristics of the previous laser ion acceleration experiments (TNSA) are that (i) laser ion acceleration has great potential, particularly in its accelerating gradient (of the order of TeV/m) and thus compactness of acceleration; (ii) however, its progress has lagged since the initial observation Clarke (2000); Maksimchuck (2000); Snavely (2000) in 2000 in enhancing its energy and other aspects with less energy laser drive, often limited to several MeV energy gain Fuchs (2006); Robson (2007); (iii) further, the energy spectra of ions remain broad Fuchs (2006), except in exceptional cases Hegelich (2006); Schwoerer (2006); Ter-Avetisyan (2006); and (iv) the efficiency remains low Fuchs (2006). On the other hand, although the numbers achieved are so far not overwhelming, some reports indicate one or two possible ways out, when, for example, the plasma density is near the critical value Matsukado (2003); Yogo (2007).

The above situation may be broadly summarized as follows. The intense laser somehow heats electrons of the solid target to high energies, which contributes to a large space charge separation on a rapid time scale of electron runaway from the surface of the target (the rear surface), which pulls ions and makes them run after the escaped electrons. The electron heating involves complex processes, both coherent and individual particle processes (such as collisions), and the original electron motion in the intense laser field is cascaded down to a thermal spectrum of electrons that drive ions as described above. Firstly, this means that the electron spectrum is broadly spread (such as Maxwellian) and is certainly limited to or less than the ponderomotively driven electron energy of $m_e c^2 \sqrt{1 + a_0^2}$. The scaling to the intensity

of the laser never greatly exceeds $\sqrt{I_L}$, as shown in the limiting energy of electrons McKenna (2007); Robson (2007). Secondly, hot electrons suddenly escape from the target, so that the ions are unable to follow the electrons with the result that some fraction of these electrons run away from the ions and the rest of them are pulled back toward the ions. The ions are unable to be smoothly accelerated; in other words, the gradual (adiabatic) acceleration process is nonexistent. This non-adiabatic nature of the ion dynamics is the underlying reason for exhibiting properties (ii) – (iv). These features arise essentially from the mismatch between the group velocity of photons and the velocity of electrons subsequently energized and that of the ions. The ions remain slow and non-relativistic, while photons and electrons are relativistic. Thus, our principal direction is first to utilize the photon energy more directly rather than cascading through multiples of collisional processes, and secondly to transfer laser energy to electron energy and to ions more adiabatically. When the solid target is too thick, most ions remain stationary and the momenta of photons are spread over broadly. Thus we should limit the number of ions influenced by laser acceleration. Secondly, in order that the twofold interaction process of laser to electrons and from electrons to ions becomes more gradual, we need to slow down the photons and electrons and make them match the sluggish ions, at least initially, until they reach high speed Mako (1984). Laser electron acceleration Tajima (1979) may be easier in this sense, because light electrons at rest may be more easily trapped by the speeding photon-driven waves, whose velocity is near c , whereas the trapping velocity width Esaray (1995) is $\sqrt{e\Phi/m_e} \sim c$, where Φ is the ponderomotive potential. This ponderomotive potential is capable of inducing the wakefield with amplitude of the order of $E_w \sim m_e \omega_p c / e$. This is why in the laser electron acceleration the fast wakefield $v_{gr} \sim c$ can still trap stationary electrons (“self-injection”). Meanwhile for ions $\sqrt{e\Phi/m_i} \ll c$, and this value can only become $\sim c$, when $a_0 = \mathcal{O}(m_i/m_e)$ (ultrarelativistic), where m_i is the mass of ions. More importantly, in order to trap ions, the trapping velocity width of ions is much smaller than that for electrons

$$v_{i,tr} \sim \sqrt{\frac{m_e}{m_i} a_0} Q c, \quad (3)$$

where Q is the ion charge in units of e (electron charge). Thus, in the regime of our interest $1 \leq a_0 \ll m_i/m_e$ the accelerating structure has to move at a velocity within this $v_{i,tr} \ll c$. For ions to obtain net energy gain, the ion velocity needs to be within the trapping separatrix, which is situated over the velocity band that is centered at the phase velocity v_{ph} of the accelerating structure (we will call it a “bucket” later in Sec. 2.4.3) with a trapping width $v_{i,tr}$. Ions outside of this band (either above or below) simply oscillate in energy, but obtain no net energy gain. Even when the bucket velocity v_{ph} increases in time, ions that are trapped deeply enough may be kept trapped and, therefore, continue to gain energy from the bucket. This is the principle of gradual acceleration. Either when the velocity v_{ph} increases too suddenly or when ions are outside of the trapping separatrix, ions spill out or are left out of the accelerating structure. In order to accomplish the first goal, one way is to adopt a very thin foil so that the mass contained in this foil is tiny. Alternatively, a diluter medium such as a dense gas or matter with clusters could be used. In order to accomplish the second goal, the most direct way to do so is to choose the density of the target material to result in a vanishing group velocity of photons such that ions can respond adiabatically. In this regard, it further helps if we can control the velocity of the accelerating structure to match the accelerated ion velocity. These considerations lead us to consider to look at very thin foil targets and alternatively at matter at or close to the critical density.

Another consequence of this general consideration entails a strategy to slow down the electron motion after they are emitted from the target. This may be done by providing a concave geometry of the surface Tajima (2005) or other target preparations. One of the recent hopes is to employ circularly polarized laser pulses (CP). These, unlike the linearly polarized laser pulses, do not have sudden high frequency ($2\omega_L$) motions by linearly polarized photons, but only result in a smooth ponderomotive acceleration of the target Klimo (2009); Macchi (2005); Qiao (2009); Robinson (2008); Rykovanov (2008). If the CP ideally works, the ponderomotive force on electrons induces and matches the electrostatic force generated between the charge separated ions and electrons, and this keeps the overall dynamics smooth and adiabatic. A somewhat extreme and earlier rendition of this concept may be that of the radiation dominant acceleration by Ashour-Abdalla et al. Ashour-Abdalla (1981) and Esirkepov et al. Esirkepov (2004), where the laser photon pressure drives electrons to relativistic speed that drags ions also to relativistic speed closely following the electrons.

2.3 CAIL and RPA experiments

In this section we present recent experimental progress on laser ion acceleration, which shows marked improvements over experiments in the regime of TNSA: in (i) the total conversion efficiency of laser energy into ion energy, (ii) the maximum observed ion energies, and (iii) the production of monoenergetic peaks in the ion energy spectra. We compare these results to previous experiments, which are based on the TNSA mechanism, established over the last 10 years in experimental and theoretical efforts. In TNSA one has observed for certain laser parameters, e.g. 1 PW lasers with 500 fs, maximum conversion efficiencies of 12 %, or one has observed maximum proton energies of 58 MeV Snavely (2000) and by filtering out small target regions could produce quasi-monoenergetic ion spectra Hegelich (2006); Schwoerer (2006); Ter-Avetisyan (2006). At present, similar experimental values of efficiency, energy, etc. can be obtained with much smaller lasers. The formerly predicted laser parameters based on TNSA Robson (2007) to reach ion energies of 240 MeV for protons or 450 MeV/u for carbon ions for medical therapy facilities have been rather big and the laser would end up costly. The new regime, where coherent dynamics of electrons in accelerating ions by laser (CAIL) plays a significant role, can yield scaling laws that lend to a prospect that short-pulse, high-intensity lasers with high repetition rate may drive ion beams competitive with classical radio-frequency accelerator systems.

2.3.1 Laser ion acceleration with ultra-thin foils (CAIL)

In order to realize the CAIL regime, one wishes to employ nanometer thick target foils together with high-intensity short pulse lasers. In search of ultra-thin free standing foils that withstand strong ion and electron bombardment, DLC foils appear to be eminently suited. They have a very high tensile strength, large hardness, good heat conduction, high heat resistance and, when used as stripper foils, they show a large survival rate for ion bombardment. With a special production technique free-standing foils with 75 % sp^3 bonds – diamond-like bonds – can be produced.

The thickness of the DLC-foils has been characterized by means of an atomic force microscope (AFM) with an accuracy close to 0.5 nm Tajima (2009). Furthermore, the detailed depth composition – showing also front layer contaminations – was measured via Elastic Recoil Detection Analysis (ERDA) Tajima (2009).

A second ingredient in these laser acceleration experiments Henig (2009); Steinke (2010) is an ultra-high contrast of the laser pulses to avoid the preheating and expansion of the target before the interaction with the main laser pulse. The intensity of prepulses and the amplified

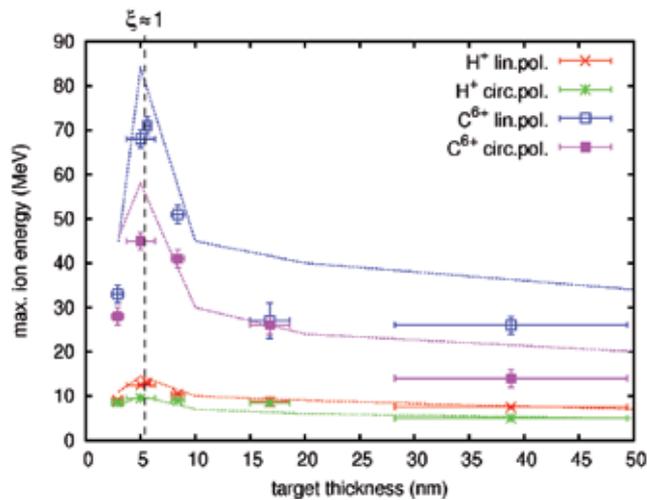


Fig. 2. Maximum cutoff ion energies as a function of target thickness in the regime of CAIL experiments Henig (2009). Theoretical curves are from the CAIL theory. Observed values and theory (CAIL) are in good agreement over a broad parameter range. The optimum condition is realized at a thickness parameter $\sigma \approx a_0$ Tajima (2009).

spontaneous emission (ASE) pedestal are characterized with a 3rd order auto correlator, yielding typical values of 10^{-7} at 10 ps before the main pulse. This value was further improved by a recollimating double plasma mirror, which lets the low intensity prepulse pass through, while it reflects the high intensity part of the pulse. In this way an estimated contrast of $\sim 10^{-11}$ was achieved. For the longer laser pulses the contrast was improved by Self-Pumped Optical Parametric Amplification (SPOPA) Shah (2009), using nonlinear optical effects and thus avoiding the 50% energy loss of the double plasma mirror.

Ultra-thin foils in two regimes have been investigated so far: (i) for laser pulses of 45 fs duration at the laser of the Max-Born Institute (MBI) in Berlin and (ii) for laser pulses with 700 fs duration at the Trident laser in Los Alamos. Laser accelerated ions were measured with a Thomson parabola spectrometer.

2.3.2 Characteristics with ultra-thin targets

Let us now discuss jointly all results for ultra-thin targets in comparison to the thicker targets results, where TNSA is the dominant acceleration mechanism.

In Fig. 3 we compare the conversion efficiency from laser energy to ion energy. At the optimum target thickness and 45 fs laser pulses, an optimum conversion efficiency of 10% for laser energy into ion energy was obtained by integrating all protons above 2 MeV and all carbon ions above 5 MeV for the linearly polarized laser. Correspondingly, $\sim 9\%$ was obtained for circular polarization. For the 700 fs experiment a lower efficiency of about 2% was observed. The values are shown in Fig. 3 in the comparison of the CAIL results with efficiencies for thicker foil targets (TNSA). We show the general trends of the TNSA mechanism by theoretical results from the fluid model Fuchs (2006), which describes the experimental data quite well. In addition, we show specific experimental results for the ASTRA laser Spencer (2003), the RAL PW laser McKenna (2004) and the NOVA PW laser Snavely (2000). We observe approximately a 50 fold increase in conversion efficiency for thin targets with the 45 fs pulses compared to TNSA at the same laser intensity, also taking our

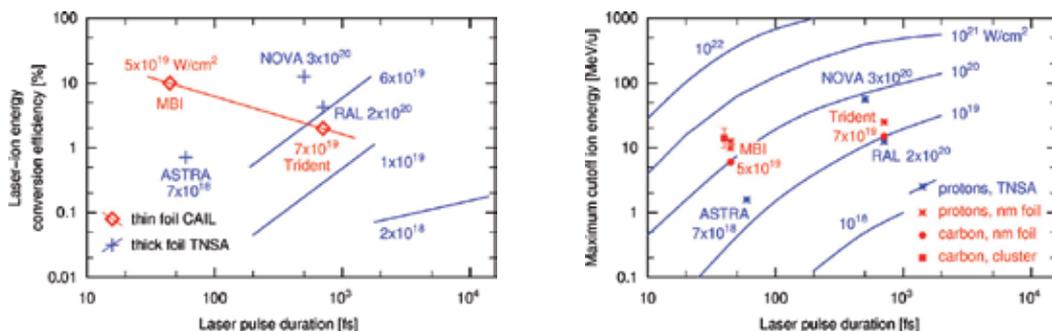


Fig. 3. Left: Conversion efficiency of laser energy to ion energy comparing results from thick targets and the TNSA mechanism to measurements with ultra-thin targets in the regime of CAIL (red diamonds and line). For the TNSA mechanism smooth curves from the fluid model by J. Fuchs Fuchs (2006) are shown together with some experimental points: ASTRA Spencer (2003), NOVA Snavely (2000), RAL McKenna (2004). Right: Maximum cutoff energies of ions given in MeV/u as a function of laser pulse duration. The energy gain by CAIL experiments is embedded with red dots in the predicted curves of TNSA. Note that in shorter pulses, energies by CAIL are more than an order of magnitude higher than for TNSA. Here also the results from the cluster target of Sec. 3.1.3 are shown Tajima (2009).

own measurements at larger target thicknesses into account. For 700 fs pulse duration the efficiencies of thick target TNSA results and thin target results are comparable. If one increases the pulse duration, for short pulses the optimum target areal electron density $\sigma \approx a_0$, while for longer pulses σ has to be significantly larger to reach the maximum cutoff energy. Here one first has to reach the relativistic transparency of the target by expanding the target (see Sec. 2.4.2), explaining in part the reduced conversion efficiency. For somewhat shorter laser pulses and cold adiabatic RPA, a 60% conversion efficiency has been predicted theoretically for higher laser intensities in an idealized 1D PIC simulation Robinson (2008). Experimentally, the optimum conditions depend on many parameters such as optimum laser focusing to prevent heating of the walls of the bulged out target Klimo (2009).

In Fig. 3 we compare the maximum cutoff ion energy for protons and carbon ions between measurements with ultra-thin targets and μm thick targets, where the TNSA mechanism dominates. In Fig. 3 for TNSA only proton energies are shown for model calculations, which reproduce the experiments quite well Fuchs (2006). Approximately an increase by a factor of 10 is observed for the short laser pulses of 45 fs in the cutoff energies between TNSA and CAIL. An overall increase in energy occurs for both processes for longer laser pulses. At the PW level the proton energies for TNSA vary from 58 MeV Snavely (2000) to 13 MeV McKenna (2004) for similar pulse energies of 500 J and 400 J. Thus it is difficult to obtain a good comparison with the results of ultra-thin targets. For the carbon ions and the longer pulse of 700 fs, a factor of 4 higher energies were observed for a factor of 4 smaller pulse energies, pointing to a clear advantage of the ultra-thin targets in CAIL. For the short pulses of 45 fs again an order of magnitude improvement is seen for the ultra-thin targets (CAIL).

2.3.3 Cluster target experiments

Fukuda et al. Fukuda (2009) explored a different path of laser ion acceleration for hadron therapy: They use a gas jet target mixed with submicron clusters. The target consists of a He gas jet with a density of $1.5 \cdot 10^{19} \text{ cm}^{-3}$, into which solid-density CO₂ clusters with an average diameter of several 100 nm are dispersed at a cluster density of $3 \cdot 10^9 \text{ cm}^{-3}$. This constitutes

an average density at or near the critical density. A well-formed self-channeling phenomenon coincides with the detection of high energy ions. They observe in their ion spectrometers rather high ion energies in the range of 10 – 20 MeV/u for carbon, oxygen, or helium ions with a small divergence angle of 3° Fukuda (2009; 2011). This maximum energy value is much higher than expected from TNSA.

It is noted that the average density near the critical density may have played an important role, perhaps similarly to Matsukado et al. Matsukado (2003) and Yogo et al. Yogo (2007). In these experiments the enhancement of ion energies was noted when the density is in the neighborhood of the critical value. Thus the acceleration with clusters has commonality with the dynamics observed in the long pulsed thin target (Sec. 2.4.2) after the laser burns through the target (see also Sec. 2.4.2), when the density becomes critical through relativistic transparency. Near the critical density, as we noted, the group velocity of photons is small. In recent simulations Kishimoto (2009) the maximum ion energy is observed to scale with the pulse length, with the intensity fixed, and inversely proportional to the size of the clusters. Thus the nano-structured targets may provide an enhanced coupling of laser and ions Kishimoto (2000). Higher efficiencies may be obtained by increasing the pulse energy, the contrast of the laser and using much smaller clusters with higher density.

Recently, ion acceleration up to 50 ± 25 MeV/u has been achieved using the cluster-gas target with the 20 TW (40 fs, 800 mJ) J-KAREN laser system Fukuda (2010) and five times more power than the earlier experiment. If we combine the two experimental results, an extrapolation shows that a 100 TW-class Ti:Sa laser is capable to generate 200 MeV/u ions. In addition, particle-in-cell simulations have delivered an energy scaling of ions generated by the magnetic vortex acceleration in near-critical density plasmas Nakamura (2010), providing a possible acceleration mechanism for the cluster-gas targets. The scaling suggests that a 100 TW-class laser is capable to generate 200 MeV protons, consistent with the experiments.

Therefore, both experiments and theory predict that 100-TW class Ti:Sa lasers, which are achievable using the present laser technologies, reach the ballpark of 200 MeV ions applicable to medical use. Such experiments will be conducted in the near future.

2.4 Towards more efficient and gradual acceleration

have been motivated by much thicker the TNSA regime electrons are first accelerated impinging relativistic laser pulse and they penetrate the target target at the rear side, field that points normal to was the origin of the terminology of the TNSA Most electrons are forced to turn around quasi-stationary assumed to follow a thermal studies of the conventional Most of the theories for thicker targets are based on TNSA Andreev (2008); Ceccotti (2002); Mora (2003; 2005); Passoni (2004; 2008). Though this mechanism is widely used in the interpretation of the experimental results, it does not apply to the ultrathin nanometer scale targets because the direct laser field and partially transmitted laser pulse play an important role in electron dynamics and the energetic electrons oscillate coherently, instead of showing chaotic thermal motion. In order to design a compact accelerator with a modestly intense laser for medical applications we discuss here, it is desirable to understand the new emerging regime that is promising for this purpose.

2.4.1 Efficient energy gain in the CAIL regime

In case of a thin target, electron motions maintain primarily those organized characteristics directly influenced by the laser field in the CAIL regime, rather than chaotic and thermal motions of electrons resulting from laser heating on the front surface, where the laser is either absorbed or reflected for the TNSA regime.

It may be instructive to compare conceptual differences of the regime of TNSA and that of CAIL. See Ref Tajima (2009) for details. In the TNSA case the laser interacts primarily at the front surface of the target, while in all cases of the CAIL interaction takes place at the rear surface. In the regime of TNSA electrons that have gained energy at or near the front surface propagate through the target and escape from the rear surface with a broad energy spread. In a model problem Mako (1984); Tajima (1978) in which the electron beam (with a delta-function energy spectrum) enters from the metallic surface that may be regarded as the rear surface, the energy gain was analyzed. In CAIL, once the laser penetrates the target and electrons gain energy from the laser, the electron dynamics in the presence of the rear surface is once again similar to this process.

In an ultra-thin target, the laser electromagnetic fields largely sustain coherent motions of electrons. As partially or fully penetrated laser fields in addition to the laser fields in the target, the electron motion under laser fields is intact and is characterized by the transverse field. The electron energy consists of two contributions, the kinetic energy of (organized) electrons under the laser and the ponderomotive potential of the partially penetrated laser fields that help sustain the electron forward momentum. Following the analysis of Ref. Mako (1984); Tajima (2009), the maximum ion energy is

$$\varepsilon_{\max,i} = (2\alpha + 1) Q \varepsilon_0, \quad (4)$$

where ε_0 is given by Eq. (1). The enhancement of the ion energy gain in Eq. (4) in the CAIL is due to the factor $(2\alpha + 1)$ compared with the equivalent TNSA energy gain Eq. (1). In Eq.(4) we see that the ion energy is greater if the coherence parameter of electrons α is greater: (a) the energy gain of the present case is several times higher than that of TNSA; (b) the energy gain maximizes at the optimum thickness of $\sigma \approx a_0$ mentioned earlier in Sec. 2.1 for CAIL, as opposed in a much thicker target for TNSA. These features are also seen in Fig. 4 later.

2.4.2 Relativistic transparency

Understanding the dynamics of the laser pulse and the target evolution is important when the pulse is longer (\sim hundreds of fs) than tens of fs and / or the target is thicker than a few nm. We now consider the cases when the target is thicker ($(\sigma/a_0) \gg 1$) than when it is immediately influenced by the laser fields, but still σ/a_0 is less than for TNSA. In this case the laser does not immediately penetrate through the target. We can delineate at least three stages. The first stage is similar to the situation we described above for $\sigma \approx a_0$. The laser just impinges on the thin surface layer of the dense target. The second stage starts when the target begins to expand by the laser interaction, primarily in the direction of laser propagation until the plasma becomes relativistically transparent at time t_1 . After this relativistic transparency time t_1 , the plasma expands in all three dimensions. The third stage begins when the plasma becomes underdense at time t_2 (the classical transparency time) and lasts until the pulse is over.

One of the distinguishing features of the thin target CAIL regime, as compared with TNSA, is the presence of the relativistic transparency time t_1 before the pulse length τ , so that the laser pulse emerges or interacts with the entire target before the pulse is gone. We find this time to be Yan (2009a)

$$t_1 \cong \left(\frac{12}{\pi^2} \right)^{1/4} \frac{N^{1/2}}{a_0^{1/2}} (\tau d / C_s)^{1/2}. \quad (5)$$

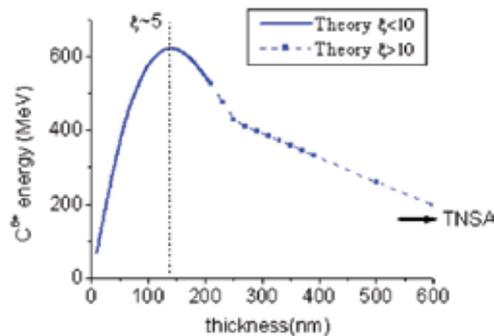


Fig. 4. The maximum ion energy driven by the laser pulse as a function of target thickness in the regime of CAIL. The optimum is reached at $\zeta = (\sigma/a_0) \sim 5$. Energies for thicker targets decrease from this value, eventually leading to the value often found in the TNSA regime (μm or more in this graph). C^{6+} energy gain estimated from Eq.(7) as a function of target thickness and with $\alpha \cong 3$. (For a given laser pulse length at 700 fs and laser amplitude $a_0 = 20$). The contribution of the electron energy gain after the relativistic transparency has been reached is dominant Tajima (2009). The experimental points by Hegelich et al., Hegelich (2011) closely follow the theoretical prediction

Here the sound speed $C_s \cong (Qm_e c^2 a_0 / m_i)^{1/2}$ and $N = n_0 / n_c$. The relativistic transparency time t_1 in Eq.(5) is approximately the geometrical mean of the laser pulse length τ and the traverse time over the target by the sound speed.

Yin et al. Yin (2007) have found in their 3D simulation that for long pulse irradiation the pulse exhibits an epoch of burn-through (and relativistic transparency). This phenomenon is when the laser penetrates the target and eventually emerges from the rear end of the target. This corresponds precisely to the second period between t_1 and t_2 and in fact most of the acceleration takes place shortly after t_1 . We now characterize the physical processes including these phenomena. Beyond time t_1 , the plasma is relativistically transparent so that the laser can now interact with the (expanded) target plasma in its entirety. It can also now expand in three dimensions. For 3D isotropic expansion, it takes time Δt during which the normalized density reduces from γ to 1 Yan (2009a) as:

$$\Delta t = \frac{Nd(\gamma^{1/3} - 1)}{\gamma C_s} \frac{1}{\sin((\pi/2\tau)t_1)}. \quad (6)$$

Now the time t_2 , when the plasma becomes underdense, is given as $t_2 = \Delta t + t_1$.

We examine the physical situation at time t_1 , when the laser pulse has penetrated the entire target with the relativistic transparency and we may regard that the laser begins to drive the entire plasma electrons from this already expanded target. An expression in a closed form for the ion energy gain between time t_1 and t_2 in the case of a laser pulse with a duration longer than the relativistic transparency (rt) time t_1 has been obtained expanding the idea in Eq. (4) as:

$$\varepsilon_{\max,i,rt} = (2\alpha + 1) Q\bar{\varepsilon}_0 \left((1 + \omega_L(t_2 - t_1))^{1/2\alpha+1} - 1 \right). \quad (7)$$

In Fig. 4 we plot the total energy gain in the case of carbon ions from this formula as a function of target thickness. Once again the optimum thickness, for which the ion gain is maximum, is sharply realized in the CAIL regime. Towards the TNSA regime the ion energy

decreases substantially. Thus theory has guided the experiments that (remakably) agree with the predictions Hegelich (2011).

It is noteworthy to consider how the photon pulse behaves right after the relativistic transparency time t_1 . The group velocity of the laser pulse $v_{gr} = c \sqrt{1 - \omega_p^2/\omega_L^2}$ vanishes at $t = t_1$. At this moment the ponderomotive structure of photons is stationary, which pushes electrons as well as ions effectively forward. This is because the heavy and sluggish ions can respond easily to this stationary potential. As the laser penetrates further and the plasma density decreases below $n_{cr} \cdot \gamma$, the group velocity begins to increase. In our model case Yan (2009a) of the laser temporal structure of $a_0 \propto \sin^2((\pi/2\tau)t)$, the group velocity increases as

$$v_{gr}(t) \sim 2c \cot((\pi/2\tau)t_1) \sqrt{(\pi/2\tau)(t - t_1)}. \quad (8)$$

This means that the speed of the accelerating structure – made up of the electron layer driven by the laser ponderomotive force and the ion layer that is attached to the former by the electrostatic force – is picking up quickly from zero. This suggests that if we can slow down the photon group velocity, the rate of increase of the photon group velocity and thus the accelerating structure is reduced and, therefore, the adiabatic nature of acceleration becomes more pronounced. Such may be accomplished by increasing the density of the plasma behind the solid target by a further material. Nakamura et al. Nakamura (2010) have investigated near critical density acceleration, which is related to this point as seen in Eq.(8).

2.4.3 Radiation Pressure Acceleration (RPA)

Mono-energetic ion beams are one of the important requirements of ion beam therapy. The Coherent Acceleration of Ions (CAIL) by linearly polarized pulses can efficiently accelerate ions to higher energy by using nanometer targets. Recently, theoretical attention has focused on the use of circularly polarized (CP) laser pulses in the CAIL regime to accelerate high density ion bunches at the front surface of thin foils. For CP pulses, the ponderomotive force has no oscillating component as discussed; hence, electrons are steadily pushed forward, inducing a charge separation field which can accelerate ions. It is expected to provide a more adiabatic interaction so that mono-energetic ion beams may be realized, in this case in the regime called Radiation Pressure Acceleration (RPA). There is a regime of phase stable acceleration in the interaction of a CP laser with a thin foil in a certain parameter range, where the proton beam is synchronously accelerated and bunched like in a conventional radio frequency (RF) accelerator. This synchronous acceleration leads to the acceleration regime in which the position of ions is well tied with the accelerating structure made up of the laser ponderomotive potential and the electron layer. Therefore, ions may be trapped in this accelerating bucket, in which they may show a phase-stable behavior. That is, ions exhibit phase stable oscillations (synchrotron oscillations).

A simple model to elucidate the bunch formation in the phase stable acceleration has been considered Yan (2009a). We introduce the relative ion position $\zeta = (x_i - x_r)$ with the compressed electron layer $-l_s/2 \leq \zeta \leq l_s/2$, where $x_r = D + l_s/2$ represents the position for the center-of-mass reference particle. The force acting on a test ion is given by $F_i = q_i E_m (1 - (x_i - D)/l_s)$. Thus, the equation of motion for the proton is

$$\frac{d^2 x_i}{dt^2} = \frac{Qe E_m}{m_i} \left(1 - \frac{x_i - D}{l_s} \right). \quad (9)$$

Eq.(9) shows that the center of mass x_r moves with constant acceleration as

$$x_s = (1/2)(d/2)\omega_{pi}^2 t^2, \quad (10)$$

where $\omega_{pi}^2 = 4\pi e^2 Q n_0 / m_i$, d and n_0 are the original foil thickness and electron density. The phase motion (ζ, t) around x_r is showing oscillatory motions:

$$\ddot{\zeta} = -\Omega^2 \zeta, \quad \Omega^2 = \frac{Qe4\pi en_0 d}{m_i l_s}, \quad (11)$$

where Ω is called the frequency of the synchrotron oscillation motion in longitudinal direction Chao (1999). This means that the center of mass accelerates with a constant rate (in the nonrelativistic regime) as in Eq.(10), while the individual ions oscillate around the center trapped by the bucket.

The bucket velocity width may be determined from Eq.(11):

$$v_{i,buc} = \zeta \Omega = \sqrt{\frac{m_e}{m_i}} Q a_0 c N^{-1/4}. \quad (12)$$

This bucket size is close to (and slightly less than) the trapping velocity width from Eq.(3). As long as $N > \gamma$ (or $t \leq t_1$), $v_{i,buc} \leq v_{i,tr}$, while for $N < \gamma$ ($t > t_1$) $v_{i,buc} \geq v_{i,tr}$ and some of the ions in the bucket begin to spill over.

The energy spread of trapped ions in the bucket of the accelerating structure with respect to the energy ε_r of the reference particle is

$$\Delta\varepsilon/\varepsilon_r = 2\zeta_0 \Omega / \sqrt{2m_i \varepsilon_r}. \quad (13)$$

If we take $\zeta_0 = l_s/2$ and $\varepsilon_r = 400$ MeV, the energy spread will be less than 4%, which agrees well with the simulation results.

In 1D simulations the plasma is kept cold and the target is pushed forward as a whole, so an ideal mono-energetic ion beam can be generated. A quasi-monoenergetic carbon ion beam with 17% energy spread has been observed in recent experiments Henig (2009): $\Delta\varepsilon/\varepsilon_r \sim 17\%$ is about 3 times higher than the estimation by Eq.(13) because of the multi-dimensional effects. In real situations typically the laser intensity is not uniform, the transverse profile tends to bend the flat target, and foil electrons are heavily heated by the oblique incident laser, in spite of the CP pulse. If and when electrons become hot or the laser leaks through, the bucket begins to collapse and the energy spread drastically increases.

2.4.4 Improved acceleration schemes

When the electron dynamics is slow enough that ions evolve less suddenly, i. e. adiabatically Chao (1999), the final energy gain of electrons (and thus that of ions) may not be that of the instantaneous energy dictated by the expression of ε_0 . For example, we have remarked a case with a circularly polarized pulse. In the latter, for example, the pulse should cause less electron energy gain than in the linearly polarized case. Therefore, the cloud of electrons cannot instantaneously shoot out of the foil, but rather leaves the target gradually. This renders a possibility that the electron energy is not only proportional to the field strength (as proportional to a_0), but also to the time during which electrons are accelerated by $v \times B$ if this is much longer than the laser period. When electrons substantially co-move with the laser pulse, this time can be proportional to a_0 or some fraction of it, leading to a proportionality greater than a_0 such as a_0^2 . We anticipate more results to come in advancing the ion energy by laser acceleration spurred by the current theoretical understanding of the physics.

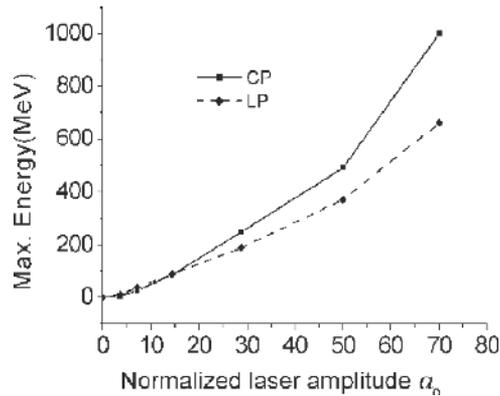


Fig. 5. Maximum proton energy versus a_0 obtained in 2D PIC simulations. In the modest a_0 regime ($15 \geq a_0 > 1$) the energy gains by LP and CP are not much different, while that by CP is much greater than by LP for large a_0 ($a_0 \geq 15$). The exponent to a_0 for CP seems to increase from less than 2 in $a_0 \leq 15$ towards 2 in $a_0 \geq 15$.

In PIC simulations, an a_0^2 scaling is observed in Rykovanov (2008) using circularly polarized (CP) laser pulses. However, this is only a 1D simulation and bending and boring effects are not considered. With 2D simulations, the scaling with the normalized vector potential a_0 is only $a_0^{1.1}$ in the linear polarized case (and the fit looks much better than Eq. (1), while for the circular case the maximum proton energy scales with $a_0^{1.6}$ at the lower intensity for $a_0 \leq 30$. It tends to be closer to an a_0^2 scaling for larger a_0 values (see Fig. 5), including the highly relativistic regime Esirkepov (2004). This tendency of having less energy (or a smaller exponent to a_0) in the 2D simulation than the one shown in the idealized 1D model, may be due to several reasons. One is the bending or bulging (the convex shaping as viewed from the rear surface, where ions are emitted) of the thin target by the impinging laser pulse. This causes the excess plasma electron heating by the obliquely incident laser electric fields and thus contributes to hot electrons that run away from the target leaving ions far behind, yielding non-adiabatic electrons and thus ion dynamics. Secondly, once the laser ponderomotive potential penetrates through the thin target, the till then slow motion of the ponderomotive potential now begins to pick up its speed, as the density of the plasma seen by the laser is less than the relativistic transparent density (in terms of the timing of the interaction corresponding to time t_1). Once the laser increases its group velocity in this less dense region, only electrons can keep up with photons, while ions are left behind. When this develops, we see that the nicely closed phase space circle is now skewed, eventually leading to a collapse of the bucket. (See details in Ref. Tajima (2009), there Figs. 18 and Fig. 19).

In order to further improve these situations, we envision to counteract the convex bulging by instituting a concave shape to the target. This little manipulation may improve the energy enhancement by a factor of a few Tajima (2005). A recent simulation demonstrates this Wang (2010). It should also help if we adopt a cone Kodama (2002) in front of the foil, which can collect the laser power to intensify the radiance of the laser. Wang et al. Wang (2011) have suggested a gas medium (in front of the thin foil) that leads to the enhanced laser intensity. To further arrest the collapsing trapping bucket by the accelerating photons after they transmit through the thin target, we could let them slow down again by adding supplementary target material such as dense gas/clusters, a foam target or a mesh of carbon nanotubes behind the rear surface. Such would decrease the group velocity of the laser after it passes through the

thin solid foil and increase the interaction time between the laser pulse and the plasma and therefore the accelerating time for ions. With special target manufacturing techniques (e.g. concave as looked from the rear surface) or hemispheric targets, it may be possible to increase the power of a_0 in ion energy that is important as an outlook, because at present we need a much bigger laser if we want to reach the medical energy of 200 MeV/u. If these additional measures can enhance the ion acceleration time, it will be perhaps possible to reduce the necessary laser intensity and still reach the same energies, contributing to a less demanding laser power for the necessary energy regime.

Even if the necessary energies are reached, there are many additional requirements a therapy system needs to satisfy, as was discussed in Sec. 1. These are by no means easy tasks and pose challenges for us researchers. On the other hand, we do have a host of new ways to manipulate intense ion and laser beams by harnessing the relativistic dynamics of the laser and its plasma interaction Mourou (2006). For example, our ability to simultaneously generate an ion beam with coherent X-rays Tsakiris (2006) from the laser-thin target interaction can allow us to perform unprecedented accurate X-ray diagnosis such as phase contrast imaging Pfeiffer (2006). This would give us the additional ability of accurate imaging simultaneous with therapy. With the increasing ability to detect smaller tumors, the laser driven ion therapy method with the imaging guidance and with on-line dose verification techniques Bolton (2010); Kormoll (2011) may become a suitable clinical option for safe treatment of small tumors Murakami (2008).

3. Medical radioisotopes with high specific activity produced in photonuclear reactions

The laser-driven γ beams are spurring the possible clinical usage of a novel class of radioisotopes that are very useful for nuclear medicine but are not easy to obtain otherwise. These radioisotopes may be delivered to specific cells/DNA/proteins/peptides of the tumor with a specific vector drug, where cancer cells may be killed by their radioactivity. This method is not hampered by the beam scattering of ion therapy in Sec. 2, nor restricted by non-metastasis, which are the general limitations of EBRT.

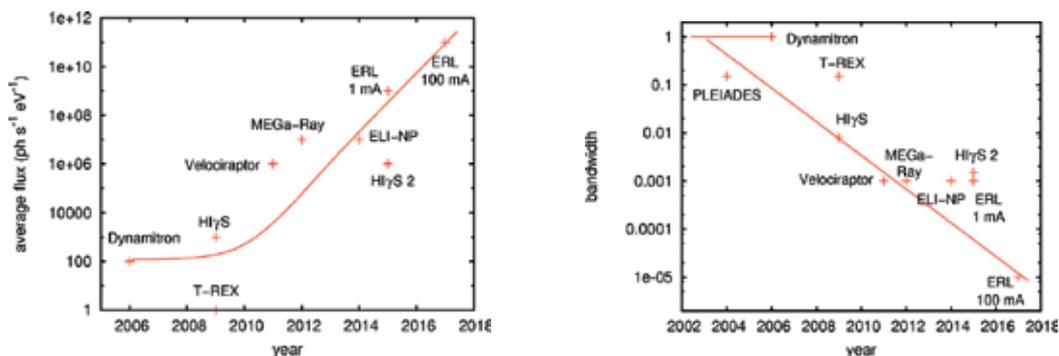


Fig. 6. Progress in flux of γ beams (left) and (right) progress in bandwidth $\Delta E_\gamma / E_\gamma$ of high-energy γ beams (≈ 10 MeV) for different (existing and planned) γ beam facilities.

In a new development the intense, high repetition rate, diode pumped lasers in combination with intense, brilliant, relativistic electron beams allow to produce very intense, brilliant γ beams via Compton back-scattering. Thus in about 2 years from now the MEGa-ray project

at LLNL Barty (2010) will have γ beams, which have $10^{(4-6)}$ times higher flux than the best existing γ beams and it can be anticipated from ongoing developments that in 5-10 years even 10^4 times more intense γ beams will become available, which will allow to produce many new medical radioisotopes for diagnostics and therapy. This is illustrated in Fig. 6. The new γ beams will also have a much smaller band width $\Delta E_\gamma/E_\gamma$, allowing to address individual nuclear levels with strong population. Here, on the one hand, by (γ, γ') photoexcitation new nuclear isomers can be produced, which decay frequently by many conversion and Auger electrons, allowing for a short-range killing of tumor cells in the surrounding 10-200 μm range after they have been transported to the overexpressed acceptors of the cancer cells. But also by $(\gamma, xn + yp)$ photonuclear reactions many new medical radioisotopes can be produced. We will discuss in detail many new specific radioisotopes. As an example we here want to mention so-called "matched" pairs for diagnostics and therapy of the same chemical element. Here pairs like: $^{44}\text{Sc}/^{47}\text{Sc}$, ^{61}Cu or $^{64}\text{Cu}/^{67}\text{Cu}$, $^{86}\text{Y}/^{90}\text{Y}$, ^{123}I or $^{124}\text{I}/^{131}\text{I}$ or $^{152}\text{Tb}/^{149}\text{Tb}$ or ^{161}Tb are of special interest, where one of the isotopes was so far difficult to produce by classical methods. Here the basic idea is to use bioconjugates Schiepers (2006), that show a high affinity and selectivity to bind to peptide receptors or antigens, that are overexpressed on certain cancer cells compared to normal cells. As shown in Fig. 7 the suitable radioisotope is placed into the chelator end of the bioconjugate. These therapies are called Peptide Receptor Radio Therapy (PRRT), when peptides are used as bioconjugates or radioimmunotherapy (RIT), when antibodies are used. This therapy allows to fight diseases, which are not localized or cancer types with multiple metastasis. Once the suitable radioisotopes have been produced the main task stays with radiochemistry and radiopharmaceutics to build the proper bioconjugates to reach the cancer cells in the optimum way. While we are pushing for the treatment of very small tumors in laser-driven ion therapy, with the new therapeutic medical radioisotopes one is going for shorter range emitted radiation (α particles, low-energy electrons) only killing cancer cells and cancer stem cells in the immediate surrounding, where the bioconjugate was delivered.

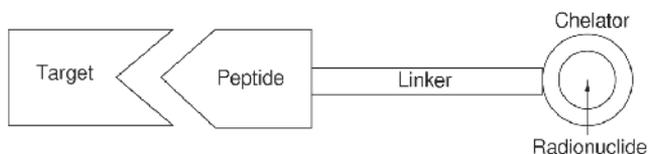


Fig. 7. Schematic picture explaining how a specific radionuclide can be transported to a specific peptide receptor.

3.1 Brilliant γ beams

In the next years γ beams, presently under construction, will become available with that much increased flux, that a good production of medical radioisotopes becomes possible. These γ beams are produced via Compton back-scattering of laser photons from a relativistic electron beam. photonuclear physics is the High-Intensity γ -ray Source (HI γ S) at Duke University (USA). It uses the Compton back-scattering of photons, provided by a high-intensity Free-Electron Laser (FEL), in order to produce a brilliant γ beam. The γ intensity in the energy range between 1 MeV and 160 MeV amounts to 10^8s^{-1} with a band width of about 5% Weller (2009). There are plans to upgrade the facility by using infrared laser light in a longitudinal enhancement cavity to increase the intensity to $2 * 10^{12}\text{s}^{-1}$ and the band width to about 0.1 - 0.2 % Wu (2011). Besides the storage ring approach an approach with a electron linac is pursued. Here the project T-REX with a normal conduction S-band electron linac was

recently terminated Albert (2010; 2011). Here a new brilliant Mono-Energetic Gamma-ray (MEGa-ray) facility at Lawrence Livermore National Laboratory (USA) is based on a normal conducting 12 GHz electron linac and should yield already in the beginning of 2013 for γ energies between 0.5 and 4 MeV a γ intensity of 10^{13}s^{-1} with an energy band width of down to 10^{-3} and a brilliance of $10^{22}/[\text{mm}^2\text{mrad}^2\text{s} 0.1\%\text{BW}]$ Barty (2010). Using the same accelerator technology, at the upcoming Extreme Light Infrastructure - Nuclear Physics (ELI-NP) facility in Bucharest, a γ beam will become available until 2015, providing about the same γ intensity and band width in the energy range of 1-25 MeV ELINP (2010). Even more efficient interactions between electron bunches and laser pulses from diode pumped lasers are under investigation, which will improve the γ flux from the presently given value of $10^6/(\text{eVs})$ by two orders of magnitude.

At present, great efforts are also invested all over the world to realize highly brilliant γ beams based on the Energy Recovery Linac (ERL) technology. The Energy Recovery Linac (ERL) requires a new type of superconducting electron accelerator that provides a highly brilliant, high-intensity electron beam. The main components of an ERL are an electron injector, a superconducting linac, and an energy recovery loop. After injection from a highly brilliant electron source, the electrons are accelerated by the time-varying radio-frequency field of the superconducting linac. The electron bunches are transported once through a recirculation loop and are re-injected into the linac during the decelerating RF phase of the superconducting cavities. So the beam dump has to take the electron bunches only with low energy, while the main part of the electron energy is recycled. At ERLs highly brilliant γ beams can be created by Compton back-scattering of photons with high energy (0.1-100 MeV), again recirculating the photons in a high finesse cavity with MW power or using ring-down cavities to overcome the small Compton cross section. ERL technology is pioneered at Cornell University (together with Thomas Jefferson National Laboratory) Bilderback (2010a;b); Liepe (2010), where an ERL is presently under construction for a 5 GeV, 100 mA electron beam. $> 10^{16}\text{s}^{-1}$ in an energy range of 0.5 - 25 MeV. Such a facility would provide a brilliant pulsed γ beam with a narrow band width much smaller than 10^{-3} and very high flux of $(10^{10} - 10^{11})/(\text{eVs})$. Since due to thermal Doppler-broadening the nuclear levels have typical widths of a few eV, good yields for medical isotopes are expected. For the new γ beams with better band width and small opening angle, we may use arrays of refractory γ lenses Schroer (2005); Vaughau (2011). In this way we could focus the γ beams to very small spot size (10 nm) for the first time and it becomes feasible to always use enriched targets for medical radioisotopes with high specific activity.

3.2 Presently used nuclear reactions to produce medical radioisotopes

Today the most frequently employed nuclear reactions for the production of medical radioisotopes are:

1. **Neutron capture** Neutron capture (n,γ) reactions transmute a stable isotope into a radioactive isotope of the same element. High specific activities are obtained, if the (n,γ) cross section is high and the target is irradiated with a high neutron flux. Neutrons most useful for (n,γ) reactions have energies from meV to keV (thermal and epithermal neutrons) and are provided in the irradiation positions of high flux reactors at flux densities of 10^{14} n/(cm^2s) up to few 10^{15} n/(cm^2s). If the neutron capture cross section is sufficiently high (e.g. 2100 barn for $^{176}\text{Lu}(n,\gamma)^{177}\text{Lu}$), then a good fraction of the target atoms can be transmuted to the desired product isotopes, resulting in a product of high specific activity.
2. **Nuclear fission** Fission is another process used for isotope production in nuclear reactors. Radiochemical separation leads to radioisotopes of "non-carrier-added" quality, with

specific activity close to the theoretical maximum. Fission is the dominant production route for the generator isotopes ^{99}Mo and ^{90}Sr , for the β^- emitting therapy isotope ^{131}I and for the SPECT isotope ^{133}Xe .

3. **Charged particle reactions with p, d or α ions** Imaging for diagnostic purposes requires either β^+ emitters for PET (mainly ^{18}F , ^{11}C , ^{13}N , ^{15}O , ^{124}I , or ^{64}Cu), or isotopes emitting gamma-rays with suitable energy for SPECT (about 70 to 300 keV), if possible without $\beta^{+/-}$ emission to minimize the dose to the patient. Thus electron capture decay is preferred for such applications, e.g.: ^{67}Ga , ^{111}In , ^{123}I , ^{201}Tl . Usually these neutron-deficient isotopes cannot be produced by neutron capture on a stable isotope (exception ^{64}Cu). Instead, they are mainly produced by charged-particle induced reactions such as (p,n), (p,2n),... High specific activities of the final product are achievable, if the product differs in chemical properties from the target (i.e. different Z) and can be chemically separated from the remaining bulk of target material. Thus Z must be changed in the nuclear reaction, e.g. in (p,n), (p,2n), (p, α) reactions. The energies of the charged particle beams for such reactions are usually in the range of 10 to 30 MeV and can be supplied with high currents (0.1 to 1 mA) by small cyclotrons.
4. **Generators** Another important technique is the use of generators, where short-lived radionuclides are extracted "on-tap" from longer-lived mother nuclides. Here the primary radioisotope (that was produced in the nuclear reaction) has a longer half-life than the final radioisotope (that is populated by decay of the primary radioisotope and is used in the medical application). The primary radioisotope is loaded onto the generator and stays there chemically fixed. The final radioisotope will grow in and can be repetitively eluted and used.
5. **Photonuclear reactions** The inverse process to (n, γ), namely (γ ,n), also allows producing neutron deficient isotopes, but conventional γ ray sources do not provide sufficient flux density for efficient production of radioisotopes with high total activity and high specific activity. Therefore, this process played no role until now.

4. Specific radioisotopes produced in photonuclear reactions

We now discuss in detail the different γ -induced reactions and specific radioisotopes that can be produced by photonuclear reactions, that are enabled by the aforementioned breakthroughs of brilliant γ beam technology. In Tables 1 and 2 we show estimates of the achievable specific activities for thin targets for a γ flux of 10^{14} per s, corresponding to a flux density of 10^{18} $\gamma/(\text{cm}^2 \text{ s})$ for a beam cross-section of $(0.1\text{mm})^2$. With a bandwidth of 10^{-3} , this results at 10 MeV in a spectral flux density of 10^{14} $\gamma/(\text{cm}^2 \text{ s eV})$. With γ lenses the beam cross section could be improved by 10^4 and a better bandwidth is expected. We compare these to thin-target yields obtained by thermal neutron capture at a typical flux density of 10^{14} $\text{n}/(\text{cm}^2 \text{ s})$ in high flux reactors. Note that alike for the potential beam parameters of γ beam facilities, there is also a wide range of flux densities available at the irradiation positions of high flux reactors. Some positions provide flux densities of several 10^{12} to 10^{13} $\text{n}/(\text{cm}^2 \text{ s})$, while few special reactors have positions that even exceed 10^{15} $\text{n}/(\text{cm}^2 \text{ s})$, namely SM3 in Dimitrovgrad Karelín (1997), HFIR in Oak Ridge Knapp (2005) and the ILL's high-flux reactor in Grenoble. Since hitherto no γ beams with sufficiently small bandwidth were available to exploit resonant excitation, there are obviously no such measured cross-sections. Presently, we can only estimate a lower bound using the averaged cross-sections measured at bremsstrahlung facilities Carroll (1991;a;b); data (2010); Neumann (1991). For cases where no measured cross-sections are available, we interpolate experimental cross-sections of the same reaction

channel on nearby elements, taking into account the energy above the reaction threshold. We have submitted a proposal to the HI γ S facility to measure the expected strong resonant gateway states for radioisotope production, which frequently can be predicted from known neighboring nuclei.

Even when using conservative assumptions, the estimated specific activities are promising for specific isotopes.

The total radioisotope activity achievable in a nuclear reactor can be relatively high since, thick (several cm) and large (several cm²) targets can be used if the cross-sections are not too high (leading to self absorption and local flux depression). Multiple irradiation positions allow producing various radioisotopes with activities of many TBq.

For the γ beam we estimate the total activities by integrating to one interaction length, i.e., where the initial γ -beam intensity has dropped to $1/e = 37\%$ of its intensity. Higher total activities can be achieved with thicker targets at the expense of lower specific activity and vice versa. The total interaction cross-section is usually dominated by the atomic processes of Compton effect and pair creation, but not for γ beams with very small bandwidth. We conservatively consider any γ ray as lost after interaction. In reality, part of the Compton scattering goes forward under small angles and the γ rays that have lost little energy can still induce photonuclear reactions. The usable target thickness ranges from 20 g/cm² for heavy elements to 40 g/cm² for light elements, i.e., in total only few mg target material are exposed to the small area of the γ beam. With non-resonant reactions of the order of 0.1 TBq activity can be produced per day, corresponding to tens (for β^- therapy isotopes) to thousands (for imaging isotopes and therapy with alpha emitters) of patient doses.

4.0.1 Isomers of stable isotopes via (γ, γ') reactions

For various applications in nuclear medicine longer-lived nuclear isomers that decay by emission of gamma rays and/or conversion electrons to the respective ground state are of

Iso- tope	Exc. energy keV	Isomer Spin & parity	$T_{1/2}$ d	I_{is}/I_{gs}	Ground state		$\sigma \cdot \Gamma$		Sep. Act. γ	Act. 1/d GBq	R_γ fraction of max.	Sp.Ac. high flux reactor GBq/mg	$R_\gamma/R_{(n,\gamma)}$
					Spin & parity	Nat. abun. %	at 4 MeV eV·b	at 6 MeV eV·b					
⁸⁷ Sr	389	1/2 ⁻	0.12	3.5	9/2 ⁺	7	3.9	8.7	10	110	2·10 ⁻⁵	0.57	18
¹¹⁵ In	336	1/2 ⁻	0.19		9/2 ⁺	95.7	18	67	58	603	3·10 ⁻⁴		
¹¹⁷ Sn	315	11/2 ⁻	13.8	0.04	1/2 ⁺	7.68	3.2	8.8	7.5	0.6	0.0025	0.003	2400
¹²³ Te	248	11/2 ⁻	119	0.13	1/2 ⁺	0.89	42	68	55	0.6	0.17	0.2	280
¹²⁵ Te	145	11/2 ⁻	57.4	0.17	1/2 ⁺	7.07	70				0.08	0.48	
¹²⁹ Xe	236	11/2 ⁻	8.9	0.10	1/2 ⁺	26.4						0.2	
¹³¹ Xe	164	11/2 ⁻	11.8	0.10	3/2 ⁺	21.2						0.2	
¹³⁵ Ba	268	11/2 ⁻	1.2	0.08	3/2 ⁺	6.59	13	60	44	33	1.5·10 ⁻³	0.045	1000
¹⁷⁶ Lu	123	1 ⁻	0.15	2.0	7 ⁻	2.59	140	350	2.0	1800	1.2·10 ⁻³	5.5	36
¹⁹⁵ Pt	259	13/2 ⁺	4.02	0.09	1/2 ⁻	33.8	30	140	72	17	0.012	0.019	3800

Table 1. Longer-lived nuclear isomers produced in (γ, γ') reactions. The relative population of the respective isomer in thermal neutron capture on $A-1$ target isotopes is given as I_{is}/I_{gs} where known experimentally. Experimental integrated cross sections for population of the isomer by (γ, γ') reactions at 4 MeV and 6 MeV were taken from Carroll (1991;a). The fraction of the maximum specific activity R_γ produced in (γ, γ') reactions is put in relation to the one obtained with (n, γ) reactions $R_{(n,\gamma)}$ at a thermal neutron flux of 10^{14} n/(cm² s) in the last column.

interest, if they can be produced with high specific activity. Table 1 shows a selection of such isomers.

Most usual production methods, e.g., via (n,γ) reactions, result in relatively low specific activity, since the dominant part of the production proceeds directly to the nuclear ground state that has a nuclear spin closer to that of the $A-1$ target isotope. However, the fact that all these isomers are actually populated via thermal neutron capture reactions on low-spin $A-1$ target isotopes proves that pathways populating the high-spin isomers from higher-lying, low-spin compound nucleus resonance levels of lower spins must exist. In Ref. Lendoux (2006) the population of high-spin isomers relative to the ground state was studied for resonances in (n,γ) reactions. An energy dependence of the isomeric ratio was observed. One may expect that this energy dependence would become even more pronounced if the reactions were excited with a primary beam of smaller bandwidth that populates more selectively states which decay mainly to the isomeric level of interest.

Also photoexcitation (γ, γ') experiments with bremsstrahlung beams were performed on a series of stable targets and showed strong population of isomeric levels Carroll (1991;b); Neumann (1991). The observed energy dependence of the isomer activation yields indicates that few gateway states are responsible for efficiently populating the isomers.

Moreover, photoexcitation with small-bandwidth γ rays allows the selective excitation of individual levels or groups of levels that decay preferentially to the nuclear isomer, thus enhancing the specific activity of the isomer. Only in few cases the energies of such (groups of) levels are already known. Note that relatively low gamma ray energies may be sufficient for such a pumping to isomeric states. In ^{125}Te a $7/2^+$ state at only 402 keV excitation energy can serve as gateway state for pumping from the $1/2^+$ ground state to the $11/2^-$ isomer at 145 keV NuDAT (n.d.).

We will estimate the achievable specific activity at the example of ^{115}In , for which the required transition energies, branching ratios and transition strengths are already experimentally known, even if this isomer has presently no application in nuclear medicine.

Experimental data on isomer population by (γ, γ') reactions have so far been obtained with bremsstrahlung spectra of large bandwidth. The integrated cross-sections at γ energies of 4 and 6 MeV, respectively, are of the order of 10 to 100 b·eV.

Many potential gateway states that could serve for pumping nuclei from their ground state to isomeric levels are expected to exist, but they still need to be identified by dedicated high resolution measurements from excitation energies of few hundred keV up to close to the particle separation energy. These measurements have to be performed with the new γ beams for each of the isotopes for variable energy windows, in order to determine the best excitation-deexcitation path to the isomer. Presently existing γ -ray beam facilities only marginally provide sufficiently monochromatic γ -ray beams to search for suitable resonance regions. A systematic investigation will require Compton backscattering facilities such as MEGa-ray (LLNL) or ELI-NP.

Selecting γ ray energies providing strong pumping to the isomeric state will improve the achievable specific activity correspondingly. Even multiple excitations of the path to the isomer are possible. Due to the missing energy match, no significant back-pumping from the isomer to the ground state will occur.

maximum specific natural abundance and assume that finally will result in a total conversion of the ground than presently

Two examples of long-lived isomers with important medical applications are discussed in the following:

1. ^{195m}Pt : Platinum compounds such as cisplatin or carboplatin are known to be cytotoxic and are frequently used for chemotherapy of tumors. Labeling these compounds with platinum radiotracers allows for in-vivo pharmacokinetic studies and tumor imaging, e.g., to monitor the patient-specific uptake and optimize the dosing individually Dowell (2000). Failure to demonstrate the tumor uptake of the chemotherapy agent by nuclear imaging helps to exclude those “non-responding” patients from unnecessary chemotherapy treatment. ^{195m}Pt has 4 days half-life and emits a 99 keV gamma ray that can be used for imaging by SPECT or gamma cameras. ^{195m}Pt emits also low-energy conversion and Auger electrons. Hence, when used in higher activities, it could be suitable for a combined chemo- and radionuclide therapy. Unfortunately, ^{195m}Pt is destroyed by (n,γ) reactions with a very high cross section of 13000 barn. Therefore the specific activity achievable by neutron capture on ^{194}Pt is seriously limited.
2. ^{117m}Sn : Also, ^{117m}Sn emits low-energy conversion and Auger electrons, making it promising for radionuclide therapy. In addition it emits a 159 keV gamma ray for imaging. It has been shown that ^{117m}Sn can be used for pain palliation in bone metastases of various cancers. Due to its soft electron energy spectrum, it has less side effects on the bone marrow than other radioisotopes with more penetrating radiation Bishayee (2000).

These two isomers appear at present most interesting for nuclear medicine applications. The specific activity and total production per day could be significantly improved with still to be found better gateway states. A detailed search for suitable gateway states at an upcoming γ -beam facility with small bandwidth is underway.

4.1 Radioisotopes via the (γ,n) reaction

When excited well beyond the neutron binding energy, a nucleus readily loses a neutron. Competing reactions such as deexcitation by gamma ray emission are far less probable.

1. $^{99}\text{Mo}/^{99m}\text{Tc}$: The presently most used radioisotope for nuclear medicine studies is ^{99m}Tc . Its 140 keV γ ray is ideal for SPECT imaging. With a relatively short half-life of 6 h and the quasi-absence of beta particles, the radiation dose to the patient is sufficiently low. ^{99m}Tc is conveniently eluted in non-carrier-added quality from simple and reliable ^{99}Mo ($T_{1/2} = 66$ h) generators that can be used for about one week. Various technetium compounds have been developed for a multitude of nuclear medicine applications Schiepers (2006). The combination of these advantages explains why ^{99m}Tc is used in about 80% of all nuclear medicine studies. Until recently five nuclear reactors were used to produce about 95% of the world needs of ^{99}Mo by neutron-induced fission of highly enriched ^{235}U targets. Recently the two reactors that used to produce the majority of the ^{99}Mo supply had extended shutdowns, leading to a serious $^{99}\text{Mo}/^{99m}\text{Tc}$ supply crisis Lewis (2009); Raloff (2009). A facility providing $10^{15}\gamma/\text{s}$ could produce via $^{100}\text{Mo}(\gamma,n)$ reactions several TBq per week. Since the present request is 3000TBq per week, many such facilities would be required to assure the worldwide ^{99}Mo supply.

This example demonstrates that the new production method by γ beams is not intended to compete with large-scale production of established isotopes. The advantage of γ beams for radioisotope production lies clearly in the very high specific activity that can be achieved for radioisotopes or isomers that are very promising for nuclear medicine, but that are presently not available in the required quality or quantity.

2. $^{225}\text{Ra}/^{225}\text{Ac}$: Alpha emitters are very promising for therapeutic applications, since the emitted alphas deposit their energy very locally (typical range of one to few cancer

Product isotope	$T_{1/2}$ d	Target isotope	Rct.	E_γ MeV	σ b	Spec. act. γ beam GBq/mg	Activity per day GBq	R_γ fraction of max.	Spec. act. HFR GBq/mg	$R_\gamma/R_{(n,\gamma)}$
^{47}Ca	4.5	^{48}Ca	(γ,n)	19	0.09	1100	400	0.05	0.9	1200
^{64}Cu	0.5	^{65}Cu	(γ,n)	17	0.09	830	1150	0.006	4	200
^{99}Mo	2.8	^{100}Mo	(γ,n)	14	0.16	960	350	0.06	0.08*	12000
^{103}Pd	17	^{104}Pd	(γ,n)	17	0.05	290	16	0.1	1.8	160
^{165}Er	0.4	^{166}Er	(γ,n)	13	0.3	1100	1100	0.016	4.7	230
^{169}Er	6.9	^{170}Er	(γ,n)	12	0.3	≈ 800	130	≈ 0.2	0.8	1000
13	0.3	≈ 200	30	≈ 0.5	170					
^{186}Re	3.7	^{187}Re	(γ,n)	15	0.6	≈ 1400	320	≈ 0.2	35	40
^{225}Ra	14.8	^{226}Ra	(γ,n)	12	0.2	≈ 300	30	≈ 0.2		
^{47}Sc	3.4	^{48}Ti	(γ,p)	19	0.02	250	100	0.009		
^{67}Cu	2.6	^{68}Zn	(γ,p)	19	0.03	260	115	0.01		
^{44}Ti	60 y	^{46}Ti	($\gamma,2n$)	27	0.01	≈ 0.5	0.008	≈ 0.1		
22	0.02	≈ 25	0.7	≈ 0.1						
^{84}Sr	($\gamma,2n$)	25	0.02	140	5.6	0.06				
^{224}Ra	3.7	^{226}Ra	($\gamma,2n$)	16	0.1	≈ 50	10	≈ 0.01		

Table 2. Estimated production rates of radioisotopes produced in (γ,n), (γ,p) or ($\gamma,2n$) reactions. Experimental cross sections were taken from data (2010), estimated cross sections are marked in italics. The fraction of the maximum specific activity produced in (γ,x) reactions R_γ , is put in relation to that obtained with (n,γ) reactions $R_{(n,\gamma)}$ at a thermal neutron flux of 10^{14} n/(cm² s) in the last column. *: For comparison we show the values for ^{99}Mo produced by $^{98}\text{Mo}(n,\gamma)$. However, usually ^{99}Mo is produced by fission with much better specific activity.

- cell diameters) with high linear energy transfer (LET) and, hence, high probability for irreparable double strand breaks. An alpha emitter coupled to a cancer cell specific bioconjugate can be used for targeted alpha therapy to treat disseminated cancer types (leukemia), micro-metastases of various cancers or to destroy chemo- and radiation-resistant cancer cells (e.g., glioblastoma). One promising alpha emitter is ^{225}Ac ($T_{1/2} = 10$ days) that decays by a series of four alpha decays and two beta decays to ^{209}Bi .
- ^{169}Er : ^{169}Er decays with 9.4 days half-life by low-energy beta emission (100 keV average beta energy). These betas have a range of 100 to 200 μm in biological tissue, corresponding to few cell diameters. The short beta range makes this isotope very interesting for targeted radiotherapy Uusijaervi (2006).
 - ^{165}Er : ^{165}Er is one example for an isotope that decays mainly by low-energy Auger electrons. Their range is shorter than one cell diameter. Hence, these Auger emitters have to enter the cell and approach the cell's nucleus to damage the DNA and destroy a cell. Coupled to a bioconjugate that is selectively internalized into cancer cells it can enhance the ratio for dose equivalent delivered to the tumor cell with respect to normal cells. This should result in an improved tumor treatment with less side effects.
 - ^{47}Sc : ^{47}Sc is a promising low-energy beta emitter for targeted radiotherapy. Scandium is the lightest rare earth element. Most established labeling procedures for valence III metals (Y, Lu, ...) can be applied directly for Sc. Its 159 keV gamma line allows imaging of ^{47}Sc distribution by SPECT or gamma cameras. Alternatively, the β^+ emitting scandium isotope ^{44}Sc can be used for PET imaging as a "matched pair". Carrier-free ^{47}Sc can be produced by $^{50}\text{Ti}(p,\alpha)$ or $^{47}\text{Ti}(n_{\text{fast}},p)$ reactions followed by chemical separation. The

- alternative production via $^{46}\text{Ca}(n,\gamma)^{47}\text{Ca} \rightarrow ^{47}\text{Sc}$ is uneconomic due to the extremely low natural abundance of ^{46}Ca .
- ^{64}Cu : ^{64}Cu is a relatively long-lived β^+ emitter ($T_{1/2} = 12.7$ h) with various applications in nuclear medicine Anderson (2009). ^{64}Cu -ATSM is a way to measure hypoxia of tumors. Hypoxia is an important effect influencing the resistance of tumor cells against chemo- or radiation therapy. ^{64}Cu can also act itself as therapeutic isotope due to its emission of β^- (191 keV mean energy) and low-energy Auger electrons.
 - ^{186}Re : ^{186}Re is a radioisotope suitable for bone pain palliation, radiosynovectomy and targeted radionuclide therapy. Rhenium is chemically very similar to its homologue technetium, thus known compounds that have been developed for imaging with ^{99m}Tc can also be labeled with ^{186}Re and used for therapy. ^{186}Re is currently either produced by neutron capture on ^{185}Re , resulting in limited specific activity, or by $^{186}\text{W}(p,n)$ reactions followed by chemical Re/W separation. Enriched ^{187}Re targets should be used to minimize contamination of the product with long-lived $^{184,184m}\text{Re}$ by $^{185}\text{Re}(\gamma,n)$ reactions.

4.2 Radioisotopes via the (γ,p) reaction

Even when excited beyond the proton binding energy, a nucleus does not necessarily lose a proton. The latter is bound by the Coulomb barrier, leading to a suppression of the proton loss channel. Only for an excitation well beyond the proton binding energy, the proton gains enough kinetic energy for tunneling efficiently through the Coulomb barrier. However, such excitation energies are usually also above the neutron binding energy or even the two-neutron binding energy. Hence neutron emission competes with proton emission and the cross sections for (γ,p) reactions may be one order of magnitude lower than the competing channels. Thus, the achievable specific activity (specific activity with respect to the target mass) is limited for (γ,p) reactions. However, the product isotope differs chemically from the target since it has one proton less ($Z_{\text{product}} = Z_{\text{target}} - 1$). After irradiation, a chemical separation of the product isotope from the target can be performed, ultimately resulting in a high specific activity that is only compromised by competing reactions leading to other isotopes of the product element (such as (γ,np) , $(\gamma,2n)EC/\beta^+$, etc.) or product burn-up by (γ,n) .

- ^{47}Sc : Besides the $^{48}\text{Ca}(\gamma,n)^{47}\text{Ca} \rightarrow ^{47}\text{Sc}$ reaction, ^{47}Sc can also be produced via the $^{48}\text{Ti}(\gamma,p)^{47}\text{Sc}$ reaction. The established Sc/Ti separation schemes can be employed for the chemical processing. Compared to the $^{47}\text{Ti}(n,p)$ way here the direct production of disturbing long-lived ^{46}Sc (via $^{46}\text{Ti}(n,p)$ or $^{47}\text{Ti}(\gamma,p)$, respectively) can be limited more easily, since ^{48}Ti is the most abundant titanium isotope and can be enriched more easily to high abundance. However, the irradiation times have to be kept relatively short to prevent excessive formation of ^{46}Sc impurity by $^{47}\text{Sc}(\gamma,n)$ reactions.
- ^{67}Cu : ^{67}Cu is also a promising beta-emitter for targeted radiotherapy. Alike ^{47}Sc it has a sufficiently long half-life for accumulation in the tumor cells when bound to antibodies and its 185 keV gamma ray allows imaging with SPECT or gamma cameras. Together with the PET imaging isotopes ^{61}Cu or ^{64}Cu , it forms a “matched pair”. The usual production routes $^{68}\text{Zn}(p,2p)$, $^{70}\text{Zn}(p,\alpha)$, or $^{64}\text{Ni}(\alpha,p)$ are all characterized by low yields. The former requires energetic protons ($\gg 30$ MeV from larger cyclotrons) and the latter two methods use expensive enriched targets with low natural abundances.
- Isotopes with higher Z: In principle, also heavier β^- emitters used for radionuclide therapy such as ^{131}I , ^{161}Tb or ^{177}Lu could be produced by (γ,p) reactions (on ^{132}Xe , ^{162}Dy

or ^{178}Hf targets respectively). However, for higher Z the increasing Coulomb barrier leads to small production cross sections.

thyroid problems. produced by thermal neutron-induced fission of enriched ^{235}U targets. be produced via $^{132}\text{Xe}(\gamma, p)$ reactions. particularly useful thin xenon gas targets. The handling of highly active stream is strongly reduced. Strongly enriched ^{132}Xe can be obtained. gas target is straightforward, e.g. xenon gas cell with sterile water established t $^{124}\text{Xe}(p, 2n)$ the thermal stress of the due to the Moreover the full recovery essential far cheaper than ^{124}Xe .

short-lived ^{225}Fr that ^{225}Ra . This reaction can be used simultaneously to production.

4.2.1 Radioisotopes via the $(\gamma, 2n)$ reaction

1. ^{44}Sc : ^{44}Sc is a promising metallic PET tracer that emits a 1157 keV gamma-ray quasi-simultaneously with the positron. With a suitable detection system (Compton telescope plus PET camera), a triple coincidence (gamma rays of 511 keV, 511 keV, and 1157 keV) can be detected Grignon (2007). Hence, for each triple-event the point of emission is derived instead of the usual line-of-response, leading to improved position resolution at reduced dose to the patient. Moreover, ^{44}Sc forms a "matched pair" with ^{47}Sc , a therapy isotope discussed above. ^{44}Sc can be obtained from $^{44}\text{Ti}/^{44}\text{Sc}$ generators where the parent isotope ^{44}Ti is very long-lived ($T_{1/2} = 60$ years). Despite the very favorable properties of ^{44}Sc , this isotope is not yet used in clinical routine, since the generator isotope ^{44}Ti is difficult to produce and therefore prohibitively expensive until now.

conveniently eluted on-site from generators and used for various. The great interest in ^{68}Ga rapidly rising demand for presently produced by $^{69}\text{Ga}(p, 2n)$ reactions that alternative production path.

β^+ emitter is continuously $^{82}\text{Sr}/^{82}\text{Rb}$ of heart and brain. imaging with ^{99m}Tc . PET imaging and the present $^{99}\text{Mo}/^{99m}\text{Tc}$ supply $^{82}\text{Sr}/^{82}\text{Rb}$ generators. produced in $^{85}\text{Rb}(p, 4n)^{82}\text{Sr}$ reactions. few large cyclotrons or linear accelerators exist world-wide that (≥ 60 MeV) for efficient $^{85}\text{Rb}(p, 4n)^{82}\text{Sr}$ reaction. by photonuclear $^{84}\text{Sr}(\gamma, 2n)^{82}\text{Sr}$ high specific activities are reached not to compromise sufficiently high per day

2. $^{224}\text{Ra}/^{212}\text{Pb}/^{212}\text{Bi}$: Via $^{226}\text{Ra}(\gamma, 2n)$ reactions the isotope ^{224}Ra ($T_{1/2} = 3.66$ d) from the thorium chain can be obtained, where the noble gas ^{220}Rn isotope can be extracted easily. The α emitter ^{212}Bi ($T_{1/2} = 60$ min) in this decay chain or its mother isotope ^{212}Pb are also considered for targeted alpha therapy, e.g., for malignant melanoma metastases Hassfjell (2001); Mia (2005).

With the new γ beam facilities like ELI-NP or MEGa-ray LLNL we will search for optimum gateway states for all these new medical radioisotopes, making the production cross section much more reliable.

5. Conclusion

The advance of intense laser technology Mourou (2006) is changing the reach of laser-driven approaches of radiotherapy, both in the EBRT and in the endoradiation therapy, taking advantage of the new brilliant γ beams. In terms of EBRT, the laser-driven ion beam therapy awaits the rapid progress in the understanding of laser ion acceleration, both theoretically and experimentally. Theoretically, the recent year's research now directs us where the "sweat spots" in reaching 100-200 MeV ion/nucleon with a 100-200 TW laser. Experimentally, so

far often with less than 100 TW lasers we now see convergence of experimental results with theory of more efficient regimes such as CAIL/RPA. Thus when near future experiments are expected to embark on PW lasers, these experiments should reveal not only sufficiently high ion energies mentioned above, but also the parameters and configurational dependencies for the "sweat spots". We then can optimize the overall best spots of operation. In going to smaller tumors (\leq cm regime), it is important to develop the image guided irradiation with pencil beams (e.g. Ref. Sutherland (2010)), the online dosimetry with sufficiently agile novel methods of detection such as prompt γ detection from the proton beam collisions with tissue nuclei Kormoll (2011). Such guidance, irradiation, and dose confirmation can make up an active feedback dose delivery by a radio-oncologist Murakami (2008) first time ever. This approach should lead to more accurate dose control over small tumors. This concept matches with the desire of the radiooncologist Molls (2009) to treat early small tumors, but also opens the avenue through which the laser-driven particle therapy can find niches when this method is ill-suited for larger tumors. The fledgling irradiation demonstrations by laser-driven proton beams on in vivo cells have shown, so far, the radiological effectiveness not far different from conventional accelerators Kraft (2010); Yogo (2009).

For the production of medical radioisotopes for nuclear medicine, we develop generalized nuclear models of doorway states for optimum production and start testing them with present γ beams of improved band width, compared to formerly published broad band width measurements. Even in the old measurements very strong variations of the production cross sections between different isotopes had been observed Carroll (1991), pointing to a nonstatistical cross section behavior. Thus we expect to find with MEGa-ray (LLNL) and ELI-NP in the next 3-5 years improved resonant cross sections, making this alternative way of medical radioisotope production more favorable and economical. The resonantly enhanced strong cross sections, with their narrow width compete much more favorably with the always present atomic cross sections. With these new intense, brilliant γ beams and γ optics the area of *nuclear photonics* starts, where nuclear collective doorway states are not only interesting from the perspective of nuclear modelling, but reach applicational importance like in nuclear medicine.

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Ion Channels: Novel Functional Hubs in Leukemia

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1. Introduction

Leukemia (from the Greek “leukos” (white) and “haima” (blood) is a type of cancer of the blood or bone marrow, characterized by an abnormal increase of white blood cells. Leukemia is a broad term, covering a spectrum of hematologic diseases, which can be distinguished by peculiar clinical and pathological characteristics. The first distinction is between acute and chronic forms. Moreover, according to which kind of blood cell is affected, leukemias are subdivided into lymphoblastic (a terminology limited to address the acute forms), lymphocytic and myeloid leukemias. Combining these two classifications provides four main categories: Acute Lymphoblastic Leukemia (ALL), Chronic Lymphocytic Leukemia (CLL), Acute Myeloid Leukemia (AML) and Chronic Myeloid Leukemia (CML). These broad categories are further subdivided into several subcategories, classically identified through morphological criteria (Greer et al., 2008).

During the last thirty years, the widespread introduction of molecular biological concepts and methods in oncology has provided the important notion that cancer cells are mutants. These cells sometimes carry somatic mutations. In other cases they bear either amplified or inactivated tumor-related genes. These latter effects can be also caused by epigenetic mechanisms, instead of mutation events *per se*. However, the picture is considerably complicated by the observation that aberrant extracellular signals can promote oncogenesis even in a benign cell physiological context. In other words, the most malignant neoplastic phenotypes do not arise in a strictly cell autonomous manner, and their manifestation cannot be understood solely based on tumor cell genomes. The ability of malignant cells to proceed along the invasion-metastasis pathway may be acquired, at least in certain cases, through their interaction with the tumor microenvironment, without the requirement to undergo additional mutations beyond those that were needed for primary tumor formation (Hanahan & Weinberg, 2011).

This scenario also applies to hematologic malignancies, where analysing the genes comprising the expression signature has provided important insights into the biology of leukemias. This has led to reshape the classification criteria for subcategories identification and risk stratification, as well as develop novel drugs addressing leukemia-specific processes. Further insights came from the discovery that the strict relationship between leukemia cells and the bone marrow microenvironment strongly determines malignancy and response to therapy. This relies on the fact that bone marrow cells emit survival signals

that strongly determine the development of the leukemic disease. CML has been for long, and is still, the “poster child” of translational medicine. The discovery of the Philadelphia chromosome and the subsequent finding of the BCR-ABL chimeric gene led to a unique understanding of the biology of the disease that spurred the development of targeted therapy, as well as methods for the molecular monitoring of the disease. These achievements have shaped a therapeutic framework that is the envy of oncology (American Society of Hematology [ASH] Education Program Book, 2010). CLL, despite being the most common leukemia with a clinical description dating back to the mid-nineteenth century, still remains a rather enigmatic disease. Nonetheless, considerable progress in understanding the biology of CLL occurred in the last few years. In particular, important advances have been made in identifying inherited and acquired genetic mutations, the role of B-cell receptor signaling and the tumor microenvironment. CLL resulted to be a disease dependent on the interplay of inherited, environmental and host factors (ASH, 2010). The picture is even more complex in acute forms, where different types of gene alterations, mainly translocations, have been observed that often lack a clear biological and clinical interpretation (ASH, 2010). ALL is the most common childhood leukemia form, and its therapy has improved substantially with the use of risk-directed treatment and improved supportive care. Current ALL trials have focused on improving the outcome of a few subtypes that remain refractory to treatment, such as infant ALL with specific gene translocations, MLL rearrangements, hypodiploid ALL, or poor early responders (ASH, 2010). By contrast, most of the AML cases continue to pose a therapeutic challenge. AML forms show marked differences in survival following intense chemotherapy based on age, blast cell morphology and cytogenetic abnormalities. However, although therapeutic advances have lagged over the past two decades, recent work has provided an array of new prognostic factors in AML, which is driving our understanding of the disease biology and the development of new therapeutic targets (ASH, 2010).

Here, we review the growing experimental and preclinical evidence that indicates that ion channels should be included among the genes whose expression is altered in leukemias. Channel dysfunction can have a strong impact on hematopoietic cell physiology and signaling, with ensuing effects on the onset and progression of the leukemia disease. These effects depend on the widespread roles of ion channels in modulating cellular functions that contribute to determine the clinical features and the therapeutic responses of hematologic diseases, such as proliferation, differentiation and apoptosis. In addition, many ion channels are at the same time effective sensors of extracellular signals and transducers of these signals into cellular regulatory cascades. From a pharmacologic standpoint, because ion channels are membrane proteins, they can be easily accessed by extracellular ligands or peptides (toxins), which offers clear advantages for treatment. For these reasons, we believe that ion channels represent promising targets for cancer therapy, which may open a novel pharmaceutical and clinical field.

2. Overview of the relevant ion channel and aquaporin types

Ion channels are integral membrane proteins that provide an aqueous pathway for ions to cross the energetically unfavourable barrier constituted by the plasma membrane. Ion channels are generally either permeable to cations or anions. Moreover, they can be more or less specific for different ions. The main stimuli for channel opening (activation) are either change in membrane potential (voltage-gated channels) or ligand binding (ligand-gated

channels). In the following paragraphs, we summarize the main structural and physiological features of the channel types that will be mentioned subsequently.

2.1 Voltage-gated cation channels (VGC)

VGCs belong to a large molecular family that comprises K_V (Voltage-gated K^+ channels), Na_V (Voltage-gated Na^+ channels) and Ca_V (voltage-gated Ca^{2+} channels). The K^+ channel types are named K_V1 to K_V12 , with subtypes named $K_V1.1$, etc. (Gutman et al., 2005). They are tetrameric channels, with each subunit containing six transmembrane domains (S1 to S6). S4 is rich of amino acid residues with alkaline side chains and is thought to be the voltage sensor (Börjesson & Elinder, 2008). S5 and S6 are linked by the so-called pore (P) loop, which gives a fundamental contribution to ion selectivity and contains the K^+ channel 'signature' GYG (Alam & Jiang, 2011). Both the N- and C-termini are intracellular. The cytoplasmic domains contain consensus sequences for phosphorylation and the N-terminus determines interaction with regulatory proteins and other subunits. Besides the classical role in mediating the repolarizing phase of action potentials, many other functions have been found to be exerted by K_V channels, which are a very diversified molecular family. These functions cannot be reviewed here, but a few relevant examples will be described later. The structure of Na_V and Ca_V is similar to that of K_V , except that the four independent subunits observed in K_V are instead four homologous repeated domains of the same polypeptide. The main physiological role of Na_V channels is shaping the rising phase of action potentials, whereas Ca_V channels control Ca^{2+} entry during action potentials, exocytosis, muscle contraction and the many other physiological processes that are modulated by $[Ca^{2+}]$. In all voltage-gated channels, auxiliary subunits regulate the channel properties and control both membrane targeting and interaction with other proteins (Catterall, 1992, 2000).

2.2 Inward rectifier K^+ channels (K_{IR})

These channels preferentially carry inward K^+ currents, because the outward currents are blocked by intracellular cations obstructing the pore, particularly cytosolic polyamines and Mg^{2+} . Block is increasingly more effective as V_m depolarizes. K_{IR} channels are tetrameric proteins structurally related to the VGC family. However, each subunit only contains two transmembrane domains (M1 and M2). These are respectively homologous to S5 and S6 and are connected by a P-loop (Nichols & Lopatin, 1997). K_{IR} channels contribute to regulate the resting V_m in a range not too far from the K^+ equilibrium potential, thus controlling for example excitability in resting conditions, the cardiac action potential repolarization and the extracellular K^+ buffering exerted by glial cells.

2.3 Ca^{2+} -activated K^+ channels (K_{Ca})

These tetrameric K^+ channels are formed by subunits collectively named K_{Ca} and structurally related to K_V , with the typical S1-S6 module (Wei et al., 2005). In $K_{Ca1.1}$, a transmembrane segment named S0 precedes the S1-S6 module. $K_{Ca1.1}$ are also named BK ('Big') because of a particularly high single-channel conductance. BK are activated by both depolarization and $[Ca^{2+}]$. $K_{Ca1.1}$ typically determines the Ca^{2+} -dependent after hyperpolarization observed in certain neurons. K_{Ca4} and K_{Ca5} present similar overall features, but their precise physiological roles are still matter of debate. K_{Ca4} channels are not voltage-dependent, because of S4 neutralization and are activated by Na^+ instead of Ca^{2+} . K_{Ca2} (or SK, after Small conductance K^+ channels) and K_{Ca3} (or IK, after Intermediate

conductance K^+ channels) are also not voltage-dependent, because of partial neutralization of S4. They are activated by $[Ca^{2+}]$ around 0.5 μM , through binding to the calmodulin proteins tightly associated with each subunit. These channels can regulate cell firing, the vascular tone and, as discussed below, are also implicated in cell proliferation and neoplasia.

2.4 Transient Receptor Potential (TRP) channels

TRP channels are homo- or hetero-tetramers of subunits structurally related to the VGCs, with the typical S1-S6, a P loop and cytoplasmic N- and C-termini. They are generally permeable to cations; the permeability to Ca^{2+} is extremely variable between subtypes. TRP are important sensors of the cell's environment and can respond to many chemical as well as physical stimuli (including temperature). In mammals, six main subfamilies are known: TRPA, TRPC, TRPM, TRPV, TRPP and TRPML (Venkatachalam & Montell, 2007). TRP channels are widely distributed in mammalian tissues, and are implicated in a wide spectrum of functions (Nilius et al., 2007). Besides the classic roles as sensory transducers, increasing evidence implicates TRP channels in developmental functions. Ca^{2+} influx through these channels can in fact regulate axon guidance and neuronal survival (Talavera et al., 2008).

2.5 Two-pore domain K^+ channels (K_{2P})

K_{2P} channels were divided into six subfamilies: TWIK (tandem of pore domains in a weak inward rectifying K^+ channel), TREK (TWIK-related K^+ Channel), TASK (TWIK-related Acid-Sensitive K^+ Channel), TALK (TWIK-related alkaline pH-activated K^+ channel), THIK (tandem pore domain halothane-inhibited K^+ channel) and TRESK (TWIK-related spinal cord K^+ channel). The systematic nomenclature is KCNK followed by a specific number for each subtype. Each subunit is formed by intracellular N- and C-termini that comprise four transmembrane domains (TMS1-TMS4). Each subunit contains two P loops (which explains the name K_{2P}), one between TMS1 and TMS2 and the other between TMS3 and TMS4. The functional channel is a dimer of two-pore domain containing subunits. K_{2P} are believed to behave as background K^+ channels, i.e. channels mostly open in resting conditions, and thus give a substantial contribution to V_{rest} . Accordingly, they present weak voltage-dependence and rectification. More specific physiological roles, including those in neoplastic cells, are still debated (Enyedi & Cziriák, 2010).

2.6 Ionotropic purinergic receptors

Purinergic receptors can be metabotropic or ionotropic receptors activated by adenosine (P_1) or ATP (P_2). In particular, P_2 receptors comprise the ionotropic P_{2X} receptors. Seven subunits have been identified (P_{2X1} to P_{2X7}) that can form homo- or heterotrimeric channels typically activated by extracellular ATP. P_{2X} receptors have intracellular N- and C-terminus and two transmembrane domains connected by a long extracellular loop involved in subunit association. The extracellular channel portion contains the ATP binding crevice plus modulatory sites. The C-terminus has very variable length and probably controls both the channel's desensitization kinetics and receptor trafficking. The open channel is permeable to cations, including Ca^{2+} . P_{2X} receptors exert physiological functions in many tissues, including the adult and developing nervous system, the respiratory, gastrointestinal, cardiovascular and genitourinary systems (Köles et al., 2007).

2.7 Voltage-gated Cl⁻ channels

These are ion channels permeable to anions and gated by membrane depolarization. Nine subunits (CLC-1 to CLC-7, plus CLC-Ka and CLC-Kb) are expressed in mammals. CLC-1, CLC-2, CLC-Ka, and CLC-Kb are certainly Cl⁻ channels, activated by membrane depolarization and permeable to different anions. The native channel comprises two identical subunits and contains two independent pores. Each subunit contains 18 α helical segments (A to R), with intracellular N- and C-termini. The segments A-I are homologous to J-R, but the two half-subunits have opposite orientation in the membrane. CLC channels are expressed in a variety of cells, where they regulate membrane excitability, cell volume, pH and transepithelial Cl⁻ flux. They are also expressed in the organelles' membranes. CLC-3, CLC-4 and CLC-5 are expressed on the membrane of intracellular vesicles and are thought to function as Cl⁻-H⁺ antiporters. However, evidence about CLC-3 is controversial. The physiology of CLC-6 and CLC-7 is also unclear (Zifarelli & Pusch, 2007).

2.8 Interplay between channels expressed in the plasma membrane and intracellular compartments: The role of CRAC

Ion channels are also widely distributed in intracellular organelles and vesicles, where they control transmembrane fluxes implicated in neurotransmitter loading into synaptic vesicles, cytoplasmic Ca²⁺ homeostasis (and the related physiological processes), mitochondrial and nuclear function. Ion transport across the intracellular membranes is tightly coupled to the fluxes between the cytosol and the extracellular space. The CRAC channels (Ca²⁺ release-activated Ca²⁺ channels) have a pivotal role in such interplay in that they mediate calcium influx from the extracellular compartment depending on the state of intracellular Ca²⁺ stores. The full physiological response of CRAC channels depends on interaction between the plasma membrane protein ORAI1 (or CRACM1), which forms at least part of the Ca²⁺ pore, and STIM1 (stromal interaction molecule 1), which is expressed in the endoplasmic reticulum membranes. When Ca²⁺ decreases in the intracellular stores, STIM1 and ORAI1 form a complex that stimulates Ca²⁺ influx from the extracellular space. This process is implicated in the cellular processes that are regulated by Ca²⁺ and the related pathologies. For review see Parekh (Parekh et al., 2010).

2.9 Aquaporins

Aquaporins (or water channels) are integral membrane proteins that form a specific transmembrane pathway for water. Several aquaporins subtypes are known in mammals, formed by subunits named AQP0-AQP12, with different tissue distribution. They form tetrameric channels, with each subunit containing 6 transmembrane domains and one pore. Both the N- and C-termini are intracellular. Aquaporins control osmotic fluxes in a variety of physiological conditions, from cell volume alteration to transepithelial flux. Some subtypes form selective water channels, with scarce selectivity for ions and other small solutes. However, recent evidence shows that other AQP subtypes are also permeable to small solutes different from water, such as glycerol, urea, CO₂, NO, NH₃ and others. This considerably extends the range of possible functions of these membrane channels (King et al., 2004).

3. Expression and function of ion channels in leukemia cells

Work carried out in the early eighties led to the discovery of ion channels in lymphocytes and suggested specific channel roles in lymphocyte activation and function (Fukushima &

Hagiwara, 1983; DeCoursey et al., 1984; Matteson & Deutsch, 1984; Fukushima et al., 1984; Chandy et al., 1984). In particular, work done in M. Cahalan's research group indicated that K^+ channels could regulate mitogenesis, in T cells. Subsequent work from this and other groups clarified the differential expression of K^+ channels in T lymphocyte populations and how they control T cell activation (Cahalan et al., 1985; Beeton & Chandy, 2005; Krasznai, 2005). These cells turned out to express delayed rectifying K^+ channels ($K_v1.3$) and intermediate conductance Ca^{2+} -dependent K^+ channels ($K_{Ca3.1}$) (Douglas et al., 1990; Logdson et al., 1997; Wulff et al., 2000). The K^+ channel-dependent hyperpolarization facilitates the Ca^{2+} influx induced by antigen binding. The consequent stimulation of intracellular Ca^{2+} - and PKC-dependent pathways triggers proliferation (reviewed in (Chandy et al., 2004)). A similar scheme may apply to transformed cell lines. K^+ currents seem often to be necessary during proliferation, although which kind of channel is involved depends on the cell type and the stimulus inducing leukemia cell entry into the mitotic cycle. Early evidence was obtained in the myeloblastic leukemia cell line ML-1. When proliferating, these cells express functional K^+ channels sensitive to 4-amino-pyridine (4-AP), which are instead suppressed after inducing macrophage differentiation (Lu et al., 1993). Treatment with 4-AP makes ML-1 cells arrest in G1, with no evidence of cell differentiation (Xu et al., 1996). Therefore, K^+ channels in ML-1 cells appear to be strictly linked to the cell cycle control. Consistently, K^+ currents are inhibited when cells are arrested in G1 by serum deprivation, and restored on serum re-addition or EGF application (Wang et al., 1997). The process depends on channel phosphorylation (Xu et al., 1999). The possible effects of K^+ channel activation on Ca^{2+} fluxes have not been tested in ML-1 cells, but were demonstrated in a rat basophilic leukemia cell line, RBL-1, which expresses K_{IR} channels. These probably maintain a favourable driving force for Ca^{2+} influx through CRAC channels (Straube & Parekh, 2002), in agreement with the early hypothesis based on work in T cells. Besides CRAC channels, other Ca^{2+} permeable channels have been studied in leukemia, and their role is still under study. For example, K562 cells, i.e. a human cell line obtained from a patient with CML in blast crisis, have been recently shown to co-express TRPV5 and TRPV6, two channel proteins that physically interact in these cells (Semenova et al., 2009). The same two channels were also detected in the lymphoblastic leukemia Jurkat cell line. Their expression pattern and high Ca^{2+} permeability indicate an important role in controlling Ca^{2+} homeostasis and probably in malignant transformation of blood cells (Vasil'eva et al., 2008). In other cases, the relation between K^+ channels and Ca^{2+} flux is more complex, with Ca^{2+} producing feedback on K^+ currents themselves. For example, the human Daudi cell line, a model of B-lymphoma, expresses functional $K_v1.3$ and $K_{Ca3.1}$. Specific block of $K_{Ca3.1}$ inhibits cell cycle, whereas the opposite occurs when these channels are up regulated by serum addition (Wang et al., 2007).

An extensive study of the K^+ channel transcripts in primary lymphocytes and leukemias as well as several hematopoietic cell lines has been carried out by Smith and colleagues (Smith et al., 2002). In particular, they tested $K_v1.3$, $K_v10.1$, $K_v11.1$ and $K_v12.2$. Among these, only $K_v11.1$ turned out to be significantly up regulated in cancer cells. Expression was however not related to proliferation *per se*, because it was not observed in proliferating noncancerous lymphocyte types such as activated tonsillar cells, lymphocytes from Sjögren's patients and Epstein-Barr virus-transformed B cells. Conversely, our group has found the $K_v11.1$ transcript and the corresponding channel protein ($K_v11.1$, better known as hERG1) and currents (I_{hERG1}) in AML cell lines and in a high percentage of primary blasts from AML

patients. In this case, the block of I_{hERG1} , by applying specific hERG1 blockers, led cells to pause in G1. However, this was not the sole effect of hERG1 blockers; in fact, hERG1-blocking drugs also impaired AML cell migration through fibronectin. Hence, hERG1 also regulates cell migration and invasiveness in myeloid leukemias. This effect was mediated by a signaling mechanism triggered by cell adhesion, centered on Akt and modulated by hERG1 channel activity (Pillozzi et al., 2007). Similar results were obtained in childhood B-acute lymphoblastic leukemia (B-ALL) (Pillozzi et al., 2007). Both B-ALL cell lines and primary B-ALL cells expressed functional hERG1 channels, and hERG1 inhibition impeded the bone-marrow induced, integrin-dependent, protection against chemotherapeutic drugs, thus restoring a substantial apoptotic cell death. The hERG1 role in cancer cell biology is thus very complex. Mechanistic hypotheses based on current evidence are discussed later. Another member of the same Kv family, Kv 10.1 or EAG1, has long been related to cancer biology. Although the physiological expression of EAG1 is restricted to the brain, this channel is frequently and abundantly expressed in many solid tumors (Stühmer & Pardo, 2010). Until recently, it was assumed that EAG1 was not expressed in hematologic malignancies; however, Pardo and coworkers found a significant EAG1 expression in myelodysplastic syndromes, CML and almost half of a cohort of AML samples. In these cells, EAG1 blockade inhibited both proliferation and migration, both in AML cell lines and cultured AML primary samples (Agarwal et al., 2010).

The regulatory complexity is considerably increased by the fact that, in other contexts, the effects of K^+ channels directly modulate cell differentiation, instead of cell cycle. This was formerly observed in Friend erythroleukemia cells (MELC), which express Ca^{2+} -dependent K^+ channels (BK_{Ca}) (Arcangeli et al., 1987a, 1987b). These are transiently activated when differentiation is stimulated by cell adhesion onto fibronectin (Arcangeli et al., 1991; Becchetti et al., 1992), or by application of classical inducers of erythroid differentiation (Arcangeli et al., 1989). Similar effects were observed in THP-1 human monocytic leukemia cells. Undifferentiated THP-1 cells express K_{DR} channels. When differentiation to macrophages is induced by phorbol esters, K_{DR} expression is turned off, whereas BK_{Ca} and IRK are turned on (DeCoursey et al., 1996). hERG1 was also shown to be relevant to mediate osteoclastic differentiation in a pre-osteoclastic leukemia cell line, FLG 29.1 cells. In these cells differentiation may be induced by integrin-mediated adhesion to fibronectin as well as by treatment with phorbol esters. In both cases, the hERG1 blockade inhibited cell differentiation, which in these cells is witnessed by the increased expression of the calcitonin gene and by the up regulation of the $\alpha_v\beta_3$ integrin, both markers of osteoclastic differentiation (Hofmann et al., 2001). A full discussion of the K^+ channel effects on differentiation is outside the scope of the present review. We limit ourselves to exhort the reader to keep in mind the possible complementary effects exerted by channel modulation on the proliferation and differentiation branches of cell signaling.

While no study is available on K^+ channel expression in true hematopoietic stem cells (HSCs), K_{IR} currents have been observed in primitive hematopoietic precursor cells (HPCs) ($CD34^+ CD38^-$) stimulated with the combination of interleukin-3 (IL-3) and stem cell factor (SCF) (Shirihai et al., 1996). The biophysical features of whole cell currents suggested that several K_{IR} channel types were co-expressed. In fact, later work showed that both strongly rectifying (K_{IR} 4.3) and weakly rectifying (K_{IR} 1.1) channels are present in these cells. The expression of both K_{IR} types seems essential for the generation of committed progenitors *in vitro*, as inhibition of the expression of either suppresses the generation of progenitor cells

from IL-3 and SCF-stimulated umbilical cord blood CD34⁺ CD38⁻ cells (Shirihai et al., 1998). More recently, the *Kv11.1* transcript was detected in circulating CD34⁺ cells upon cell cycle induction by IL3 (interleukin 3), GM-CSF (granulocyte-macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor) and SCF (Pillozzi et al., 2002). As illustrated in more detail below, *K_v11.1* (i.e. hERG1) associates with the β_1 integrin in cord blood CD34⁺ cells. This interaction is essential for proper BM engraftment of these HPCs. Finally, the *K_v11.1* transcript was recently detected in CD34⁺/CD38⁻/CD128^(high) leukemic cells (Li et al., 2008), i.e. in the stem cell population that is critical for perpetuation of the leukemia disease (Li et al., 2008). On the whole, it is conceivable that *K_v* channels, and in particular hERG1, is relevant for normal and leukemic hematopoietic stem cells.

A novel player in this field is represented by the family of aquaporins (AQPs). A member of the family, AQP5 turned out to be overexpressed in CML cells, where it promotes cell proliferation and inhibits apoptosis, perhaps through an effect on cell volume control. In addition, the AQP5 expression increased with the emergence of imatinib mesylate resistance (Chae et al., 2008) (see also the paragraph below). Another member of the family, AQP9, has been shown to play a role in drug uptake and modulation of drug sensitivity in leukemia (Bhattacharjee et al., 2004) (see Chapter 6). Another channel, which has been recently reported to be expressed in the Jurkat cell line (Pottosin et al., 2008), is the TWIK-related spinal cord K⁺ (TRESK) channel, belonging to the double-pore domain K⁺ channel family.

As detailed in the general session on ion channels, P_{2X7} receptors are widely distributed in a variety of cell types and are involved in diverse biological effects. A sustained high level of extracellular ATP was detected in a tumor microenvironment which implied the involvement of abnormal signaling in tumour cells mediated by P2 family receptors. Besides being detected in solid tumors (Solini et al., 2008), high expression of the P_{2X7} receptor was observed in B-cell CLL (Adinolfi et al., 2002), acute leukemias and myelodysplastic syndromes (Zhang et al., 2004; Chong et al., 2010). Moreover, a series of P_{2X7} polymorphisms have been discovered, and their impacts on P_{2X7} functions and prognosis were studied (see also paragraph 3.2). For example, a N187D substitution was found in the J6-1 leukemia cell line, which displayed a lack of P_{2X7}-mediated calcium response upon BzATP stimulation (Zhang et al., 2004). It was also shown that K562 leukemia cells bearing this hyposensitive mutant displayed a proliferative advantage over wild-type P_{2X7}, both *in vitro* and in a nude mouse model. Furthermore, an increased angiogenesis and intratumoral inflammation could be detected in tumor masses formed by K562 cells bearing this mutant (Chong et al., 2010). Finally, P_{2X7} receptors were also functionally expressed in murine erythroleukemia cells, and the activation of these receptors seems to be important in the induction of apoptotic death and release of microparticles by these cells (Costantinescu et al., 2010).

An interesting debate, partially solved by the work of Huber's group (Kasinathan et al., 2007), involves chloride channels, and in particular ClC-3 channels. In this work, fluorescence microscopy revealed an intracellular localization of ClC-3 protein in K562 cells. Oxidation, on the contrary, increased the expression of ClC-3 protein into the plasma membrane, suggesting a role of plasma membrane-inserted ClC-3 in the oxidation-stimulated anion current observed in these cells (Kasinathan et al., 2007). Oxidation not only affects anion currents in K562 cells but also activates the non-selective cation channel TRPM2, resulting in an increase of intracellular free Ca²⁺ concentration, which in turn activates SK4 K⁺ channels on the plasma membrane and may trigger apoptosis. An oxidation-induced co-activation of the ClC-3-dependent anion permeability results in loss of

KCl (and osmotically obliged H₂O) and thus in cell shrinkage, suggesting that the ClC-3-dependent anion permeability *per se* may generate apoptotic volume decrease. Another intriguing role of anion conductance emerged from the work of Soriani and coworkers (Renaudo et al., 2007). In particular, they studied the relationships between sigma receptors and volume-regulated chloride currents (VRCC). Sigma receptors are intracellular proteins that were first postulated as opioid receptors on the basis of pharmacological and behavioural studies. Sigma-1 receptors were functionally coupled with membrane potassium channels in the pituitary (Aydar et al., 2002; Soriani et al., 1999a). Subsequently, it was reported that the activation of sigma-1 receptors by highly selective ligands provoked the arrest of the cell cycle progression in the G1 phase, in cancer cells. This effect was partly linked to the inhibition of voltage-dependent potassium channels (Renaudo et al., 2004). More recently, they also demonstrated that the sigma-1 receptor modulates VRCC and cell volume regulation properties in leukemic cells leading to an alteration of cell proliferation and apoptosis (Renaudo et al., 2007).

4. Genetics and epigenetics of channel expression in leukemias

It is clear that most human neoplastic cell types show altered expression of a variety of ion channels and that these exert different functional roles. However, there does not seem to be a specific channel-tumor correlation. This conclusion is in broad agreement with current genetic evidence, because no clear cancer-related mutation in any channel-encoding gene has been reported so far. Therefore, it would appear that ion channels are not so much involved in tumor causation, but are implicated in the different stages of neoplastic progression. A partial exception is *KCNRG*, which encodes a K⁺ channel-regulating protein that has been proposed to be a tumor suppressor gene (Ivanov et al., 2003). A missense mutation at the codon 92 of *KCNRG* is often present in human hepatocellular carcinomas, positive for the Hepatitis B virus (Cho et al., 2006). In B cell-CLL *KCNRG* has been detected in the minimal common deleted region (CDR) of the 13q14 chromosomal deletions. The latter is the most common abnormality in CLL (Liu et al., 1997; Dohner et al., 1999); deletions at 13q14.3 are associated with the longest survival. Rearrangements and/or deletions in the region of 13q14.3 are also found in other types of hematopoietic malignancies, including mantle cell lymphomas (Rosenwald et al., 1999) and multiple myelomas (MM) (Harrison et al., 2003; Elnenaï et al., 2003). In the majority of these non-CLL cases, 13q14 deletions are associated with a poor chemotherapy response profile. The CDR encompasses an area containing *DLEU1*, *DLEU2*, *RFP2*, and *KCNRG* as well as microRNAs miR-15a and miR-16-1 (Liu et al., 1997; Tyybakinoja et al., 2007; Kapanadze et al., 1998; Calin et al., 2002; Baranova et al., 2003). *KCNRG* is located within the 3' end of the largest transcript of *RFP2* (Ivanov et al., 2003). Due to its effect of interfering with the normal assembly of the K⁺ channel proteins by binding to their tetramerization domain, thereby, causing the suppression of Kv currents, it has been hypothesized that *KCNRG* may exert a tumor suppressor effect relevant to CLL and MM. Another frequent genetic alteration in leukemia is represented by translocations. The *RUNX1* (previously known as *AML1* or *CBFA2*) gene, located on chromosomal band 21q22, encodes the alpha subunit of the heterodimeric core-binding factor (CBF). Located at the C-terminus of *RUNX1*, a transcriptional regulation domain is required for the transcriptional activation or repression of genes relevant to myeloid and lymphoid development. *RUNX1* acts as a key regulator of hematopoiesis and is frequently targeted by mutations and chromosomal translocations in leukemias (Redaelli

et al., 2004). The t (1;21)(p22;q22) was first reported in a case of a possible therapy-induced leukemia (Rosenwald et al., 1999); in this case the partner gene of *RUNX1* was identified in the *CLCA2* gene, which then resulted to be a novel fusion partner of *RUNX1* in adult patients with a therapy-related AML.

The frequent overexpression of channel-encoding genes in human cancers seems to be often caused by gene amplification. This has been demonstrated for *KCNK9*, in breast (Mu et al., 2003) and colorectal cancers (Kim et al., 2004), and *CACNA1E* (Ca_v 2.3), in Wilms' tumours (Natrajan et al., 2006). In other cases, epigenetic mechanisms have been invoked. Among these, a paradigmatic example is aberrant promoter methylation of the growth regulatory genes. This mechanism is probably a common alternative to gene inactivation, in human cancers. Evidence along this line is available for channel-related genes, such as *CLCA2*, whose promoter region is frequently inactivated by hypomethylation, in breast cancer (Li et al., 2004). Moreover, methylation of *KCNH5* is observed in about 80% of NSCLC tissue, but only in 14% of non-cancerous tissue (Feng et al., 2008). Finally, inactivation of *CACNA1G* by aberrant methylation of its 5' CpG island has been reported in AML, gastric cancers and colorectal cancers (Toyota et al., 1999). More puzzling remain the genetic mechanisms underlying AQP5 overexpression in CML. No genomic amplification was detected, whereas methylation analysis of the AQP5 promoter region suggested that promoter demethylation might be relevant, although this fact was proven in head and neck and lung cancer cell lines, while validated data are still lacking in leukemias.

Another genetic mechanism that could explain the alterations of ion channel encoding genes in leukemias, involves micro (mi) RNAs. MiRNAs are naturally occurring 18- to 25-nucleotide RNAs that are cleaved from 70- to 100-nucleotide hairpin precursors by a complex protein system that includes the RNase III Droscha and Dicer, members of the Argonaute family. Mature microRNAs typically hybridize to the 3' untranslated regions of protein-coding messenger RNAs (mRNAs) and cause their post-transcriptional repression and/or degradation (Ambros, 2004; Bartel, 2009). MiRNAs regulate normal cell homeostasis and are involved in many physiologic processes, including hematopoiesis (Garzon & Croce 2008; Vasilatou et al., 2010; Havelange & Garzon, 2010). Recently, dysregulation of miRNAs has been shown in many types of solid tumors and leukemias (Calin & Croce, 2006). Direct involvement of miRNAs in cancer has been suggested by a study demonstrating that several miRNAs are localized in genomic regions associated with cancer, such as breakpoint regions in chromosome aberrations involving oncogenes or tumor suppressor genes, minimal regions of loss of heterozygosity, minimal regions of amplification, and at loci close to fragile sites and integration sites of the human papilloma virus. Several functional studies confirmed the important role of miRNA deregulation in hematologic malignancies, mainly B-CLL and AML. Ion channel encoding genes are among the target genes of miRNAs: for example two miRNAs often dysregulated in CLL, miR15a and miR16-1 have, among their target genes, genes encoding K_v and water channels, in particular *KCNH8* (i.e. *K_v12.1*) and aquaporin 11, respectively. *KCNH3* (i.e. *K_v12.2* or *elk-2*), another K_v encoding gene strictly related to *KCNH8* and functionally similar to *K_v11.1*, is also one of the target genes of miR221, often deregulated in CLL. *K_v11.1* is also negatively regulated by miR133, which is one of the miRNAs down-regulated in a specific clinicopathological subgroup (t (8:21)) of AML. This fact could explain the frequent increased expression of *K_v11.1* transcript in AML. Growing evidence also suggests that tumors tend to express splice variants or alternative transcripts of channel-encoding genes, although the significance for cancer progression is still uncertain. The *hslbBK* splice variant of *gBK* has been detected in gliomas (Olsen et al.,

2005) and the *herg1b* alternative transcript of $K_v11.1$ is overexpressed in human leukemias and neuroblastomas (Pillozzi et al., 2007; Crociani et al., 2003). Another splice variant of the $K_v11.1$ transcript, which encodes for a C-terminus deleted $K_v11.1$ protein, named *herg1b_{USO}*, is also overexpressed in several leukemias cell lines, and exerts a post-translation control on the plasma membrane expression of the full length $K_v11.1$ protein (Guasti et al., 2008).

5. Ion channels in primary leukemias: A novel biomarker?

As better detailed in chapter 1, leukemia is a disease with marked heterogeneity in both response to therapy and survival. Cytogenetic, age, and performance status have long determined prognosis and therapy. The advent of molecular diagnostics has heralded an explosion in new prognostic factors: microarray technology can now identify unique gene expression signatures associated with prognosis. Similarly miRNA expression, single nucleotide polymorphism arrays, and DNA methylation signatures have recently described important new prognostic subgroups in the single leukemia categories. We may be close to a time when we will be able to use these prognostic factors and technologies to identify new targets for therapy and to determine who may benefit from that therapy, and ultimately change how we treat individual patients with leukemia. It is mandatory at this time, to transfer these concepts to the ion channels, after a 20 years work mainly focused to study ion channel expression and function identification. Indeed some recent papers aim at delineating the impact of single ion channels, or of an “ion channel signature” as biomarkers in leukemias. Following are some examples: $K_v11.1$ expression was correlated with a more aggressive AML phenotype both *in vitro* and *in vivo*. In a cohort of patients affected by AML, $K_v11.1$ expression was associated with a higher probability of relapse and a shorter overall survival (Pillozzi et al., 2007). This was one of the first clinical and prognostic applications of an expression screening for a K_v channels. Subsequently, EAG1 was found to be expressed in myelodysplastic syndromes, CML and AML. Interestingly, channel expression in AML patients strongly correlated with increasing age, higher relapse rates and a significant shorter overall survival. Multivariate Cox regression analysis revealed EAG expression levels in AML as an independent predictive factor for reduced disease-free and overall-survival; such association further stresses the impact of K_v channels of the EAG family as biomarkers in AML (Agarwal et al., 2010). Moon and co-workers (Chae et al., 2008) found evidence that AQP5 might be associated with the progression of CML. Indeed, CML patients diagnosed at accelerated or blast crisis phase showed significantly higher level of AQP5 expression than those diagnosed at chronic phase, while CML patients who gained imatinib mesylate resistance at chronic phase exhibited significantly higher level of AQP5 expression than those who gained resistance at accelerated or blast crisis phase. Furthermore, AQP5 expression increased with the appearance of imatinib mesylate resistance (see also chapter 7.2). A recent paper reported the results of a study in which the expression of P_{2X} receptors in blood mononuclear cells from Chinese pediatric leukemias patients was determined. P_{2X1} , P_{2X4} , P_{2X5} and P_{2X7} were simultaneously over expressed in leukemias compared to controls. The co-expression of P_{2X4} and P_{2X7} was a common feature of leukemic samples, and the highest expression of P_{2X7} was detected in relapsed patients, whereas a concomitant decrease of P_{2X4} , P_{2X5} and P_{2X7} was observed after chemotherapy (Chong et al., 2010). This aspect has a clear relevance also for the chemoresistance, which is described in chapter 6.

Some studies also reported altered expression of ion channels and transporters in primary lymphomas. For example upregulation of KCNN3 ($K_{Ca2.3}$) was observed in germinal center B-like diffuse large B cell lymphoma (DLBCL), whereas KCNA3 ($Kv 1.3$) was upregulated in activated B-like DLBCL (Alizadeh et al., 2000). Moreover, in the lymphoma cell line Daudi, expression of $K_{Ca3.1}$ has been described and its activity may account for cell malignant growth and proliferation (Wang et al., 2007). Data obtained by a microarray study in pediatric brain tumors indicate a marked deregulation of ion channels and transporters. Hence, we suggest to focus on the “ion channels and transporters” term when analyzing microarray gene expression data (Masselli et al., unpublished).

6. Outline of the functional role of ion channels in cancer

Ion channels are generally involved in many processes necessary for cell proliferation, adhesion to substrate and motility. For example Ca^{2+} fluxes control the cell cycle machinery and the secretion of cytokines and growth factors. Moreover, ion channels participate in regulating the cell volume changes that normally occur during mitosis, cell migration, etc. However, altered expression or function of different channel types are also thought to determine more specific aspects of malignancy. In leukemic cells, these alterations contribute to determine the poorly differentiated phenotype, invasiveness, transendothelial migration and chemoresistance (Arcangeli et al., 2009). In this context, a common occurrence is ion channel involvement in the signaling pathways that are related to the modulation of cell adhesion to the extracellular matrix.

6.1 V_m in cancer cells

As is well known, proliferating cells tend to be depolarized as compared to non cycling cells. Such a difference may depend on regulated channel expression. For instance, developing glial cells generally express outward rectifying K_v , subsequently substituted by inward rectifier K^+ channels that, in mature cells, determine the typical hyperpolarized state of adult glia (Sontheimer et al., 2008). Why expression of different K^+ channels produces such a different effect on V_m ? The reason is that proliferating and tumor cells usually undergo slow and low amplitude V_m changes, compared to excitable cells. Therefore, what matters more are the steady state K^+ channel properties, which determine the fraction of open channels at a certain stable V_m . During the neoplastic transformation, the process exemplified by glia differentiation often reverts in that cancer cells tend to express a variety of K^+ channels that either carry little current at strongly negative V_m , such as $K_v10.1$ and $K_v1.3$ (unless they undergo overexpression, see below) or channels whose maximal steady state probability of being open is obtained at relatively depolarized V_m (e.g. -40 mV for $K_v11.1$). In some instances, the neoplastic condition seems a partial reversion to a state normally occurring during development, but the evidence about this is still limited.

However, considering the average V_m is simplistic, because oscillations are observed in either the expression or regulation of several ion channel types during the cell cycle phases, with ensuing alterations in V_m (Arcangeli & Becchetti, 2006). In general, it is unclear if these V_m changes regulate the downstream signals or are by products of alterations in channel activity/expression that the cell controls for reasons not necessarily linked to V_m . For example, specific channel subtypes can exert specific signaling roles. Current evidence indicates that there is probably no unique explanation. Several examples suggesting a spectrum of possible mechanisms are illustrated below.

6.2 Hyperpolarization can stimulate Ca^{2+} influx

Intracellular Ca^{2+} signals are known to regulate cell cycle in both normal and cancer cells. Ca^{2+} channels are in fact widely expressed in cancer cells, in which they are also likely to modulate cell movement and migration. Original work in T lymphocytes indicated that a hyperpolarization produced by increased K^+ channel function facilitates the Ca^{2+} influx necessary for T cell activation. Analogous results were more recently obtained in breast cancer cells, where growth factors applied in G1 can lead to such an over-expression of $\text{K}_v10.1$ to lead to cell hyperpolarization and G1-S transition. Such a hyperpolarization triggers a positive feedback in that Ca^{2+} influx stimulates $\text{K}_{Ca3.1}$, which maintains a tonic hyperpolarization that further sustains the Ca^{2+} signal that regulates the cyclins and the cyclin-dependent kinases (Ouidid-Ahidouch & Ahidouch, 2008).

6.3 Control of cell volume and motility

Mammalian cells undergo oscillatory volume changes during both cell cycle and migration (Habela & Sontheimer, 2007; Boucrot & Kirchhausen, 2008). The interplay of K^+ and Cl^- channels is very important in the early phases of cell volume regulation. A full review of this topic cannot be given here (Chandy et al., 2004; Nilius, 2007) but it is clear that altered control of these processes may affect proliferation as well as tumor invasiveness, as has been shown in gliomas.

6.4 Ion channels and intracellular signaling

Ion channels are also implicated in different aspects of leukemia malignancy, such as the lack of differentiation, invasion and transendothelial migration, chemoresistance (see also Table 1). The role of ion channels in such processes can be attributed to signaling mechanisms, which are often related to the modulation of adhesive interactions with the extracellular matrix.

6.4.1 Signaling mechanisms

Cell proliferation in mammalian cells can be triggered by growth factor (GF) binding to specific receptors, usually protein tyrosine kinases that autophosphorylate upon ligand binding. This process typically turns on a kinase cascade that converges onto phosphorylation of the extracellular signal-regulated protein kinase 2 (ERK2) mitogen-activated protein (MAP) kinase. Once activated, this protein translocates to the nucleus and phosphorylates an array of specific transcription factors (Arcangeli & Becchetti, 2006). A similar mechanism is also triggered by cell adhesion. In particular, the integrin-mediated cell adhesion to the extracellular matrix modulates, in proper context, cell migration, proliferation, differentiation and prevents apoptosis (Juliano, 2002; Arcangeli & Becchetti, 2006). The integrin linked kinase (ILK) and the focal adhesion kinase (FAK) are pivotal factors in these cascades. Once activated, they recruit further signaling components, thus leading to the activation of MAP kinases, of the phosphoinositide-3 kinase (PI3K), and small GTPases such as Rho A, Rac 1 and CDC42 (Arcangeli & Becchetti, 2006). Adhesion signals greatly overlap with those activated by GF and cytokine receptors. In some cases, such an overlap depends on the formation of macromolecular complexes between integrins and the other membrane receptors, to form signaling platforms at the adhesive sites. These platforms can also include ion channels, with ensuing crosstalk between the different components (Arcangeli & Becchetti, 2006). For example, T lymphocyte activation leads $\text{K}_v1.3$

channels to associate with β_1 integrins and activate them (Levite et al., 2000), an interaction also observed in melanoma cells (Artym et al., 2002). A macromolecular complex between β_1 integrin subunit and hERG1 (K_v 11.1) forms in several neoplastic cells (Cherubini et al., 2005). The β_1 /hERG1 complex localizes at the adhesion sites, probably within caveolae/lipid rafts, and recruits FAK, Rac1 and PI3K. FAK phosphorylation, Rac1 and PI3K activities turned out to depend on hERG1 currents (Cherubini et al., 2005). Moreover, we noticed that in AML cells the β_1 /hERG1 complex also includes the VEGF receptor 1 (Flt-1). The macromolecular complex regulates signaling downstream to Flt-1 (MAP kinase and PI3K) and thus AML proliferation and migration (Pillozzi et al., 2007). In childhood B-ALL, the β_1 /hERG1 complex is triggered by adhesion onto human bone marrow stromal cells (MSC), and comprises the chemokine receptor CXCR4. We determined the signaling pathways activated by the components of the β_1 integrin/hERG1/CXCR4 complex in B-ALL cells and we found that ILK was the first effector to be activated after engagement of CXCR4, integrin activation and/or culture of leukemia cells on MSC. MAP kinase and PI3K/pAkt pathways (downstream effectors of ILK activity) were also activated in B-ALL cells cultured on MSC. The activation of both signaling pathways depended on β_1 integrin activation, as it was inhibited by a blocking antibody. Importantly, both ILK activity and ERK1/2 and Akt phosphorylation were strongly reduced in B-ALL cells cultured on MSC after blocking hERG1 channels by two specific blockers. When cultured on MSC, leukemia cells are protected from apoptosis induced by chemotherapeutic drugs (see below). Another example, involves the K_v channels regulated by GFs in ML-1 myeloblastic leukemia cells (Xu et al., 1999; Guo et al., 2005), as well as for the VGSCs controlled by NGF (Brackenbury et al., 2007) and EGF (Uysal-Ongonen et al., 2007; Ding et al., 2008). But signals can also flow in the opposite direction: for example the 4-AP-sensitive K⁺ channels control ML-1 proliferation through multiple signal transduction processes, such as phosphorylation of ERK 1/2 and Akt (Guo et al., 2005).

6.4.2 Non-conductive roles

As discussed above, some effects of ion channels on neoplastic progression are clearly a consequence of changes in ion fluxes. Less attention has been so far devoted to possible modulation of intracellular pathways by enzymatic roles of channel proteins or conformational coupling with their proteins, ultimately converging onto the transcriptional regulation of cancer-related genes. These mechanisms may well accompany the typical effects on ion flow. Some VGCs have been in fact shown to behave as bifunctional proteins that, besides gating ion fluxes as usual, exert an ion conduction-independent control of several intracellular responses (Iwasaki et al., 2008; Wang et al., 1999). An example with oncological implications is the voltage sensor-containing phosphatase (Ci-VSP) of *Ciona intestinalis*. This consists of an ion channel-like transmembrane domain, followed by a phosphatase domain highly homologous to PTEN, a tumor suppressor of human cancers (Iwasaki et al., 2008). Recent review of some of these non-conducting functions is found in (Levitan, 2006). Although a similar behaviour has not been clearly demonstrated to occur in cancers and leukemia, it could be relevant in prospecting new therapeutic strategies.

7. Ion channels as therapeutic targets in leukemia

Using extracellular ion channel blockers for oncologic therapy would limit harmful metabolic effects and allow relatively easy calibration of doses. Moreover, thanks to the

modern electrophysiological methods and recent insight into ion channel structure, the mechanism of action of channel blockers is often understood in depth, which facilitates rational therapy and design of new compounds. These can be tested both in heterologous systems and *in vivo*, which allows to predict or study some of the side effects. Although ion channels are very suitable targets for drug screening and rational therapeutic strategies, they are still scarcely used to target non excitable cells such as the neoplastic, because of the risk of serious side effects. A classical example is $K_v11.1$. As fully discussed elsewhere (Arcangeli et al., 2009), several indications suggest that $K_v11.1$ could be an effective target for cancer therapy. However, this channel type also regulates the repolarization phase of the cardiac action potential. Therefore, $K_v11.1$ block can lead to the so-called *torsade de points* (TdP), i.e. ventricular arrhythmia that may lead to lethal ventricular fibrillation (Witchel et al., 2000). Therefore, hERG1 blockers are generally considered unsafe for pharmacological treatment in humans. Another drawback of the most common hERG1 blockers is that they act on the cytoplasmic face of the channel.

However, even in cases as unfavourable as this, a way out of the trouble is provided by the availability of many different inhibitors that act with different mechanisms. More explicitly, several $K_v11.1$ blockers are not torsadogenic, although the reasons for such differences between different compounds are still poorly understood. In general, recent work suggests several methods to obtain better tissue specificity even when selective blockers for channel subtypes are not available. Alternatively, ion channels can be targeted to deliver tracers or cytotoxic compounds. The following paragraph summarizes such evidence with a focus on leukemic cells.

7.1 Targeting ion channels in leukemias: Experimental evidences

As described above, different papers have reported that ion channels inhibitors can affect different biological aspects of leukemic cells, both *in vitro* and *in vivo*. Such evidences are summarized in Table 1, in which are shown the specific drug and ion channel targeted as well as the biological aspect of leukemia influenced.

	LEUKEMIA	ION CHANNEL	DRUG (or molecular tools)	References
PROLIFERATION	AML	$K_v 11.1$	E4031, WAY 123.398	Pillozzi, 2002 Pillozzi, 2007
	AML, CML myelodisplastic syndrome	EAG1	astemizole, imipramine, mAb56	Agarwal, 2010
	CML	AQP5	siRNA	Chae, 2008
	AML (ML-1 cell line)	K^+ currents	4-AP	Xu, 1996
	B-lymphoma	$KCa 3.1$	TRAM-34	Wang, 2007
MIGRATION/TEM	AML	$K_v 11.1$	E4031, WAY 123.398	Pillozzi, 2007
DIFFERENTIATION	AML-M5A	$K_v 11.1$	WAY 123.398	Hofmann, 2003
CHEMIORESISTANCE	B-ALL	$K_v 11.1$	E4031, WAY 123.398, sertindole, erythromycin.	Pillozzi, 2011
APOPTOSIS	CML	AQP5	siRNA transfection	Chae, 2008
	APL	AQP9	overexpression	Bhattacharjee, 2004

Table 1. Pharmacological and molecular modifiers able to affect ion channels involved in different biological aspects of leukemia disease. (AML: acute myeloid leukemia; CML: chronic myeloid leukemia; ALL: acute lymphoblastic leukemia; APL: acute promyelocytic leukemia).

Several types of K⁺ and Cl⁻ channels have been shown to be potential targets for cancer treatment (Arcangeli et al., 2009). In leukemia cells, most of the relevant results concern EAG and hERG1, both of which belong to the K_V channel family and share 47% of the amino acid sequence. They are expressed in different forms of leukemias and have been implicated in cell cycle progression and proliferation in these and other cancers (Arcangeli et al., 2009). Inhibition of these channels reduces proliferation both *in vitro* and *in vivo*, which indicates that modulating their activity could produce beneficial effects on patients. In fact, several studies on immunodepressed mice show that blocking EAG and hERG1 channels inhibits progression of the disease. In fact, we also determined whether hERG1 inhibitors could improve leukemia treatment in murine models of B-ALL. In a first set of experiments, NOD-SCID mice were inoculated with 697 cells and treated daily with E4031 (20mg/Kg) for two weeks, starting one week after the inoculum. At the end of treatment, some of the mice were sacrificed and the degree of bone marrow, peripheral blood and extramedullary organs invasion by B-ALL cells was quantified. E4031 treatment significantly reduced the leukemia burden and the liver and spleen infiltration by leukemic cells, with significant prolongation of mice survival. In a second set of experiments, we tested the effects *in vivo* of the combined treatment with E4031 and dexamethasone on REH cells, which have been reported to be resistant to corticosteroids *in vivo*. Mice were treated for two weeks with dexamethasone, E4031, or both. E4031 reduced bone marrow engraftment of REH cells, similarly to what was observed for 697 cells. This effect was related to an increased apoptosis of B-ALL cells, and was higher than that produced by dexamethasone. Treatment with dexamethasone and E4031 together nearly abolished bone marrow engraftment while producing substantial apoptosis. A marked reduction in leukemic cell infiltration of the spleen in mice treated with dexamethasone plus E4031 was also observed. These data clearly indicate that hERG1 blockers can treat the leukemia disease *in vivo*, both alone and in combination with classical chemotherapeutic drugs and are capable of reverting drug resistance *in vivo*. These drugs also exerted clear antileukemic activity (see details below) and thus represent promising candidates for further studies aimed at paving the way to clinical trials.

Because EAG and hERG1 channel are structurally related, any drug acting on EAG channel may also block hERG1 channels and thus have arrhythmogenic effects. Therefore, irrespective of whether one intends to use pharmacological blockers of EAG and hERG1, to avoid side effects it will be necessary to either develop specific inhibitors for different subtypes or carefully test each inhibitor for the effects on each channel type. These and other molecular tools have been already applied to specifically target EAG in cancer cells, and particularly 1) chemical blockers; 2) monoclonal antibodies; 3) small interfering RNA (siRNA). The challenge with the latter approach is designing an appropriate vehicle for transport and delivery of siRNA to the target organ, something that is currently the subject of intense research. All of these methods can be used in conjunction with chemotherapeutic agents or can be exploited to improve survival in chemoresistant diseases.

As to Cl⁻ channels, the activation and the subsequent hyperpolarization triggered by the antiparasitic ivermectin at low micromolar concentrations preferentially induces cell death in AML cell lines and primary patient samples compared to the normal hematopoietic cells. Ivermectin also delayed tumor growth in 3 independent mouse models of leukemia and synergized with cytarabine and daunorubicin, that also increase reactive oxygen species production. Although the specific channel activated by ivermectin has not been clearly

identified, the thorough knowledge about the toxicology and pharmacology of ivermectin, this compound could be rapidly advanced into clinical trial for leukemia. (Sharmeen et al., 2010).

7.2 Ion channels and leukemia chemoresistance

Chemoresistance is recognized clinically as the development of tumor resistance to a wide variety of anticancer drugs following exposure to a single drug. Resistance to multiple drugs could arise from cellular defenses that broadly limit access of the agent to a cellular target, or prevent the cell from entering apoptosis following injury. The development of resistance to a wide spectrum of cytotoxic drugs frequently impedes the successful treatment of acute and chronic leukemias either at the initial presentation or following primary or subsequent relapses (O’Gorman et al., 2001). The majority of leukemias in fact respond to initial treatment; however, relapse is common, indicating resistance of leukemic cells to current therapies. Several mechanisms may account for this phenomenon, including failure of the drug to reach and/or affect its intracellular target or failure of the cell to undergo apoptosis in response to chemotherapy (Ross et al., 2000). There is emerging evidence that also extrinsic components mediated by the microenvironment play a pivotal role in survival and drug resistance of leukemic cells. It is believed that environment-mediated drug resistance is a transient state whereby leukemic stem cells are protected through signals from the niche, which eventually leads to the selection of secondary genetic changes and outgrowth of cells that acquired multiple mechanisms of pharmacologic resistance (Meads et al., 2009).

In addition to the direct relationship between transporters and drug substrates, indirect mechanisms may also modulate chemosensitivity. For example, transporters and channels can affect chemosensitivity by providing nutrients to cancer cells or modulating the electrochemical gradient across membranes, thereby, modifying apoptosis pathways or the efficiency of drug diffusion along electrochemical gradients into cells (Huang et al., 2006).

Several genes that encode subunits of Na^+ , Cl^- , K^+ , and other cation channels correlated with drug activity, confirming that ion channels can modulate drug response—possibly by affecting the cell’s resting potential or by providing key metal ion cofactors. We do not know at this point whether these gene products mediate drug transport directly or affect sensitivity and resistance by indirect mechanisms. Ion channels modulate electrochemical gradients generated by ion pumps and ion exchangers. Maintenance of a strong electrochemical gradient is vital to the cell and a potentially strong influence on drug activity. K^+ and Cl^- leakage currents tend to polarize cells, whereas Ca^{2+} and Na^+ channels depolarize them. These two types of flux would be expected to have opposite effects on drug equilibration across cell membranes. However, Ca^{2+} flux is also important in apoptotic signaling, as noted above, so the net effect on drug potency is difficult to predict.

The main ion channels whose activity is related to the establishment of chemoresistance in leukemia cells are mitochondrial voltage-dependent anion channels (VDAC), aquaporins and hERG1. The VDAC, located at the outer mitochondrial membrane, play a central role in the regulation of apoptosis. VDAC is the constituent of the mitochondrial permeability transition pore (PTP), which mediates the release of apoptogenic factors such as cytochrome c from the intermembrane space into the cytosol (Shimizu et al., 1999). Three VDAC genes have been identified in human, *VDAC1*, *VDAC2* and *VDAC3*. *VDAC1* binds to Bcl-2 and its homologues, such as Bax, Bak and Bcl-XL to regulate the opening of PTP (Abu-Hamad et al., 2009). Cells deficient in *VDAC2* but not cells lacking the more abundant *VDAC1* are more

susceptible to apoptotic death. VDAC has been suggested to be one of the biological targets of arsenic trioxide (As_2O_3), an anticancer drug for acute promyelocytic leukemia (APL) (Zheng et al., 2004). One member of the aquaporin family, AQP9, has been shown to play a role in drug uptake and modulation of drug sensitivity in leukemia (Bhattacharjee et al., 2004). AQP9 has broad transport specificity, including water, glycerol, urea, carbamides, purines, and pyrimidines, but its physiological function is still unknown. Increased expression of AQP9 in leukemic cells K562 and HL60 increases uptake of and sensitivity to As (III) and Sb (III), the trivalent arsenic drug trisenox. Moreover primary APL cells expressed AQP9 significantly (2-3 logs) higher than other AML, which might explain their exquisite As_2O_3 sensitivity. AQP7 also transports As (III) and Sb (III), but to a lesser extent than AQP9. Arsenic drugs often cause significant toxicity during treatment, showing marked individual variability. Individual variability in expression of AQP7 and AQP9 may partly explain the differential response and toxicity to arsenic therapy (Liu, 2002). Based on these observations, we suspect that AQP5 may be conferring a growth advantage in the process of CML progression. Furthermore, it has been reported that AQP5 may play a role in developing imatinib mesylate resistance, irrespective of other known major resistance mechanisms such as *bcr-abl* mutation or amplification (Chae et al., 2008).

As anticipated in paragraphs 6.4.1 and 7.1 our group studied the molecular mechanisms underlying the protection by MSC in B-ALL. We evidenced that coculture with MSC induced the expression of a plasma membrane signaling complex in B-ALL cells, constituted by hERG1 channels, the β_1 integrin subunit and the chemokine receptor CXCR4. Interaction of integrins with their ligands on MSC layers is critical to the formation of the complex. We next tested the effects of coculturing B-ALL cell lines with MSC on chemosensitivity and the role of the β_1 integrin/hERG1/CXCR4 complex. B-ALL cell lines cultured with or without MSC were exposed to doxorubicin, prednisone and methothrexate, drugs commonly used to treat ALL. Classical hERG1 blockers, E4031 or Way 123,398, were tested on cells cultured on MSC and treated in combination with each drug. It emerged that MSC protected B-ALL from the apoptosis induced by all the drugs tested, although to different degrees depending on the drug and cell line. MSC-induced resistance was almost completely abrogated when ALL were treated with hERG1 blockers. We also tested the effects of sertindole (an antipsychotic) and erythromycin (an antibiotic), which are known to block hERG1 channels but do not cause cardiac arrhythmia and can be used clinically. The two drugs were added to B-ALL cells cultured on MSC, along with doxorubicin. Both drugs reverted MSC-drug induced chemoresistance in all the B-ALL cell lines tested, at levels even greater to those obtained with the hERG1 blockers E4031 and Way 123,398. Hence, the activity of hERG1 channels inside the β_1 integrin/hERG1/CXCR4, whose activation is triggered by culture on MSC, mediates the MSC- induced drug resistance to apoptosis, and that different hERG1 blockers can overcome drug resistance. These results were corroborated by the studies in murine models of B-ALL reported above. We can conclude that hERG1 channels are upstream regulators of the MSC-triggered pro- survival signals in B-ALL, and that administration of hERG1 blockers could improve chemotherapy responses in patients with ALL (Pillozzi et al., 2011).

8. Conclusion

The evidence we have reviewed shows that certain ion channel types exert important regulatory roles in leukemic cell physiology. These functions are implicated in the neoplastic

progression and thus appear to be potential target for therapy, as is also suggested by recent work in murine models. For the reasons discussed above, particularly the possibility of serious side effects, ion channels are still somewhat neglected as pharmacologic targets in oncology. However, we believe they should receive more attention because they present considerable advantages in terms of thorough mechanistic understanding and clinical potential, not only for leukemias. In fact, clinical trials are currently in progress for testing the efficacy of targeting specific channel types in several tumors such as glioblastoma and urinary bladder carcinoma. Therefore, more widespread efforts should bring novel pharmacological applications of ion channel for treating oncohematologic diseases as well as other cancers

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Targeted Therapies in Hematological Malignancies

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1. Introduction

Tumorigenesis has a complex pathogenesis; with multiple genetic, proliferative, apoptotic and differentiation pathways aiding in development and growth of a tumor. Conventional chemotherapy has been the backbone for cancer treatment. The chemotherapeutic agents act on different phases of cell cycle of rapidly proliferating cells. Subsequently, in addition to effect of cancerous cells they also affect rapidly multiplying gastrointestinal lining cells, bone marrow and hair follicles leading to mucositis, diarrhea, various degrees of myelosuppression and alopecia. The search for less toxic agents has been ongoing for decades. Targeted therapy refers to directing a drug to a target that is either specific to or over expressed on the malignant cell. The concept of targeted therapy dates back to Paul Ehrlich who used the term “magic bullets” to describe a therapeutic agent which killed the microorganism but left the patient unharmed. Today the concept of targeted therapy is the most rapidly evolving field in drug discovery and cancer treatment.

One of the major hallmarks of successful targeted treatment is use all trans-retinoic acid (ATRA) for acute promyelocytic leukemia (APL). APL is distinguished by translocation between chromosome 15 and 17 (PML-RAR- α) which halts the differentiation at promyelocyte stage. ATRA induces the differentiation of promyelocytes into mature myeloid cells. Use of ATRA has improved response rate and survival rate in APL which was previously associated with a significantly worse outcome. In this chapter we review other major targeted agents. Due to limited space we emphasized primarily on chronic myeloid leukemia, multiple myeloma and lymphoma.

2. Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is other example for successful targeted therapy treatment. The initial description of chromosome abnormality was first described by Nowell and Hungerford in two patients who were noted to have a loss of the long arm of chromosome 21 or 22 (later confirmed to be chromosome 22 and designated Philadelphia chromosome (Ph))(Nowell and Hungerford 1960; Nowell and Hungerford 1961). Subsequently, Rowley discovered that there is a reciprocal translocation between chromosome 9 and 22 in these patients(Rowley 1973). As a result of the translocation, cellular oncogene ABL on chromosome 9 and a segment of chromosome 22, the breakpoint cluster region BCR, fuse and cause activation of tyrosine kinase which is capable of inducing

the disease in mice. This established the fusion protein as the cause of malignant transformation in this disease. This led to the development of small molecule inhibitors of the mutant kinase and led to the discovery of Imatinib mesylate (Gleevec; Novartis, Basel, Switzerland).

Imatinib was the first tyrosine kinase inhibitor (TKI) to be developed and approved for CML in 2002. It was developed on the concept of designing synthetic compounds with chemical structures that are able to compete with the binding site in the kinase domain. Imatinib has demonstrated higher response rates (RR) and better tolerability compared to interferon and low dose cytarabine (International Randomised Study of Interferon and ST1571 (IRIS) trial) (Santos and Quintas-Cardama 2011). Recent update of IRIS trial reported that at 8 years follow up 83% had complete cytogenetic response (cCR) and overall survival (OS) was 85% among patients with newly diagnosed, untreated CML in chronic phase (American Society of Hematology, 2009, Abstract 1126). Authors did report that 45% of patients in Imatinib arm discontinued treatment and one of the major reasons was development of resistance (Deininger M et al 2009). Over expression of the BCR-ABL1 oncogene, mutations in BCR-ABL1 that obstruct imatinib binding, alternative signaling pathways which reduce levels of transporters responsible for imatinib uptake have been associated with resistance. Second generation TKI approved in for patients with imatinib-resistant CML or imatinib intolerant are dasatinib (Sprycel; Bristol-Myers Squibb) and nilotinib (Tasigna; Novartis) and are highly active against most BCR-ABL1 mutations. Moreover, recently results of two phase III randomized trials comparing them to imatinib as first-line treatment showed higher major cytogenetic response (MCyR), major molecular response (MMR) and reduced rates of transformation to accelerated phase (AP) or blast phase (BP) (Kantarjian, Giles et al. 2007; Hochhaus, Baccarani et al. 2008). As a result, U.S. Food and Drug Administration (FDA) approved both dasatinib and nilotinib as first line therapies for patients with CML in chronic phase (CP).

There are patients who continue to experience poor outcomes despite the development of second generation TKIs. T315I mutation is the one of major cause of this resistance. Furthermore, none of the first and second generation TKIs eliminate leukemic stem cells (LSCs). A fraction of LSCs persist in a quiescent state and are seen in patient with CML in cCyR. It is believed that they are responsible for CML relapse upon discontinuation of imatinib therapy.

2.1 Newer agents in CML treatment

New drugs have been developed with special interest in agents active in highly TKI-resistant CML (Table 1).

3. Multiple myeloma

Multiple myeloma (MM) is a B-cell neoplasm that is characterized by clonal proliferation of terminally differentiated plasma cells. Clinical features include bone disease, hypercalcemia, cytopenias, and renal dysfunction. Our understanding of this disease has improved significantly in the past decade and has led to the development of novel targeted therapies. The interaction between MM cells and their microenvironment is the subject of intense research and several novel targets have emerged (Hideshima, Mitsiades et al. 2007).

Drug	Study Phase	Comments	Clinical trial*
Panotinib	II	Third generation TKI which acts against T315I mutation	NCT01207440
Danusertib	II	Inhibits Aurora kinase and BCR-ABL1 (includes T315I mutation)	NCT00335868
XL-228	I	Inhibits Aurora kinase and BCR-ABL1 (includes T315I mutation)	NCT00464113
AT-9283	I	Inhibits Aurora kinase and BCR-ABL1 (includes T315I mutation)	NCT00522990
DCC-2036	I	Switch pocket inhibitor	NCT00827138
BMS-214662	I	Farnesyl transferase inhibitor	NCT00006213
Panobinostat	I/II	Histone deacetylase inhibitor	NCT00686218
Omacetaxine	I/II	Homoharringtonine formulation- disrupts protein synthesis	NCT00462943 NCT00375219 NCT00006364

* Clinicaltrials.gov accessed on 4/30/11

Table 1. Lists the newer promising agents currently in preclinical or clinical phase of development for treatment of CML.

3.1 Intracellular and nuclear targets

3.1.1 Proteasome inhibitors

The proteasome is an abundant catalytic complex that is found in both the nucleus and cytoplasm of eukaryotic cells (Adams 2004). Its function is to degrade intracellular proteins, such as the cyclins, caspases, BCL2 and NF- κ B, which mediate cell cycle progression and apoptosis. The mechanisms by which malignant cells are more susceptible to proteasome inhibition than normal cells are not completely understood. One explanation is that many types of malignant cells rapidly proliferate and have one or more aberrant cell-cycle checkpoints. These cells might accumulate defective proteins at a higher rate than normal cells, which increases their dependency on the proteasome as a disposal mechanism. Inhibition of proteasome function would lead to a progressive accumulation of these proteins and could trigger apoptosis. In addition, NF- κ B activation pathway has been associated with MM and has been linked to drug resistance. Proteasome inhibition might make malignant cells more sensitive to apoptosis by death-inducing ligands (Jeremias, Kupatt et al. 1998).

Bortezomib (Velcade, Millenium Pharmaceuticals, Country) was the first proteasome inhibitor to be widely used in the management of MM. It is a boronic acid dipeptide that reversibly inhibits chymotryptic-like activity of the proteasome and blocks the NF- κ B pathway. Bortezomib treatment also leads to downregulation of transcripts that are associated with growth and survival pathways (e.g. IGF-1 pathway) and upregulation of transcripts involved in both of the main pro-apoptotic pathways (Mitsiades, Mitsiades et al. 2002). In MM cells, bortezomib inhibits DNA repair and induces p53 by phosphorylation

and degradation of MDM2, through activation of caspase-3 by caspase-8. Bortezomib also inhibits MM- bone marrow stromal cell (BMSC) interactions (Mitsiades, Mitsiades et al. 2002), expression of phosphorylated VEGF-induced caveolin-1, ICAM-1, VCAM-1 (Kastritis, Charidimou et al. 2009).

The antimyeloma activity of bortezomib was confirmed in a phase II study (CREST) in relapsed or refractory MM patients (Richardson, Barlogie et al. 2003; Jagannath, Barlogie et al. 2008). Bortezomib was administered with or without dexamethasone and a response was seen in 35% with a complete response (CR) in 7 patients and a near complete response (nCR) in 12 patients. Subsequently a phase III randomized study in relapsed MM (Richardson, Sonneveld et al. 2005) demonstrated a RR of 43% with 9% CRs. These findings led to the approval of bortezomib by the FDA for relapsed or refractory myeloma. Studies of bortezomib in front line setting have demonstrated higher RR (Harousseau, Attal et al. 2010) in the bortezomib combination arm and continued to remain higher after transplant as long as patients have achieved at least very good partial response (VGPR). This and other studies have found that bortezomib remains superior in patients with high-risk disease (elevated β 2-microglobulin, deletion of chromosome 13, t (4;14), and del p53) (Cavo M 2007; Harousseau, Attal et al. 2010). Bortezomib based regimens have also been found to be effective as front line treatments in transplant ineligible patients (Mateos, Hernandez et al. 2006).

As more data emerged about the effects of proteasome inhibition on MM cells several studies of combining bortezomib with other agents were designed. These were initially studied in the preclinical setting based on synergistic rationale and several of these are now being studied in the clinical setting.

Several second generation proteasome inhibitors are currently being studied for their role in treatment of MM. Carfilzomib is an irreversible proteasome inhibitor that binds to β 5 subunit of the 20S proteasome (Kuhn, Chen et al. 2007). Multiple pathways are implicated in the programmed cell death of MM cells when exposed to carfilzomib (Kuhn, Chen et al. 2007). Carfilzomib has been studied in relapsed/refractory multiple myeloma patients demonstrating a 26% RR (S. Jagannath 2009). In another phase II study comparing bortezomib exposed and naïve patients, the naïve patients had a greater RR (57%) (R. Vij 2009).

Other proteasome inhibitors CEP 18770, Marizomib and MLN 9708 are also currently being evaluated in phase I and II studies (www.clinicaltrials.gov ; Chauhan, Catley et al. 2005; Piva, Ruggeri et al. 2008). Marizomib (NPI-0052) is an orally active proteasome inhibitor that acts by a different mechanism to inhibit the proteasome. Marizomib has been shown to be a more potent inhibitor of the NF- κ B and other cytokines (Chauhan, Catley et al. 2005) than Bortezomib. It interferes with the chymotryptic-like, tryptic-like and caspase-like proteolytic activity of the proteasome, while bortezomib only interferes with the chymotryptic-like. It has also been shown to overcome bortezomib resistance both *in vitro* and *in vivo*. Studies are currently evaluating Marizomib as a single agent as well as in combination with bortezomib where a synergistic effect has been seen (Chauhan, Singh et al. 2008).

3.1.2 Immunomodulatory drugs

Immunomodulatory drugs (IMiDs) including thalidomide (Thal) and lenalidomide (Len) are commonly used in the treatment of MM (Rajkumar 2011). Multiple mechanisms have been proposed including effects on angiogenesis, cytokine production, direct antineoplastic

effects, anti-inflammatory effects, sensitization of MM cells to apoptosis and interaction with bone and micro-environment (D'Amato, Lentzsch et al. 2001; Mitsiades, Mitsiades et al. 2002). IMiDs may have direct antineoplastic effects by blocking signaling through NF- κ B signaling, which is universally activated in MM cells and may induce apoptosis via the caspase-8/death receptor pathway (Lacy 2011). Pomalidomide and Len also cause cell cycle arrest in plasma cells by p21 WAF-1 activation, which is p53 independent, suggesting possible efficacy in cancer with p53 mutation and deletion (Escoubet-Lozach, Lin et al. 2009). They also have potent immunomodulatory properties including augmentation of natural killer cell activity and stimulation of cytotoxic T cells (Haslett, Corral et al. 1998; Corral, Haslett et al. 1999).

Thal is a synthetic glutamic acid derivative that was the first agent in this class to be used to treat MM because of its antiangiogenic properties. In relapsed myeloma, Thal and dexamethasone have RR of 40 to 50% (von Lilienfeld-Toal, Hahn-Ast et al. 2008). Len was approved by the FDA based on results from phase III studies that showed a combination of Len and high dose dexamethasone was superior to dexamethasone alone (Dimopoulos, Spencer et al. 2007; Weber, Chen et al. 2007). The RR was 60% in the Len group as compared to 24% in the placebo group. A significant difference was also seen in the time to progression (TTP) (11.3 months vs 4.7 months) and OS (HR 0.66). Comparison of Len with high or low dose dexamethasone showed that the low dose dexamethasone is safer and associated with improved survival in patients with newly diagnosed MM (Rajkumar, Jacobus et al. 2010). A subsequent phase I/II study evaluated the combination of Len, bortezomib and dexamethasone in newly diagnosed MM and reported partial response (PR) was 100% with 74% achieving a VGPR. With a median follow up of 21 months, estimated 18-month progression free survival (PFS) and OS for the combination treatment with/without transplantation were 75% and 97% respectively. A phase III trial evaluating Len /dexamethasone vs bortezomib/ Len /dexamethasone as induction treatment in newly diagnosed MM patients who are not candidates for transplant is currently underway (www.clinicaltrials.gov). A combination of bortezomib, dexamethasone, Len and cyclophosphamide is being studied in a phase I/II trial (EVOLUTION) (Kumar, Flinn et al. 2010). The overall RR in the phase I portion of this study was 96% with a 68% VGPR or better. Len has also shown benefit as maintenance treatment after transplant (Michel Attal 2010; Philip L. McCarthy 2010). In the CALGB trial patients who were randomized to receive Len (10mg daily) had a median TTP of 42.3 months vs. 21.8 in the placebo arm.

Pomalidomide is a new Thal derivative and has been shown to be the most potent IMiD (Lacy 2011). In addition to mechanisms mentioned above, pomalidomide may have a role in preventing or treating myeloma bone disease via effects on osteoclasts (Anderson, Gries et al. 2006). A phase II study using pomalidomide and dexamethasone in a relapsed/refractory multiple myeloma showed a RR of 63% (33% CR or VGPR) (Lacy, Hayman et al. 2009). The median PFS in this study was found to be 11.6 months irrespective of risk factors. This and other studies seem to suggest that pomalidomide is active in patients that are refractory to Len and those who have high risk molecular markers.

3.1.3 Heat shock protein 90 (HSP 90) inhibitors

HSP90 is a molecular chaperone that is induced in response to cellular stress and stabilizes client proteins involved in cell cycle control and proliferative/anti-apoptotic signaling. It facilitates the folding and stability of numerous signaling molecules that control the growth

and survival of cancer cells (Whitesell and Lindquist 2005). HSP 90 is a key molecular chaperone for signal transduction proteins critical to MM cell growth and survival and drug resistance. MM cells produce a large quantity of immunoglobulins that are folded into tertiary structures in the endoplasmic reticulum. HSP90 plays a large role in chaperoning these proteins into formation and disposing of misfolded proteins. HSP90 inhibitors interrupt this chaperoning activity, which leads to accumulation of misfolded proteins, endoplasmic reticulum stress, and ultimately apoptosis (Davenport, Moore et al. 2007; Mitsiades, Hideshima et al. 2009; Chanan-Khan, Borrello et al. 2010).

Tanespimycin (KOS-953) is one of the HSP90 inhibitor and acts mainly through the inhibition of ATPase activity of HSP90. HSP90 inhibition increases the bortezomib induced apoptosis in MM cells by blocking the HSP90 stress response. Preclinical data has shown that tanespimycin may also be protective against the peripheral neuropathy associated with bortezomib. A phase I/II study evaluated bortezomib followed by tanespimycin in relapsed/refractory MM (P. G. Richardson 2009) and reported RR were 41%, 20% and 14% in the bortezomib-naïve, bortezomib-pretreated and bortezomib-refractory patients respectively. Several other HSP90 inhibitors are currently in early phase trials to evaluate their response in myeloma.

3.1.4 HDAC inhibitors

Eukaryotic DNA is packed in a high level structure called chromatin. Expression of genes is controlled by the interaction between the negatively charged phosphate groups on DNA and the positively charged amine groups on the lysine and arginine amino acids on histone terminal tails. The N-e-acetylation of lysine residues found in histones is equilibrated by two enzymes: the histone acetyl transferases (HAT) and the histone deacetylases (HDAC). HDAC inhibitors result in an increase in acetylation of histones which in turn promotes the re-expression of silenced regulatory genes. These compounds represent a family of small molecule-based anti-cancer therapies.

Currently several HDAC inhibitors are being studied as single agents or in combination with other agents mainly bortezomib and Len. Vorinostat is an oral HDAC inhibitor that is currently being used in the treatment of T-cell lymphoma. It downregulates IGF-1 and IL6 signaling pathways as well as DNA synthesis and repair enzymes (Mitsiades, Mitsiades et al. 2004). Vorinostat is also being studied in combination with bortezomib based on preclinical studies which suggest synergistic anti-MM activity.(Mitsiades, Mitsiades et al. 2004). Phase I studies combining bortezomib and vorinostat have shown (Badros, Burger et al. 2009) RR of 42% including 3 PRs among 9 bortezomib refractory patients. Further studies with vorinostat in combination with other agents are currently ongoing.

Panobinostat (LBH589) is a potent pan-deacetylase inhibitor that disrupts aggresome and HSP 90 function via inhibition of HDAC6, promoting cytotoxic misfolded protein aggregates and MM cell death. It is currently being tested in combination with other therapies for relapsed/refractory MM. In a phase Ib trial responses were observed in 68% of patients across all cohorts and in 62% of bortezomib refractory patients. (M. Alsina 2010). Several other phase I and I/II trials have shown similar responses in relapsed/refractory MM in combination with Len and melphalan (M Mateos 2010) prednisone and Thal (Massimo Offidani 2010). Currently phase II trials are ongoing comparing a combination of Panobinostat/Bortezomib/Dexamethasone vs Bortezomib/Dexamethasone in relapsed/refractory MM. Overall panobinostat in combination with other agents has shown encouraging anti-myeloma activity however further studies are needed to establish its role in treatment of MM.

Other HDAC inhibitors currently being evaluated in the treatment of MM are romidepsin, belinostat, ITF2357 and AR 42.

3.1.5 AKT

The Phosphoinositide 3 Kinase (PI3K)/protein kinase B(AKT) pathway is a central signaling pathway in several cellular functions including proliferation, growth, survival and migration. AKT is activated by PI3K and in turn activates several downstream targets. AKT activation has been reported to induce growth and survival advantage to MM cells through GSK-3 β and mTOR phosphorylation. AKT activation has been shown to be associated with advanced stage and poor prognosis in MM patients and also resistance to dexamethasone in MM cells. Targeting the PI3K/AKT pathway is being studied in hematological malignancies (Kawauchi, Ogasawara et al. 2009).

Perifosine (KRX-0401) is a synthetic novel oral alkylphospholipid that inhibits both constitutive and cytokine induced AKT activation and januskinases (JNK) activation leading to apoptosis of MM cells including those adhering to bone marrow stromal cells (BMSC) (Mitsiades, Hideshima et al. 2009). In a phase I/II study adding perifosine to bortezomib and dexamethasone treatment showed an RR of 38% and OS of 16 months in the bortezomib-refractory group and an RR of 55% and a median OS that is not reached in the bortezomib-relapsed group (Paul Richardson 2009). Currently there is a phase III trial recruiting patients with MM pretreated with bortezomib, to be randomized to bortezomib-dexamethasone and perifosine or placebo (NCT01002248).

3.1.6 Mammalian target of rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) is an intracellular kinase that controls the production of proteins through regulation of their translation. mTOR is activated by AKT and regulates cell growth, proliferation, motility, survival and metabolism. mTOR exerts its downstream effects through the formation of protein complexes called mTORC1 and mTORC2. The PI3K/AKT pathway is commonly activated in human cancer and active AKT promotes mTORC1 signaling by phosphorylating and inhibiting the tuberous sclerosis1/2 (TSC1/TSC2) negative regulatory complex(Dowling, Topisirovic et al. 2010). mTOR acts as a neoplastic switch that is frequently turned on by many mutations found in cancer, and its inhibition offers a promising target.

Temsirolimus is an analogue of the rapamycin that acts by binding to FKBP-12, an intracellular protein, and the FKBP-12-temsirolimus complex inhibits mTOR activity in the PI3K-AKT pathway. In addition to renal cell carcinoma temsirolimus is being investigated in MM and other cancers. A phase II study (Farag, Zhang et al. 2009) reported an overall RR of 38% (1 PR, 5 MR). Another phase I/II study reported 33% overall RR combining temsirolimus with bortezomib (Ghobrial, Weller et al. 2011). Other mTORs including everolimus and ridaforolimus are currently being studied in MM (www.clinicaltrials.gov).

Emerging data has shown that rapamycin analogs do not appear to be effective as monotherapies (Dowling, Topisirovic et al. 2010). Rapamycin treatment leads to hyperactivation of AKT through loss of the mTORC1/S6k1/IRS-1/PI3K negative feedback loop. In addition to this mTORC2 is rapamycin insensitive and is known to cause AKT phosphorylation. A majority of the first generation mTOR inhibitors that do not inhibit mTORC2 are thus not as effective as previously thought, but their use in combination with other agents can overcome these resistance mechanisms. This serves as the rationale for

Target	Drug	Study phase	Comments
Farnesyl transferase	Tipifarnib (Zarnestra)	II	FTI inhibitors prevent the farnesylation of Ras. Preclinical models have shown a synergism with bortezomib.
Bcl2	Obatoclox ABT737	I/II Preclinical	Bcl2 prevents cell death by inhibiting adapter molecules involved in the activation of caspases in intrinsic pathway. It is overexpressed in most human tumor types.
Aurora A Aurora B	MLN 8237 ENMD-2076 TAK 901	I/II I I	The aurora kinases regulate cell cycle transit from G2 through to cytokinesis. Aurora kinase inhibitors have been shown to inhibit MM cells.
P38 MAPK	SCIO 469	II	MAPK is a signaling protein that is important in cell proliferation. In combination with bortezomib an ORR of 32% with 9% stable disease in rel/ref MM.
Kinesin spindle protein	ARRY 520	I/II	KSP is required for cell cycle progression through mitosis. KSP inhibition arrests cells in mitosis, resulting in cell death and KSP inhibitors target proliferating cells.
Multiple kinases	Plitidepsin (Aplidin) Curcumin Sorafenib	III II I/II	Plitidepsin is a cyclodepsipeptide which induces MM cell death by activation of p38 and c-jun signaling as well as caspase activation. A phase II study showed 15% ORR.
CDK	Dinaciclib PD 0332991 Flavopiridol SNS-032 Seliciclib	I I/II I/II I I/II	Cyclin dependant kinases (CDKs) are a family of protein kinases that play a vital role in cell cycle regulation. Cyclins D1, D2 and D3 are dysregulated in all MM cells. CDK inhibitors target multiple CDKs as well as other targets including RNA polymerase II or GSK-3 β . CDKIs are being testes as single agents or in combination in MM.
PKC β	Enzastaurin	II	
MEK 1/2	Selumitinib (AZD6244)	II	MEK/ERK pathway activation in MM is critical for cell growth and survival. AZD6244 has been shown to target both MM cells and osteoclasts
Casein Kinase 2	CX-4945	I	
Telomerase	GRN163L	I	GRN163L is an antisense oligonucleotide that binds to, and competitively inhibits telomerase. This leads to telomerase shortening, cessation of MM cell growth, and promotion of apoptosis.
IKK	RTA 402 PS 1145	I Preclinical	
eIF5A	SNS01-T	Preclinical/I	

Table 2. Agents targeting intracellular and nuclear molecules.

combining mTOR inhibitors with AKT inhibitor perifosine which has shown enhanced activity in MM cells (Cirstea, Hideshima et al. 2010). Other combinations of mTOR inhibitors with MAPK inhibitors or RAF/VEGF inhibitors are currently being tested in the clinical setting. Newer mTOR inhibitors that have activity against mTORC2 may represent agents that could be used in combination with other anti-myeloma agents in the future.

Several other intracellular and nuclear targets are being studied in different stages for MM. Some of these are in clinical trials (plitidepsin) while others are still in the preclinical phase (I κ B kinase (IKK) inhibitors or SNS01-T). More agents that are being tested in multiple myeloma are shown in table 2.

3.2 Cell surface receptors, growth factors and growth factor receptors targets

3.2.1 EGFR

The epidermal growth factor (EGFR) is a member of the ErbB family of receptor tyrosine kinases and its role in carcinogenesis has been established. Upon ligand binding the EGFR homo or heterodimerizes, which in turn stimulates the tyrosine kinase activity and initiates cell signaling pathways including mitogen activated protein kinase (MAPK) pathway and the PI3 kinase pathway (Mendelsohn and Baselga 2006). EGFR has been shown to be expressed on malignant plasma cells of MM and its microenvironment. Inhibition of EGFR signaling has shown to induce apoptosis in MM cells (Mahtouk, Jourdan et al. 2004; Mahtouk, Hose et al. 2005). Cetuximab is a chimeric anti-EGFR antibody that inhibits EGFR-ligand interaction and induces cell cycle arrest, apoptosis, and antibody-dependant cellular cytotoxicity (ADCC) (Boll, Eichenauer et al. 2010). A phase II trial is currently undergoing to evaluate dexamethasone with or without cetuximab in relapsed/refractory MM (Boll, Eichenauer et al. 2010).

3.2.2 IL6

IL6 is an inflammatory cytokine that activates Jak/STAT pathway by binding to its receptor (IL6R). It acts as an antiapoptotic factor for MM cells and also confers drug resistance within the bone marrow microenvironment. IL6 also stimulates osteoclastogenesis thereby contributing to the development of osteolytic lesions. Siltuximab (CNTO 328) is a chimeric monoclonal antibody that is derived from the fusion of the murine variable IL6 binding region with human IgG constant domain. Preclinical studies have shown that CNTO 328 enhances the cytotoxic effects of bortezomib by activation of caspases 3, 8 and 9 (Voorhees, Chen et al. 2007). A phase II study combining CNTO 328 and dexamethasone in relapsed/refractory MM has shown 57% RR (3CR, 9PR). Antibodies directed against the IL6 receptor are also currently being studied in MM.

3.2.3 CS1

CS1 is a cell surface glycoprotein belonging to immunoglobulin gene superfamily that is highly expressed on MM cells (Hsi, Steinle et al. 2008). The role of CS1 is not clear but it may promote and supports MM cell adhesion to BMSCs. Anti-CS1 staining was seen in all plasmacytomas and bone marrow biopsies. More importantly CS1 staining is not seen on normal tissues including CD34 cells. Elotuzumab (HuLuc63) is a humanized monoclonal antibody that has shown to induce significant anti-myeloma activity both *in vitro* and *in vivo* (Hsi, Steinle et al. 2008; Tai, Dillon et al. 2008). A phase I/II study evaluating elotuzumab with Len and low dose dexamethasone in relapsed/refractory MM has shown that the

combination is relatively safe and a RR of 82% (64%PR, 18% VGPR) (S. Lonial 2010). Another phase I study combining elotuzumab with bortezomib (A. J. Jakubowiak 2010) demonstrated a RR of 60%.

3.2.4 CD40

CD40 is a transmembrane protein belonging to the tumor necrosis factor - α (TNF α) family and is highly expressed on the surface of MM cell lines and primary MM cells (Westendorf, Ahmann et al. 1995). Binding of CD40 on MM cells with its ligand ,CD40L, upregulates the expression of adhesion molecules (e.g. LFA-1 and VLA-4) which further enhances the adhesion of MM cells to BMSCs as well as IL-6 and vascular endothelial growth factor (VEGF) secretion from BMSCs (Hideshima, Mitsiades et al. 2007). CD40 activation promotes MM cell growth and migration via PI3K/AKT/NF κ B signaling. Anti CD40 monoclonal antibodies (SGN-40, CHIR-12.12) have shown anti-MM activity *in vitro* and *in vivo* (Hayashi, Treon et al. 2003; Tai, Catley et al. 2004). A phase I study of dacetuzumab (SGN-40) reported 20% stable disease (SD). Further trials using dacetuzumab in combination with other agents including Len are currently underway (Edward Agura 2009).

Several other agents directed against cell surface receptors, growth factors and growth factor receptors are listed in table 3.

4. Targeted therapy in lymphoma

Despite remarkable advances in diagnostic techniques and treatment, lymphoma remains leading cause of cancer-related mortality. Since US Food and Drug Administration (FDA) approval in 1997, rituximab has been the mainstream of treatment for non-Hodgkin's Lymphoma (NHL) expressing CD20 antibody on their surface. The surface expression of CD20 on B cell lymphoma, and the fact that most NHL's are B cell has provided development of this and many other monoclonal antibodies (mAB) in treatment of this group of malignancies.

To-date there are about six mAB based treatments for hematologic malignancies that have been approved by Food and Drug Administration (FDA) in the United States. These mAB treatments have improved outcomes and reduced toxicity compared to more conventional chemotherapy regimens. In spite of recent advances in mAB development, current treatments are not optimally effective; with relapse and resistance to chemotherapy or even mAB's seen commonly and the risk of secondary malignancies is an ongoing concern. Due to upcoming more sophisticated and modern molecular techniques new monoclonal antibodies targeting CD3, CD4, CD8, CD20, CD22, CD19, CD40, CD52, CD 74 and HLA Dr β has been developed, but only antibody to CD20 on B cells and CD52 on T cells are broadly used in clinical practice.

4.1 Rituximab

4.1.1 Diffuse large B-Cell lymphoma

Diffuse Large B-Cell Lymphoma (DLBCL) is the most frequent subtype of NHL in all countries around the world and all age groups (Jaffe ES 2001). This aggressive lymphoma is potentially curable, but carries a high risk of relapse. Addition of rituximab (R) to standard

Target	Drug	Study phase	Comments
TRAILr	Mapatumumab	II	A humanized mAb for TNF-related apoptosis-inducing ligand receptor being studied in combination with bortezomib because of its ability to induce apoptosis.
VEGF, VEGFr	Bevacizumab Vandetanib	II II	Angiogenesis plays a crucial role in MM regulated by interactions between the MM cells and the BM microenvironment.
IGF1, IGF1R	AVE 1642	I	IGF signaling pathway is important in tumor growth invasion and metastasis through the Ras/Raf/MEK and PI3K/AKT pathways. Targeting this pathway is difficult due to the cross reactivity with insulin receptors.
Hedgehog	BMS 833923	Ib	BMS 833923 is a small molecule inhibitor of Smoothened (SMO), a component of the hedgehog (Hh) pathway that plays a role in cell differentiation and proliferation. A multiple ascending dose (MAD) study is underway.
CS1	Elotuzumab (HuLuc63)	I/II	
CD 56	BB-10901 IMGN901	I I	CD 56 is a membrane glycoprotein that is expressed on 70-90% of MM cells. Humanized mAb linked to DM1, a cytotoxic maytansinoid are being studied in combination with other drugs.
PD-1	CT 011	II	
Tyrosine kinase inhibitors.	Dasatinib Dovitinib (TKI 258) Sunitinib	II II II	Several targets of TKIs including Src family kinases, PDGFR and cKIT have shown some role in MM pathology and these drugs are being studied as single agents or in combination with other agents. Dovotinib is an inhibitor of the FGF-3 receptor that is involved in 10-20% MM patients with t(4;14).
KIR	IPH 2101	II	
RANKL	Denosumab	I/II	
DKK	BHQ880	I/II	DKK is a soluble Wnt pathway antagonist that is secreted by MM cells and inhibits osteoblastic activity and its serum level correlates with osteolytic lesions.
GM2	BIW 8962	I/II	
CD 38	Daratumumab	I/II	
MUC1	ImMucin	I/II	
CD 74	Milatuzumab	I/II	
BAFF	LY2127399 AMG523	I/II Preclinical	B cell activating factor (BAFF) is derived from stromal cells and osteoclasts and its inhibition reduces tumor burden and osteolytic lesions in preclinical studies.
CXCR 4	BKT 140	I/II	
CD 138	BT 062	I/II	CD 138 is a heparin sulfate proteoglycan that serves as a receptor for EGF ligands and is overexpressed on MM cells.

Table 3. Agents targeting cell surface antigens, growth factors/cytokines and growth factor receptors.

CHOP (cyclophosphamide, hydroxydaunorubicin (doxorubicin), oncovin (vincristine), and prednisone) has transformed outcome of DLBCL, and increased cure rate by about 20%.

A: Previously untreated DLBCL

The first study was reported by Coiffier et al (Coiffier, Lepage et al. 2002) and was recently updated (Coiffier, Thieblemont et al.) in 2010. In this French study, patients with untreated DLBCL were randomized to either CHOP or R-CHOP. Event free survival (EFS) was significantly improved with addition of R. Higher CR or unconfirmed complete response (uCR) was achieved in R arm (76% Vs 63%). Survival also improved by 13% (70% compared to 57%) at 2 years in the R arm. This benefit was independent of international prognostic index (IPI) and age. Recent update of this study confirmed improvement in PFS and OS at 10years in the R combination arm (PFS 36.5%, vs 20%, OS 43.5% vs 27.6%). (Coiffier, Thieblemont et al.)

ECOG 4494 (Habermann, Weller et al. 2006) trial evaluated the role of R in induction and maintenance treatment of untreated DLBCL patients. Two year failure free survival (FFS) rate was significantly higher in maintenance R arm (MR) (76% vs 61%) however there was no significant OS benefit. In addition patients who received R in induction phase did not benefit from MR. In a secondary analysis, patients who received R-CHOP as induction had longer three year FFS (52% vs 39%) as well as OS (67% vs 58%).

Role of R in good-risk younger patients was reported by the Mabthera International Trial group (Pfreundschuh, Trumper et al. 2006). In that trial patients assigned to R-chemotherapy arm had significantly improved three year event free survival (EFS) (79% vs 59%) and OS (93% vs 84%). On long term follow up there was continued benefit in EFS (Pfreundschuh M 2010). Data in younger patients with high risk features is lacking, simply due to fact that this patient population is best served with autologous transplant in first CR (Milpied, Deconinck et al. 2004). S9704 (South West Oncology Group), a phase III randomized trial addressing that question, has completed accrual and results are expected to be presented soon.

B. Relapsed DLBCL

R has improved response rates of salvage regimens like ICE (ifosfamide-carboplatin-etoposide) (Kewalramani, Zelenetz et al. 2004) and DHAP (cisplatin-cytosine arabinoside-dexamethasone) (Witzig, Geyer et al. 2008). A recent trial evaluated R-ICE vs R-DHAP followed by autologous stem-cell transplant (ASCT) and then again randomized to R maintenance or observation (Gisselbrecht, Glass et al.). R-ICE or R-DHAP had similar 3 years EFS and OS. Prior R treatment, early relapse (<12 months) and higher IPI (2 or higher) have been shown to affect 3yrs EFS, PFS and OS. Data from R maintenance part of this trial is still maturing and may provide insight for future treatments.

4.1.2 Follicular lymphoma

Follicular lymphomas (FL) are considered incurable with standard chemotherapeutic options (Cheson et al 2007). Multiple phase III trials have shown improvement in PFS as well as OS in low-grade B cell lymphoma when treated with R.

Initial study by Czuczman et al reported 95% overall RR (55% CR, 40% PR). 74% patients remained in remission at the end of median follow up of 29 months (Czuczman et al 1999). German Low-Grade Lymphoma Study Group (GLSG) conducted a larger study with untreated advanced-stage FL randomized to R-CHOP versus CHOP (Hiddemann et

al 2005). Overall RR was higher in R-CHOP arm compared to CHOP only arm (96% vs 90%, $p=0.011$), though CR rates were not statistically significant (20% vs 17%). Importantly, after median follow up of 18 months, 28 patients relapsed in R-CHOP arm compared to 61 patients in CHOP arm, resulting in significant risk reduction by 60% and longer time to failure ($p<0.001$), as well as longer duration of response ($p=0.001$). This benefit was extended to all subgroups irrespective of IPI status and age. R in combination with interferon and chemotherapy in patients with high tumor burden was reported to have improved disease control with fewer treatments (Salles et al 2008). East German Study Group Hematology and Oncology study reported improved survival with R combination (Herold, Haas et al. 2007).

Based on the above trials R-CVP or R-CHOP has been established as a standard of care for advanced stage follicular lymphoma. R continues to be evaluated in combination with new agents.

GLSG reported significantly higher overall RR, PFS and 2 year survival rates in combining R with chemotherapy in patients with relapsed/refractory FL (Forstpointner, Dreyling et al. 2004). Another phase III trial reported similar results (van Oers et al 2006)- superior overall RR (85.1% vs 72.3%; $P < .001$) and CR rate (29.5% vs 15.6%; $P < .001$).

Recently, PRIMA study reported R as maintenance therapy, after induction treatment, improved CR rates and PFS however there was no difference in OS (Salles, Seymour et al.). Similar findings were reported in MAXIMA trial phase IIIb update at ASH 210 meeting (Fao R et al). These two trials have established a role for maintenance R in improving RR and PFS however there was no improvement in OS.

The Watch and Wait study is a randomized phase III trial which enrolled patients with asymptomatic, non-bulky stage II, III and IV FL (Ardhesna et al)(Kirit M Ardesna 2010). Primary end point of this study was to determine time to initiation of new systemic therapy, and quality of life. 462 Patients were randomly assigned with ratio of 1:1:1 to watchful waiting (arm A), R 375mg/m² weekly for four weeks (arm B) or R 375mg/m² weekly for 4 weeks followed by MR every 2 months for 2 years (arm C). Median follow-up was 34 months at the time of analysis. At three years, 49% of patients in watchful waiting did not receive any treatment, compared to 80% in R single agent (arm B) and 91% in MR (arm C). After three years, 30% of patients in watchful waiting group had not progressed compared to 60% in R single agent (arm B) and 81% in MR (arm C). Further follow-up did not reveal any difference in survival in either of the arms. Dr. Ardesna concluded that R significantly improved both the time to initiation of new therapy and PFS.

4.1.3 Rituximab in other lymphomas

Rituximab has been evaluated in other lymphomas. R was added to hyper-CVAD (hyperfractionated cyclophosphamide/vincristine/doxorubicin/dexamethasone) alternating with high-dose methotrexate/cytarabine regimen for newly diagnosed patients with mantle cell lymphoma (MCL), Burkitt lymphoma and mature B-cell acute lymphoblastic lymphoma (Fayad, Thomas et al. 2007). Overall RR for MCL was 97% (CR/uCR 87%); 5 year FFS and OS were 48% and 65% respectively after median follow-up of 4.8 months. After median follow-up of 22 months overall RR for Burkitt's lymphoma was 97% (CR 86%) with estimated 3-year OS, disease-free survival and EFS of 89%, 88% and 80% respectively (Fayad, Thomas et al. 2007). R also has shown activity in patients with relapsed indolent lymphoma or MCL in conjunction with bendamustine in a phase II

trial (Robinson, Williams et al. 2008). Overall RR was 92% (CR 41%, uCR 14%, and PR 38%). Median duration of response and PFS were 21 months (95% CI, 18 to 24 months) 23 months (95% CI, 20 to 26 months) respectively. Outcomes were similar for patients with indolent or mantle cell histologies (Robinson, Williams et al. 2008). Recently, R with bendamustine has shown significant RR including CR, as well as rapid and durable responses in patients with Waldenstrom macroglobulinemia (Treon, Ioakimidis et al. 2009).

4.2 mTOR inhibitors

Details regarding the pathway are mentioned above in MM part of this chapter. MCL is an aggressive NHL with cyclin D1 over-expression, which remains target for these mTOR inhibitors. The rapamycin analogues, everolimus and temsirolimus, are approved for treatment of renal cell cancer and have shown activity in lymphoma in pre-clinical models (Jundt, Raetzl et al. 2005).

A phase II study evaluated role of everolimus as a single agent in patients with relapsed aggressive lymphoma (DLBCL 61%, MCL 25%, FL-III 10%)(Witzig, Reeder et al.). Overall RR was 30% (20PR/3uCR). Median duration of response was 5.7 months, and 5 patients remained disease free at 12 months. A study is currently recruiting patients to determine whether everolimus plus R is safe and effective in patients with relapsed or refractory DLBCL (NCT00869999).

Temsirolimus was initially studied in relapsed MCL as a single agent (Witzig, Geyer et al. 2005). ORR was 38% (3% CR, 35% PR). The median time-to-progression was 6.5 months; with duration of response of 6.9 months. A recent phase III study randomized 162 patients with relapsed or refractory MCL to 1:1:1 to receive one of two temsirolimus regimens: 175 mg weekly for 3 weeks followed by either 75 mg (175/75-mg) or 25 mg (175/25-mg) weekly, or investigator's choice therapy from prospectively approved options (Hess, Herbrecht et al. 2009). Overall RR was 22%, 6% and 2% in 175/75-mg, 175/25-mg, and investigator's choice groups, respectively. Median PFS was 4.8 (175/75-mg), 3.4 (175/25-mg), and 1.9 months (investigator's choice groups, $p=0.0009$ vs 175/75 mg). Patients treated with temsirolimus 175/75-mg had significantly longer PFS than those treated with investigator's choice therapy ($P = .0009$; hazard ratio = 0.44); those treated with temsirolimus 175/25-mg showed a trend toward longer PFS ($P = .0618$; hazard ratio = 0.65)(Hess, Herbrecht et al. 2009). Overall RR was significantly higher in the 175/75-mg group (22%) compared with the investigator's choice group (2%; $P = .0019$). Median OS for the 175/75-mg, 175/25-mg, and investigator's choice groups was 12.8, 10 and 9.8 months respectively at the time of last update (Hess, Herbrecht et al. 2009).

Deforolimus is a novel mTOR inhibitor which was studied in relapsed/refractory hematologic malignancies (Rizzieri, Feldman et al. 2008) and is undergoing clinical studies (NCT00086125).

Preclinical studies have shown efficacy of mTOR inhibitors in Waldenstrom macroglobulinemia (WM), this was followed by a phase II trial (Ghobrial, Gertz et al.). Overall RR of 70% (42% PR, 28% minimal response, 0 CR) with estimated PFS at 6 and 12 months of 75% and 62% were reported. Everolimus was also studied in relapsed Hodgkin's lymphoma (HL) (Johnston, Inwards et al.). 19 patients with relapsed HL received everolimus with overall RR of 47% (1CR, 8PR) and a median duration of response of 7.1 months (Johnston, Inwards et al.).

4.3 Radioimmunotherapy

The efficacy of R can be augmented by “arming” the antibody with a radionuclide, toxin, or chemotherapeutic agent. In case of lymphomas, radioimmunotherapy (RIT) using CD20-targeted immunoconjugates was developed. Two available RITs are: tositumomab/iodine I-131 tositumomab (Bexxar) and Yttrium-90 -Labeled Ibritumomab Tiuxetan (Zevalin). Ibritumomab, the murine IgG1 anti-CD20 antibody that is the parent of the engineered chimeric antibody R, targets the same epitope on the CD20 antigen.

A large randomized phase III trial compared R to yttrium-90 (90Y) ibritumomab tiuxetan in 143 patients with relapsed or refractory low-grade, follicular, or transformed CD20(+) transformed NHL (Witzig, Gordon et al. 2002). Overall RR was 80% for the ⁹⁰Y ibritumomab tiuxetan group versus 56% for the R group ($P = .002$). CR was higher in the ⁹⁰Y ibritumomab tiuxetan arm compared to R groups (30% vs 16%, $p=0.04$). An additional 4% achieved an unconfirmed CR in each group. Median duration of response (14.2 mo vs 12.1 mo, $p=0.6$) or TTP (11.2 mo vs 10.1 mo, $p=0.30$) were not significantly different.

Another RIT ¹³¹I-Tositumomab was evaluated in treatment-naïve advanced stage FL (Kaminski, Tuck et al. 2005). Overall RR was 95% with CR of 75%. PFS was 59% and median PFS was 6.1 years with median follow-up of 5.1 years. Of 57 patients who had a CR, 40 remained in remission for 4.3 to 7.7 years. Southwest Oncology Group (SWOG) conducted a phase II trial (SWOG 9911) of CHOP chemotherapy followed by ¹³¹I-Tositumomab consolidation for treatment-naïve advanced stage FL (Press, Unger et al. 2003). The overall RR was 90% (CR 67%, PR 23%). 27 of the 47 fully evaluable patients converted to CR following ¹³¹I-Tositumomab consolidation. With a median follow-up of 2.3 years, the 2-year PFS was estimated to be 81%, and 2-year OS was 97%. 5-year follow-up of this trial (Press, Unger et al. 2006) reported PFS was 67% and OS was 87%. The overall RR was 91%, including a 69% CR rate. Compared to historical group of patients in SWOG database who were treated with CHOP, the estimated 5-year OS and PFS were 23% better. Molecular remission was seen in 7 patients (18%) after CHOP and 24 additional patients (63%) after tositumomab/iodine I-131 tositumomab therapy (Press, Unger et al. 2006).

Multiple other trials have compared RIT to fludarabine (Leonard, Coleman et al. 2005), CVP (Link, Martin et al.) or R-CHOP (Zinzani, Rossi et al. ; 2006) in first line treatment with excellent responses with CR between 60%-90%. Though, these treatments are very effective, they are still underutilized.

4.4 Newer-generation anti-CD20 antibodies

One of the newer generations of anti-CD20 antibodies is ofatumumab. This antibody is completely humanized IgG1 that recognized epitopes which are different than the ones recognized by R. Ofatumumab binds to a novel epitope of CD20, which encompass the small extracellular loop (residues 74 to 80) and the N-terminal region of the second large extracellular loop, including amino acid residues 163 and 166A (Teeling, Mackus et al. 2006). Coiffier et al published initial data on ofatumumab in patients with relapsed or refractory Chronic Lymphocytic leukemia (CLL) with RR of 50% and PFS of 106 days (Coiffier, Lepage et al. 2008).

Ofatumumab as a single agent was studied in patients with CLL refractory to fludarabine and alemtuzumab (FA-ref) or refractory to fludarabine with bulky (> 5 cm) lymphadenopathy (BF-ref) (William Wierda 2009). FA-ref patients had overall RR of 58%, median PFS of 5.7 months, and median OS of 13.7 months. BF-ref patients had RR 47%,

median PFS of 5.9 months and median OS of 15.4 months. Subgroup analysis continued to show response irrespective of prior anti-CD20 monoclonal antibody therapy with R, including refractoriness to fludarabine-based regimens containing R (William Wierda 2009). Ofatumumab was also proven effective in patients with treatment-naïve CLL with RR of 72% and CR of 50% (William G Wierda 2009).

Currently in development are : ocrelizumab, a 2H7 murine monoclonal antibody (Kausar, Mustafa et al. 2009); veltuzumab (Immu-106, hA20) also seeks target 2H7 (Morschhauser, Leonard et al. 2009); obinutuzumab (GA101) which recognizes a different epitope (Niederfellner, Lammens et al.)

4.5 Alemtuzumab

Alemtuzumab (Campath®/MabCampath®; Bayer Schering Pharma, Berlin) is a fully humanized IgG1-type monoclonal antibody directed against CD52, a glycosylphosphatidylinositol-anchored cell surface glycoprotein expressed on human B and T cells, natural killer cells, eosinophils and macrophages (Treumann, Lively et al. 1995).

Initial pilot study by Osterborg et al confirmed activity as well as safety of alemtuzumab in patients with CLL (Osterborg, Fassas et al. 1996). A larger phase II study showed RR of 87% with 19% CR in 41 newly diagnosed patients with CLL (Lundin, Kimby et al. 2002). In recent update median TTF was determined at 28 months (range 4 to 102+ months) (Karlsson, Norin et al. 2006).

A large phase III study (CAM307) randomized patients with newly diagnosed CLL to receive either alemtuzumab (30 mg iv tiw for 12 weeks) or chlorambucil (40 mg/m² orally once every 28 days for up to 12 cycles) (Hillmen, Skotnicki et al. 2007). Alemtuzumab arm had significantly improved ORR (83%, Cr 24%) compared to chlorambucil arm (55%, CR 2%) (p<0.0001). Median time to alternate therapy was also significantly prolonged with alemtuzumab (23.3 months vs 14.7 months, HR 0.54, p<0.0001) (Hillmen, Skotnicki et al. 2007). This trial proved efficacy of alemtuzumab over purine analogues in treatment-naïve B cell CLL, and has been approved by FDA as a first line therapy.

T cells have high expression of surface CD52 antigen, highest being in T-prolymphocytic leukemia (T-PLL) (Ginaldi, De Martinis et al. 1998). A phase II open label study treated 27 treatment-naïve Peripheral T-cell Lymphoma (PTCL) patients with CHOP in combination with alemtuzumab (CHOP-C) for 8 cycles (Gallamini, Zaja et al. 2007). CR was achieved in 17 (71%) patients. At a median follow-up of 16 months, 13 patients were disease free, with median duration of response of 11 months (Gallamini, Zaja et al. 2007).

4.6 CD30

Hodgkin's lymphoma (HL) and anaplastic large-cell lymphoma are the two most common tumors expressing CD30. Brentuximab vedotin (SGN-35) is a monoclonal antibody that delivers monomethyl auristatin E to HL cells and utilized an anti-CD30 antibody to induce cell death, improved overall RR in patients with relapsed/refractory HL who has previously undergone ASCT.

Initial phase I trial reported excellent overall RR and tumor regression (Younes, Bartlett et al 2010). This led to larger pivotal phase II single arm study in patients with relapsed or refractory HL post ASCT (Chen R et al)(Robert Chen 2010). Overall RR was 75% (34% CR, 40% PR) with tumor regression in 94% patients. Estimated 12-month OS was 88%. Currently a larger phase 3 study is ongoing to evaluate role of Brentuximab Vedotin (SGN-35) in patients at high risk of residual HL following ASCT (The AETHERA Trial) (NCT01100502).

Side effects:

The “targets” on the cancer drugs are often present in normal tissues to which leads to side effects. Due to limited space, the side effects experienced commonly by the most frequently use targeted agents are presented in the following table.

Target	Drug	Side effect
BCR-ABL	Imatinib	Nausea/vomiting, fluid retention with pleural effusions, ascites, pulmonary edema and weight gain, diarrhea, myelosuppression (neutropenia and thrombocytopenia), skin toxicity
Immunomodulatory drugs	Thalidomide	Teratogenic toxicity, constipation, peripheral neuropathy, skin rash, day time sedation with larger doses, increased risk of thromboembolic events
	Lenalidomide	Teratogenic toxicity, myelosuppression (neutropenia, thrombocytopenia that is usually reversible), increased risk of thromboembolic events, diarrhea or constipation
Proteasome Inhibitor	Bortezomib	Fatigue, malaise, weakness, nausea/vomiting, diarrhea, myelosuppression (neutropenia and thrombocytopenia that is usually reversible), peripheral sensory neuropathy, orthostatic hypotension, fever
Histone deacetylase (HDAC) inhibitor	Vorinostat	Nausea/vomiting, diarrhea, myelosuppression (anemia and thrombocytopenia), fatigue, cardiac toxicity (QTc prolongation), hyperglycemia, increased risk of thromboembolic events
mTOR inhibitor	Temsirolimus	Fatigue, pruritus and pustular, acneiform skin rash, mucositis, hyperlipidemia, hyperglycemia, rarely bowel perforation, interstitial lung disease, renal toxicity, peripheral edema
CD20 receptor	Rituximab	Infusion related toxicities including anaphylaxis, tumor lysis syndrome, skin reaction ranging from pemphigus to toxic epidermal necrolysis, lymphopenia, rhinitis and dyspnea
CD52 receptor	Alemtuzumab	Infusion related reactions, increased incidence of opportunistic infections including <i>Pneumocystis jiroveci</i> , cytomegalovirus, herpes zoster, candida, <i>Cryptococcus</i> , myelosuppression (neutropenia, rarely pancytopenia with marrow hypoplasia)

Table 4. Side effects of targeted treatment

5. Conclusion

Targeted therapies have changed the era of cancer treatment and their development is rapidly making headway. As listed above many are becoming front line therapies in combination with chemotherapy. Imatinib and ATRA have dramatically changed the course

of treatment for patients with CML or APL respectively. In addition, rituximab has improved RR and OS in patients with B-Cell lymphoma. Many other such drugs have been listed above and more drugs are in development. It is very crucial to understand the pathways and "find the right target" in specific diseases. This new knowledge about "targets" will hopefully help us choose personalized treatments for our patients one day, improving their survival with minimal impact on their quality of life.

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New Approaches Targeting Androgen Receptor Signal Pathways for Treatment of Castration-Resistant Prostate Cancer

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1. Introduction

Even orchiectomy/androgen depletion was proposed for treating prostate cancer in the middle of the last century (Attar et al., 2009a; Ross et al., 2008; Vis & Schroder, 2009), androgen deprivation remains to be the mainstay for advanced prostate cancer treatment. However, androgen depletion is usually effective for a limited duration (i.e., a median time of 2-3 years) and majority of the treated prostate cancer patients will develop unresponsiveness to the initial androgen depletion treatment and then evolves to regain the ability to grow despite low levels of androgens in the circulation (Chen et al., 2008; Mostaghel et al., 2009). These so called “castration-resistant prostate cancer (CRPC)” is almost incurable and becomes insignificantly responsive or resistant to most of anti-cancer, cytotoxic drugs except docetaxel and cabazitaxel that seem to show modest but significant improvement of patient survival time (de Bono et al., 2010; Petrylak et al., 2004; Vaishampayan et al., 2009). Evidence strongly suggests that the nuclear androgen receptor (AR) still plays an important role in CRPC and may be, in part, attributed to the resistance to cytotoxic anti-cancer drugs. Intriguingly, these CRPC patients may still respond to second line of AR-related treatment. Thus, it has been urgent to develop novel and more effective AR-related treatments for CRPC.

Acquired resistance has been the main limitation of efficacy of cytotoxic drug chemotherapy and hormonal therapy for CRPC. Recently, new molecules or agents aiming at local angiogenesis, immunotherapy, apoptosis, chaperone proteins, the insulin-like growth factor (IGF) pathway, mammalian target of rapamycin (mTOR), RANK ligand, endothelin receptors, and the Src family kinases in CRPC have been developed for treating CRPC, some of which have been either approved by US Food and Drug Administration (FDA) or entering into different phases of clinical trial (Aggarwal & Ryan, 2011; Leo et al., 2011; Macfarlane & Chi, 2010; Seruga et al., 2011). This chapter will only focus on AR related targeting approaches for CRPC treatments.

2. AR mediated actions in prostatic cells

Wild type of AR is an approximately 110 kDa protein encoded by the gene on Chromosome Xq11-12 containing 8 exons. It is a ligand (e.g., dihydrotestosterone or DHT) dependent transcription factor belonging to the nuclear steroid hormone receptor superfamily (Balk &

Knudsen, 2008; Li & Al-Azzawi, 2009; He & Young, 2009). AR protein has three main functional domains consisting of an N-terminal (NTD) transactivation-1 domain (AF-1; exon 1), a central DNA binding domain (DBD; exons 2 and 3), and a C-terminal ligand binding domain (LDB) with a ligand dependent activation function-2 (AF-2) (exons 4-8) and a short hinge region (exon 4). Normally, androgen binding to LDB in cells will induce an AR conformational change that facilitates its nuclear translocation and specific genomic DNA binding, and therefore, the consequence of the regulation of AR target gene expression.

The functions of AR can vary depending on the cellular context of cell types. In the adult prostate, both stromal and secretory epithelial cells express the same AR protein. It has been shown that castration can induce secretory epithelial cells regress due to apoptosis in rodent and human prostates (Cunha & Chung, 1981; Cunha & Donjacour, 1989; Cunha & Lung, 1978; Kurita, et al., 2001; Niu et al., 2008a; Niu & Xia, 2009; Placencio et al., 2008), but stromal or epithelial basal cell components remain unaffected. After replenishing with exogenous androgens, secretory epithelial cells will re-grow from stem cells in the basal cell layer (Heer et al., 2007; Niu & Xia, 2009; Vander Griend et al., 2008). It has been suggested that the AR in stromal cells, upon androgen stimulation, controls the out growth of the epithelial cells from basal cells by secreting a number of growth factors (Heer et al., 2007; Niu & Xia, 2009; Vander Griend et al.). The basal cells normally do not express detectable AR protein. The prostatic stem cells stimulated by surrounding stromal cells may out grow and differentiate into mature secretory epithelial cells which express AR protein. There are many known prostate-specific differentiation proteins, including prostatic specific antigen (PSA), human kallikrein-2 (hK2), prostatic alkaline phosphatase and others found to be expressed directly or indirectly through regulation by androgens via AR in these secretory epithelial cells (Niu et al., 2008b; Young et al., 2004). Thus, in the normal prostate, the role of AR in the epithelial cells seems to maintain a normal, terminal differentiation state of the cells. The results of recent studies using transgenic mouse models appear to be consistent with the above notion that the AR in prostatic luminal epithelial cells may function, partly, as a tumor suppressor. Furthermore, there are reports (Olshavsky et al., 2008; Balk & Knudsen, 2008) demonstrating that cyclin D3 is an intriguing cell cycle regulatory molecule that interacts with AR in prostatic epithelial cells and represses cell growth stimulatory activities of AR. This event may actually occur *in vivo*, because it was found that cyclin D3 was expressed in a higher level in normal or benign prostate tissues than that in prostate cancer tissues. There are also other cell cycle regulators such as p53 and RB tumor suppressor proteins (Balk & Knudsen, 2008; Chen et al., 2008; Goo et al., 2004; Shenk et al., 2001; Vis & Schroder, 2009) that have been shown to be able to interact with and regulate AR. However, their roles in regulation of AR functions in normal prostatic epithelial cells still require further clarification (see below).

Even both prostatic epithelial and stromal cells express the same AR protein, the functions of AR in stromal cells may eventually be different from that in luminal epithelial cells. In addition, studies have shown that the stromal AR in some conditions may act to promote the formation of prostate cancer (Cunha & Chung, 1981; Cunha & Donjacour, 1989; Cunha & Lung, 1978; Kurita, et al., 2001; Niu et al., 2009a; Niu & Xia, 2009; Placencio et al., 2008). For example, the mouse renal capsule tissue recombination experiments were used to demonstrate that only stromal AR is required for prostate epithelial development, therefore, the presence of epithelial AR is not required for the development of prostatic epithelia. Further, transgenic prostate cancer mouse models with cell-specific AR knock-out were used to demonstrate that the prostate stromal AR plays a more prevalent role than the

epithelial AR for the promotion of prostate cancer and early stages of cancer progression. Importantly, prostatic stroma play an important role in inducing epithelial apoptosis during castration or androgen deprivation through stromal transforming growth factor- β (TGF β) action (Kurita, et al., 2001; Niu & Xia, 2009; Placencio et al., 2008). Early studies showed that an increase of TGF β is associated with androgen ablation in normal/benign and cancer tissues (Lee et al., 1999; Kyprianou & Isaacs, 1999; Hsing et al., 1996; Brodin et al., 1999). In more recent studies, rodent prostate models were used to show that stromal TGF β was upregulated by androgen ablation which then antagonized stromal Wnt expression and reduced the paracrine effect of Wnt on neighboring epithelia (Placencio et al., 2008; Li et al., 2008). TGF β can also inhibit proliferation of malignant prostatic epithelial cells, however, it has been shown that the loss of TGF β receptors I and II was associated with high Gleason grades and with a reduce survival time in prostate cancer patients. Loss or reduction of expression of TGF β receptors I and II in prostate cancer cells has been suggested to be partially attributed to TGF β resistance in prostate cancer (Cardillo et al., 2000; Li et al., 2008). In transgenic TRAMP mouse models, the dominant-negative mutant TGF-beta receptor II expressed in mouse prostate epithelial cell seems to increase prostate tumor initiation and progression (Pu et al., 2009). Interestingly, the dysfunctional TGF-beta receptor II expression significantly enhanced AR expression at mRNA and protein levels. However, another study (Turley et al., 2007) found that the decreased or lost expression of the type III TGF-beta receptor (TbetaRIII) could be largely accounted for TGF β resistance of this cancer and its progression because the loss or reduction of this receptor was found frequently in the cancer tissues. *In vitro* and *in vivo* studies showed that TbetaRIII mediates the decrease of cell migration and invasion as well as of tumor growth in xenografts. The results of the study conclude that the lost or reduced expression of this receptor correlates well with increased clinical stages, metastatic status, and PSA recurrence of the cancer. Further studies showed the reduced expression of the receptor is probably due to the combination of the loss of heterozygosity at the TGFBR3 genomic locus around chromosome 1p32 and epigenetic control of the TbetaRIII promoter.

Increasing angiogenesis in prostate tumor areas is another important function of the AR in prostate stroma in order to support sufficient nutrients for cancerous prostate cells to grow. Reducing the expression and secretion of VEGF with subsequently decreasing micro-blood vessels by androgen deprivation can be attributable to another mechanism for castration-mediated apoptosis (Cheng et al., 2004). Interestingly, it was found that, when injected into an AR expressing stromal environment, the androgen-independent/AR-null prostate cancer cells can benefit from the presence of androgens owing to its effects on angiogenesis (Cano et al., 2007; Halin et al., 2007). On the other hand, castration of the rodent host can induce partial apoptosis of orthotopically injected AR-less prostate cancer cells due to the inhibition of expression of VEGF from the stroma and angiogenesis of tumor (Cano et al., 2007; Hammarsten et al., 2006). Castration plus administration of an inhibitor of vascular endothelial growth factor receptor 2 and epidermal growth factor receptor signaling, N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine (ZD6474), enhanced apoptosis of the prostate cancer tumors, suggesting a potential new way to treat CRPC (Cano et al., 2007; Hammarsten et al., 2006; Johansson et al., 2007). Moreover, studies also showed that, by unknown mechanisms, the AR could re-appear in stroma and epithelial areas of human prostate cancer tissues after castration therapy, therefore, this means that stromal AR could be important to the progression of CRPC and be a highly feasible target for CRPC treatment (Henshall et al., 2001; Ricciardelli et al., 2005; Wikström et al., 2007).

3. AR in malignant prostatic cells

AR in malignant prostate cells shows cell proliferative stimulation and cell survival functions that are apparently different from that of the AR in normal epithelial cells as described above. In primary prostate tumor AR responds to castration or androgen deprivation and cause tumor regression by apoptotic cell death. AR expression may be reduced during the hormonal treatments. In fact during the malignant progression, both stromal and epithelial cells in tumor areas become resistant to hormonal deprivation (Henshall et al., 2001; Wikström et al., 2007). However, AR may re-appear and has been shown to be still expressed in the majority of CRPC as well as in a number of androgen-independent prostate cancer cell lines. The AR in the prostate tumor cells draws a great deal of attention from clinicians and researchers, because it mediates not only cell growth and survival but also cell death in response to new hormonal treatments for CRPC cells (Attar et al., 2009a; Chen et al., 2008; Mostaghel et al., 2009; Ross et al., 2008; Vis & Schroder, 2009). Survival and proliferation of CRPC cells have been suggested as mediated by gain-of-function changes in the AR and AR reactivation (Attar et al., 2009a; Chen et al., 2008; Mostaghel et al., 2009; Ross et al., 2008; Vis & Schroder, 2009). Importantly CRPC seems to be able to respond to second line hormonal therapy. Experimentally knocking-down of AR expression showing the induction of cell death in CRPC cells in cell culture and xenograft systems provides strong evidence that AR target therapy for CRPC is highly feasible.

There are several mechanisms that have been widely suggested that AR still plays an important functioning role in promoting CRPC and can be used as therapeutic target for treating the cancer (Attar et al., 2009a; Chen et al., 2008; Mostaghel et al., 2009; Ross et al., 2008; Vis & Schroder, 2009). These include (1) AR mutations and splicing variations; (2) AR amplification and/or overexpression; (3) de novo androgen production; (4) overexpression of AR co-regulators; and (5) AR activation by non-steroidal growth factors, cytokines, or aberrant AR phosphorylation [please see Attar et al., 2009a; Chen et al., 2008; Mostaghel et al., 2009; Ross et al., 2008; Vis & Schroder, 2009; Steinkamp et al., 2009 for review]. Although we will not further discuss all of these mechanisms, some of them with the recent new findings will be selected for discussion in this article in order to further enlighten the point of views of these mechanisms as well as new derivative concepts of the mechanisms, by which more efficient treatments for CRPC may be developed.

4. AR and cell cycle in androgen dependent and CRPC cells

As mentioned, one major role of AR in prostate cancer cells is to promote cell proliferation, and the AR seems to be involved in the cell cycle process (Balk & Knudsen, 2008). It has been suggested that androgen activated AR may direct G1-S transition in androgen dependent prostate cancer cells by increasing motor mediated translational activities that result in cyclin D1 accumulation and CDK4/cyclinD1 assembly. In this process, S-phase progression is further enhanced by RB phosphorylation and de-repression of cyclin A expression. It has been demonstrated that AR may acts as a DNA licensing factor in androgen responsive prostate cancer cells (D'Antonio et al., 2009). "DNA licensing" process occurs in a critical period of G1 phase by a temporally coordinated binding of a number of licensing factors including origin recognition complex (Orc1-6), cell division cycle 6 homolog (Cdc6), chromatin licensing and DNA replication factor 1 (Cdt1), and mini chromosome maintenance proteins (Mcm2-7) at origins of DNA replication sites to form a pre-replication complex (pre-RC). Immuno-precipitation analysis showed that nuclear AR

was associated with the licensing proteins Orc2, Cdc-6, Cdt-1 and Mcm2. AR protein levels may be fluctuated along with cell cycle phases because the AR protein is degraded by proteasome in mitotic phase and increases in G1 phase in order to ensure next cell cycle re-entry and re-licensing. This study (D'Antonio et al., 2009) points out the critical role of AR in G1 through S phase for promoting cell growth of androgen dependent prostate cancer cells that is different from its functions in normal prostatic cells.

Nonetheless, a delicate study (Wang et al., 2009) reported recently the identification of a number of androgen regulated genes using a number of sophisticated assays including a global gene expression profiling and other analyses on an androgen-independent LNCaP sub-line to address the AR mediated proliferative function in CRPC cells. The authors suggested that, instead of promoting G1/S transition by androgen activated AR in androgen responsive prostate cancer (ARPC) cells, ligand-free AR may play a role to drive M-phase transition in androgen-independent prostate cancer (AIPC) cells. This study discovered that many of these AR up-regulated genes are M-phase related genes. Clinical tissue samples from AIPC and ARPC patients were also used for gene profiling analyses which strongly support the conclusion obtained from the above *in vitro* AIPC cell model. To further prove the differences of AR bound genes between AIPC and ARPC cells, chromatin immunoprecipitation (ChIP) plus genome-wide tiled oligonucleotide microarrays (ChIP-on-chip) was performed. The authors found that there was more AR occupancy in AR upregulated cell cycle and M-phase genes in AIPC cells than that in ARPC cells. The M-phase related regulatory genes including CDC20, UBE2c CDK1 and ANAPC10 were indeed AR binding genes in androgen-independent (AI) LNCaP cells when a direct ChIP assays was performed. These M-phase genes were shown to be up-regulated by AR in the absence of androgens and anti-sense knock down of these genes can affect cell proliferation activity. This study strongly suggested that AR without exogenous androgens can regulate M phase gene expression for cell proliferation in AIPC/CRPC cells. M-phase regulatory genes become AR's new targets, whose elevation of expression seems to be assisted with alteration of epigenetic marks in AIPC/CRPC cells. This also raises the possibilities that in addition to G1/S transition, M phase regulated by AR is also important in AIPC cells and the observed changes in epigenetic marks could increase the previously inaccessible AR binding sites in ARPC cells to become accessible by AR in AIPC/CRPC cells.

Another possible explanation of cell cycle deregulation by AR in AIPC/CRPC cells could be because of alterations of the RB pathway. Although the RB tumor suppressor alterations are not the major events in primary prostate cancer, the alterations were observed frequently in prostate cancer tissues after androgen blockade treatments (Mack et al., 1989). A study also showed that RB-deficient prostate cancer cells can increase susceptibility to certain anticancer cytotoxic agents by inducing cell death. The authors suggested that RB depletion can be developed to enhance conventional cytotoxic therapeutic intervention for a subset of prostate cancer (Sharma et al., 2007). Recently, examination of 44 CRPC tissues with both RB mRNA expression levels and gene expression signature profiling of RB function loss indicated the frequent alterations of RB coincidence with CRPC development (Sharma et al., 2010). Further analyzing 156 CRPC tissue samples from the 44 patients showed 115 of 156 (73.7%) of the specimens with negative for nuclear RB immunodetection. The authors concluded that loss of the *RB1* locus itself may be a major mechanism of RB inactivation in CRPC which was infrequently observed in primary disease (Sharma et al., 2010). Experimental evidence then demonstrated that loss of RB may enhance AR expression and functions even in the absence of androgens or the presence of anti-androgens via enhanced

E2F transcription factor 1 activity. Interestingly, RB loss/depletion can also enhance AR regulated M-phase genes like CDK1 described above, indicating the AR activities may be broadened in CRPC cells (Sharma et al., 2010; Macleod, 2010). Furthermore, the study demonstrated that RB loss/depletion enhanced expression and activities of E2F-1. The authors (Sharma et al., 2010) concluded that the RB/E2F/AR network may provide potential new avenues for developing effective therapeutic intervention for CRPC as suggested elsewhere (Knudsen & Wang, 2010).

Contrast to the above report (Sharma et al., 2010), an earlier study (Davis et al., 2006) indicated that by examining the expressions of E2F1 and AR proteins in 667 prostate tissue microarray cores E2F1 protein levels are low in benign and localized prostate cancer, moderate high in hormone native metastatic lymph nodes and significantly increased in CRPC metastatic tissues. However, the AR protein levels are strong in 83% benign prostate, 100% localized prostate cancer and 80% lymph node metastasis tissues and decreased to 40% in metastatic CRPC tissues. Additionally, forced expression of E2F1 showed inhibition of expression of AR mRNA and protein. The authors also used chromatin immunoprecipitation assays to demonstrate that the AR promoter is the target for E2F1 and the pocket protein family members p107 and p130 to bind and repress the expression of the AR gene. The reasons for the different conclusions of the two studies (i.e., Davis et al., 2006; Sharma et al., 2010) are not very clear. However, this study (Davis et al., 2006) did not examine RB status in the tissues studied.

5. AR variants or splicing isoforms in CRPC

Through previous structural and functional analyses, AR NTD that comprises nearly 60% of the coding region has been shown to exhibit constitutively active, transactivation activity (Dehm & Tindall, 2006; Dehm & Tindall, 2007). AF-1 embedded within NTD functions as a ligand-independent transcriptional activator, and there are two transactivation units TAU-1 and TAU-5 located within the AF-1. The AF-1a (a. a.101-211) and AF-1b (a. a. 253-361) domains in the TAU-1 (Dehm & Tindall, 2006) and WHITLF (a. a. 435-439) motif in the TAU-5 are the main sub-regions in mediating the ligand-independent NTD transcriptional activity (Dehm et al., 2007). AR protein becomes active without androgens in response to stimulation of several molecules such as interleukin 1 beta (IL-1 β), IL-6, bone-derived factors (Blaszczyk et al 2004; Ueda et al., 2002a; Wang & Sadar, 2006) and others via crosstalking mechanism in activating AR NTD. For example, TAB2, a sensor for inflammatory signals, in response to IL-1beta treatment was shown to interact with the AR NTD at residues 179–188 and then recruit MEKK1, which in turn mediates dismissal of the N-CoR/HDAC co-repressor complex from AR and allows derepression of AR target genes. Additionally, IL-1beta induces a switch whereby anti-androgens were able to activate the AR through AR NTD (Ping et al., 2006). Steroid receptor coactivator-1 was also shown to respond to IL-6 stimulation and bind the AR NTD in order to induce ligand-independent activation of the AR (Ueda et al 2002b). Interestingly, calpain, a calcium-dependent proteinase and highly expressed in prostate cancer cells (Libertini et al., 2007), was shown to be able to cleave the native AR into an androgen independent isoform. *In vitro* and *in vivo* analyses demonstrated that calpain removes the COOH-terminal ligand binding domain from an intact AR molecule generating a constitutively active molecule which would be highly potentially attributable to androgen independent growth of prostate cancer cells mediated by the AR isoform. However the study did not show how often this truncated AR occurs in CRPC

Recently three splicing variants of AR have been discovered in a castration resistant human CWR22Rv1 (22Rv1) prostate cancer cell line (Dehm et al 2008). One of the variants produced a full-length AR with a duplicated exon 3 (AR 1/2/3/CE3) and other two resulted in two shorter isoforms (AR 1/2/2b and AR 1/2/3/2b) devoid of ligand binding domain but with a novel exon 2b. All three isoforms are constitutively active transcription factors and able to bind an androgen responsive element as well as to promote cell growth of 22Rv1 cells in an androgen-independent manner. One of these variants may be detectable in other commonly studied cell lines. More recent study (Li et al., 2011) from the same group of the investigators indicated these aberrant variant expressions were derived from an intragenic rearrangement of an approximately 35-kb AR genomic segment containing a cluster of the above mentioned alternative AR exons. Genomic analysis of tissues from 14 CRPC patients revealed similar AR intragenic rearrangement in conjunction with AR amplification. Another study (Guo et al., 2009) reported the identification of over 20 splicing variants, many of which were derived from intronic cryptic exon splicing, in prostate cancer cells. AR3 is a major variant and encodes an about 80 kDa protein containing an intact NTD and a DBD domain but lacking a hinge region and a LBD. Some of these variants including the AR3 can be found in 22Rv1 prostate cancer cells. However, AR3 is different from the variants described in the above studies. Moreover, an AR3 specific antibody was generated, which was able to detect AR3 protein in benign prostatic stromal and basal epithelial cells but little expression in normal luminal epithelia. On the other hand, AR3 protein was expressed at higher levels in malignant prostatic epithelia and suggested to be a significant predictor for prostate cancer recurrence after prostatectomy. The study also indicated that androgen depletion forced the overexpression of AR3. Interestingly, AR3 protein was demonstrated largely in cytoplasm, however, experimental evidence showed that AR3 could act as a transcription factor via an ARE of AR regulated genes and promote cell proliferation in the absence of androgens. In addition to the variants identified in the above studies, a group of investigators (Marcias et al., 2010) recently also identified several novel constitutively active AR variants in this same CWR22R cell line, which were generated by aberrant pre-mRNA splicing or nonsense mutations. It will be interesting to determine if these new variants are frequently expressed in clinical specimens.

Another new AR variant, AR^{v567es}, initially identified from a series of human prostate cancer xenografts was also reported (Sun et al., 2010). This variant is produced by alternative mRNA splicing and contains an exact full amino acid sequence from exons 1-4 and a 10 amino acid sequence from exon 8 by skipping exons 5-7. By examining 13 CRPC patients, 10 of the patients had at least one metastasis showing positive for AR^{v567es}. The study demonstrated that AR^{v567es} protein is constitutively active and capable of forming CRPC in a xenograft model. Interestingly, this variant can bind wild type AR and therefore stabilize and increase the activities of wild type AR with or without androgens. Moreover, in a different report (Watson et al., 2010) a number of AR variants including the previously reported ones like AR3 and new ones were examined for their functions and regulation. It was found the expression of these variants was repressed by androgens and de-repressed by androgen deprivation. Some of these variants including AR3 and AR^{v567es} as described above confer CRPC growth activity but other variants such as AR-V1 may show dominant interfering activity. Unexpected finding in this study was that the CRPC growth activity of the constitutively active AR variants requires the presence of the wild type AR, because an antiandrogen (i.e., MDV 3100; also see below) or specific siRNA for only silencing wild type

AR can abolish CRPC growth activity of the AR variants tested. The authors of this study suggested that the roles of AR variants in CRPC appear to be highly complex and the requirement of wild type AR for the function of AR variants indicates the need of developing better antiandrogens for treating CRPC.

6. Other mechanisms for overexpression of AR in CRPC

As already mentioned above, there are several mechanisms that have been reported to manifest aberrant AR functions during CRPC progression. It has been considered that AR over-expression and re-expression are the major mechanisms for the progression of CRPC after hormonal therapy (Edwards et al., 2003; Holzbeierlein et al., 2004; Linja et al., 2001). Although AR gene amplification is recognized as an important way for overexpression of AR, the amplification only occurs in 20 -30% of CRPC tumors. Therefore there are other mechanisms, in addition to those being already described above, as discussed below that could be contributed to the AR overexpression in CRPC.

Immunohistostaining assays previously showed heterogeneity in the expression of AR protein increases with progression of high grade prostate cancer (Magi-Galluzzi et al., 1997; Pertschuk et al., 1995). Examining the methylation status of the 5' CpG of the AR gene promoter showed that that approximately 20-30% of CRPC tumors losing or reducing AR expression, partly, by DNA hypermethylation in the AR promoter (Jarrard et al., 1998; Kinoshita et al., 2000; Nakayama et al., 2000; Sasaki et al., 2002; Takahashi et al., 2002). DNA methylation and histone modification are two major epigenetic mechanisms that can affect gene expression in normal and malignant cells. The above studies indicated that DNA methylation could be one possibility for causing heterogeneity of AR expression CRPC. However, it is not clear whether aberrant DNA hypomethylation in the AR gene can be a mechanism for overexpression and re-expression of AR. This would be a research area of interest to pursue. Moreover, aberrant DNA methylation in other genes may affect AR function. For example, it has been demonstrated that melanoma antigen gene protein-A11 (MAGE-11) as a transcriptional regulator can enhance AR transcriptional activity by interacting through FXXLF related motifs in both NH(2)-terminal domains of AR and p300 (Askew et al., 2010; Karpf et al., 2009). This study showed that an increased DNA hypomethylation in a CpG island of the MAGE-11 5' promoter in CRPC tissues and cell lines may cause increased expression of MAGE-11 mRNA and protein which, in turn, enhances AR signal activities in CRPC.

AR mutation may not be frequent events in CRPC, however, it becomes apparent that hormone therapy such as the use of anti-androgens could be a potential selection force for AR mutations that can affect its stability, promoter preference, or ligand specificity (Attar et al., 2009; Chen et al., 2008; Mostaghel et al., 2009; Ross et al., 2008; Vis & Schroder, 2009; Steinkamp et al., 2009). Increased stability of mutated receptor may be a factor for the apparent AR overexpression.

The signal transducer and activator of transcription 5 (Stat5) has been shown to be involved in prostate cancer progression including development of CRPC (Dagvadorj et al., 2008; Tan et al., 2008; Thomas et al., 2011). Stat5 protein is capable of interacting with and increasing AR activity without the presence of androgens (Tan et al., 2008). It has been found that Stat5 expression levels correlated with high grades of prostate cancer and its overexpression

could be used to predict early prostate cancer recurrence in patients treated with radical prostatectomy (Li et al., 2004, Li et al., 2005). Moreover, Stat5 immunostaining on tissue microarray containing 143 primary prostate cancer and 20 CRPC presented significant overexpression of Stat5 in short-term and long-term androgen deprivation tissues as well as CRPC tissues compared with untreated primary prostate cancer tissues (Thomas et al., 2011). A Stat5 antisense oligonucleotide (ASO) was used for specific Stat5 knockdown and showed diminished nuclear translocation of AR and decreased AR protein stability by triggering its degradation via the proteasome pathway. The result of an *in vivo* study using Stat5 antisense oligonucleotide via ip injection showed that the Stat5 knockdown treatment significantly delayed CRPC tumor progression in a xenograft model. The study did not address whether Stat5 indeed increases AR expression in CRPC cells. However, it is conceivable that AR expression as well as functions is enhanced through its interaction with Stat5, thus, a potential mechanism for AR overexpression. Stat5 should be an effective therapeutic target by a number of means including a specific prolactin receptor antagonist G129R-Prl, small-molecule inhibitors for Jak2, and oligonucleotides or RNA interference, etc., as suggested to remove or reduce AR from CRPC cells (Liao et al., 2010).

It has been shown that NF- κ B proteins as transcription factors play critical roles in the development and progression of several types of human cancer (Baud & Karin, 2009; Shen & Tergaonkar, 2009). NF- κ B can be constitutively activated with a consequence of increasing cell proliferation and survival, reducing programmed cell death and enhancing angiogenesis and metastasis in many tumors. Because NF- κ B/p65 expression has been found to be higher in prostatic intraepithelial neoplasia and cancer tissues when compared with benign tissues, its expression levels were suggested as a predictor of PSA biochemical recurrence in certain prostate cancer patients (Chi et al., 2009; Jin et al., 2008; Ko et al., 2008; Ross et al., 2004; Sweeney et al., 2004; Zhang et al., 2009). In addition, evidence showed that activation of NF- κ B may be highly associated with the development of CRPC. NF- κ B activation can respond to several proinflammatory cytokines such as interleukin 6 and tumor necrosis factor alpha (TNF α). Intriguingly, androgen responsive and androgen independent PCa cells may respond to TNF α treatment differently. TNF α induces apoptosis in androgen responsive LNCaP cell line by activating NF- κ B with subsequent down-regulation of AR mRNA and protein. It was shown that the 5'-untranslated region of the AR gene contains a composite response element for NF-kappaB and B-myb transcription factors (Ko et al., 2008). With TNF α treatment, NF-kappaB and B-myb were enriched in this composite site with a number of co-repressors including the histone deacetylase 1, corepressor silencing mediator of retinoid and thyroid hormone receptor and the corepressor-associated scaffold protein mSin3A. On the other hand, the above complex formation did not appear at the composite site of the AR gene in two androgen independent LNCaP subline lines, C4-2 and C4-2B, which are resistant to TNF α treatment (Zhang et al., 2009). Indeed, the activated NF- κ B binds its responsive element in the 5' promoter of the AR gene and enhances AR expression in especially CRPC cells. In fact, there were eight putative NF- κ B binding sites within the -3.6 kb 5' promoter region of the AR gene detected by CHIP assays (Jin et al., 2008). This seems to suggest there may be a sub set of CRPC progressed during androgen deprivation therapy that is associated with overexpression and constitutive activation of NF- κ B as well as AR overexpression. NF- κ B would be an important therapeutic target for reducing AR in CRPC cells.

7. Overexpressed AR and AR variants in CRPC as therapeutic targets

The failure of hormonal therapy for prostate cancer has been largely attributed to overexpression of AR in recurrence of the cancer (Chi et al., 2009; Joseph et al., 2009; Norris et al., 2009; Singh et al., 2008; Tran et al., 2009; Zhou et al., 2008). Development of more effective hormonal therapeutic agents targeting overexpressed AR becomes an important research area. Many AR antagonists previously developed are mainly ligand competitors for androgens and can not overcome the over-expressed AR in CRPC.

Newer approaches have been used to develop agents that can target potential sites in the AR to abrogate the function of the mutated or over-expressed receptor in cancer cells (Chi et al., 2009; Joseph et al., 2009; Norris et al., 2009; Singh et al., 2008; Tran et al., 2009; Zhou et al., 2008; Shen & Balk, 2009). For example, it was reported that a conformation-based assay was used to screen a diverse small molecule library of $\approx 10,000$ compounds in order to select chemicals that can inhibit the AR-gelsolin interaction (Joseph et al., 2009). Among the 87 compounds that scored positive in the primary mammalian 2-hybrid-based assay with no demonstrable cell toxicity, two chemicals, D36 and D80, were selected based on their ability to inhibit agonist-mediated transcription of an MMTV-luciferase reporter in LNCaP cells. These two AR binding compounds were shown to be different from currently used AR antagonists such as casodex and hydroxyflutamide, because they do not compete with androgens for binding and do not affect AR nuclear localization. Experimental evidence showed they affect recruitments of co-activators and RNA pol II enzyme to AR and AR DNA binding ability, and subsequently loss of its ligand-induced transcription activity as well as inhibition of cell proliferation in AR overexpressing LNCaP cells.

In a study shAR lentiviral vector directed AR knockout CWR22R cells which ectopically re-express a truncated AR lacking of LBD (i.e., AR Δ LBD) were used to screen the DiverSet Chemical Library consisting of 34,000 small molecules (Gioeli, 2010; Narizhneva et al., 2009). The AR Δ LBD is a constitutively active AR variant capable of supporting androgen independent proliferation of CWR22R cells. The genome of the cells also contained integrated ARE linked luciferase reporter. In this cell based assay, compounds showing inhibited luciferase activity at least two-fold after 24 hr incubation were selected for further studies. Focusing on AR mediated cell proliferation, there were two types of compounds selected, one type caused cell death, and other reduced cell growth in cell cultures. Although the actual mechanisms by which these compounds act on AR, obviously not via AR LBD, are not known, both types of compounds can diminish *in vivo* prostate tumor growth in a xenograft model. There is no doubt that clinically relevant studies of these compounds will decide their potential clinical utility.

Also, there are two new classes of small molecules isolated from sponge (*Dysidea* sp.) extract that exhibit anti-AR activity (Andersen et al., 2010; Quayle et al., 2007; Sadar, 2011; Sadar et al., 2008). One type of these sponge compounds are small chlorinated peptide called sintokamides, and the other is EPI-001 (i.e., bisphenol A diglycidic ether.HCl.H₂O). The study showed that isolated sintokamides were able to inhibit androgen dependent AR activation in cells transfected with a native androgen response element (ARE) linked luciferase reporter plasmid. Further studies were performed in LNCaP cells transfected with plasmids for the AR NTD-Gal4DBD chimera protein and the Gal4-luciferase reporter. This system allows transactivation of AR NTD by forskolin without the presence of androgens showed that sintokamides indeed interfere with transactivation of AR NTD. Although both sintokamides and EPI-001 target AR NTD activities, the latter has been studied more

extensively using cell based assays to measure anti AR/AR NTD activities. The studies provided evidence that EPI-001 may directly bind the AR NTD not the AR LBD in test tubes. Moreover, in cells EPI-001 seems to be able to interact with the NTD of AR and inhibit both the interaction of CREB binding protein (CBP) with AR AF1 and the interaction of the AR NTD and AR C-terminal domain. EPI-001 does not inhibit AR nucleus translocation and DNA binding and does not reduce AR protein levels in cells or xenograft tumors. This compound was shown to inhibit *in vitro* and/or *in vivo* prostate cancer cells growth mediated by either wild type AR or constitutively active AR variants mentioned above but not inhibit *in vitro* and/or *in vivo* proliferation of AR null cells like PC-3.

The problem with currently clinically used AR antagonists such as bicalutamide (also known as casodex) is their low affinity to AR that can not effectively suppress AR in CRPC, especially at overexpressed states. This also seems to explain, in part, why the antagonists can become a weak agonist for CRPC that overexpresses AR. Another new approach was used to improve antagonist affinity to AR in order to effectively repress AR function at high levels (Tran et al., 2009). The investigators used a relatively high affinity and specific nonsteroidal AR agonist RU59063 as a starting compound for developing potential high affinity antagonists (Tran et al., 2009). By screening about 200 thiohydantoin derivatives of RU59063 for AR antagonism in human prostate cancer cells expressing elevated AR, two diarylthiohydantoins RD162 and MDV3100 were chosen for further biological and clinical studies. It was shown that in addition to their higher affinities than bicalutamide, these two compounds can reduce AR nuclear translocation, DNA binding to androgen response elements, and recruitment of coactivators. Oral treatment of RD162 and MDV3100 demonstrated tumor regression in mouse xenografts of human CRPC. Furthermore, MDV3100 was used in a Phase I/II clinical trial in 30 CRPC patients. Thirteen of 30 (43%) showed sustained declines (by >50%) in serum biomarker prostate-specific antigen concentrations. The second phase 1-2 study (Scher et al., 2010) was conducted with 140 CRPC patients in 5 US centers. The goals were to determine the safety and tolerability profile of MDV3100 and to establish the maximum tolerated dose. The study concluded that the drug had a well tolerable toxicity and was able to reduce prostate-specific antigen levels and circulating tumor cell counts as well as to stabilize prostate cancer metastases. It seems that this compound is underway for further clinical trials for advanced prostate cancer (Schmidt, 2011).

Other new AR LBD antagonists have also been described. For instance, similar to the above studies, a new series of thiohydantoin derivatives were generated for screening AR antagonistic activities and led to the identification of a metabolic stable compound, CH5137291 (Kawata et al., 2011; Yoshino et al., 2010). This compound also showed to repress AR nuclear translocation and AR mediated tumor growth in xenografts. It would warrant further clinical studies on human patients. BMS-641988 was, another example, identified as a promising AR antagonist, with more than ten times AR binding affinity compared to bicalutamide, in the initial preclinical studies (Attar et al., 2009b). However, phase 1 clinical trial with 61 CRPC patients showed a limited anti-cancer activity with occurrence of seizure activity in patients, eventually leading to closure of the study (Rathkopf et al., 2011).

8. Androgen synthesizing enzymes in CRPC as therapeutic targets

Hormonal therapies can effectively reduce circulating androgens by surgical or chemical castration plus adrenal steroidogenic enzyme inhibitors (e.g., ketoconazole, liarozole, and

aminoglutethamide), however, AR can still be functionally active inside CRPC tissues. In fact one of the main reasons is that androgens can be *de novo* synthesized in tumor cells of CRPC patient tissues and cell lines (Attard et al., 2009a; Cai et al., 2009; Dillard et al., 2008; Locke et al., 2009a; Locke et al., 2009b; Locke et al., 2008; Locke et al., 2010; Mohler, 2008; Mohler et al., 2011; Montgomery et al., 2008; Stanbrough et al., 2006). This relatively wide spread phenomenon is due to up-regulation of expression of certain steroidogenic/androgen synthesizing enzymes in prostate cancer cells, including FASN, CYP17A1, HSD3B1, HSD17B3, HSD17B6, CYP19A1, and UBT2B17. It is highly critical that the up-regulated steroidogenic enzymes can produce sufficient intracellular androgens to act as intracrine or paracrine ligands for AR in order to facilitate progression of CRPC. Because of the inefficiency of suppression of adrenal androgen production by using ketoconazole and aminoglutethamide, circulating adrenal androgens, dehydroepiandrosterone (DHEA) and 5'-androstenediol (ADE) could be a source for making high affinity androgens testosterone (T) and DHT in prostate cancer tissues by HSD3B, HSD17B6, AKR1C and SRD5A enzymes. Intratumor androgens can also be generated by the *de novo* or intracrine synthesis from cholesterol or other steroid precursor biosynthetic pathways in especially CRPC cells. It was found that in a xenograft CRPC progression model, the expression of proteins for cholesterol regulation including LDL-r, SR-B1, HMG-CoA reductase, ACAT1,2, and ABCA1) were deregulated, and influx and synthesis of cholesterol were increased as sources for intratumor DHT synthesis (Leon et al; 2010). A net increase of intra-tumor androgens can be achieved by either increasing reductive enzymes such as HSD17B3 and HSD17B5 or decreasing those enzymes that catalyze the reverse oxidative reaction (e.g., HSD17B2) or both. Also, pregnenolone and progesterone may be used as the initial substrates catalyzed by CYP17A in the so called "backdoor" pathway to produce DHEA and ADE, and DHT can be formed from the above products by HSD17B and SRD5A enzymes. When examining 19 local CRPC tissues, a recent study concluded that intratumoral steroid biosynthesis mentioned above may be less important than the production of intraprostatic T and DHT from adrenal androstenedione by overexpressed AKR1C3 and SRD5A1 in prostate cancer tissues for CRPC progression (Hofland et al., 2010).

How the steroidogenic related-enzymes become deregulated remains unclear presently. It is possible that as discussed above, CRPC cells could adapt to have different epigenetic responses from that of androgen dependent prostate cancer cells. For example one could speculate that because chromatin structures of the steroidogenic related-enzyme genes become more accessible for their expression in CRPC cell, however, there is no experimental evidence to support it yet. Nonetheless, a recent study showed that a proinflammatory cytokine, IL-6, increased expression of several steroidogenic enzymes including HSD3B2 and AKR1C3 at mRNA and protein levels in LNCaP cells or xenografts (Chun et al., 2009). The up-regulation appeared to be mediated through IL-6 receptor signaling pathway, as shown by abolishing the up-regulation by knocking down IL-6 receptor with a specific small interfering RNA. The study also convincingly showed that the upregulated steroidogenic enzymes by IL-6 treatment indeed increased T production in LNCaP cells.

Although an adrenal steroidogenic enzyme CYP17 inhibitor like ketoconazole has been widely used to repress adrenal and testicular androgen production as a second line hormone therapy for CRPC patients, its efficacies are not high and side-effects including hepatotoxicity, gastrointestinal toxicity, and adrenal insufficiency could be severe (Mostaghel et al., 2009; Shah & Ryan, 2010). Corticosteroid replacement has to be used with

ketoconazole treatment. A recently developed, highly selective irreversible CYP17 inhibitor, abiraterone, presents higher potency and selectivity, and lower, manageable toxicity compared to ketoconazole (Attard et al., 2008; Shah & Ryan, 2010; Yap et al., 2008). Note, even combined with prednisone, abiraterone still showed certain degrees of adverse effects (e.g., fatigue, hypertension, headache, nausea, and diarrhea). A phase I clinical trial of 21 CRPC patients with continuous administration of abiraterone showed safety of its use and its antitumor activity in up to 70% of the patients (Attard et al., 2008). A phase II study by the same group of investigators seemed to be able to confirm the previous result (Attard et al., 2009b; Reid et al., 2010). Another phase II clinical trial study (Danila et al., 2010) was performed to determine the safety and efficacy of abiraterone when used with prednisone in order to reduce the secondary symptoms such as hyperaldosteronism. The study had 58 CRPC patients who had previous hormonal therapy including antiandrogens (91%), ketoconazole (47%) and estrogens (16%) and failed on previous docetaxel treatment. The inclusion of prednisone in the abiraterone treatment seems to reduce significantly incidence of hypokalemia, hypertension, and fluid retention and the efficacy of abiraterone remain similar to previously observed. A phase III clinical trial on post-docetaxel, castration-resistant prostate cancer patients presented a significantly increased overall survival by about 3.9 months compared with the placebo group (i.e., 14.8 months vs. 10.9 months). The clinical use of this drug seems to be much closer to an approval by U.S. FDA.

There are also other new CYP17 inhibitors such as TAX700 and VN124-1 (Vasaitis et al., 2008; Vasaitis et al., 2010), currently undergoing early clinical trial evaluation. VN/124-1 (3 β -hydroxy-17-(1H-benzimidazole-1-yl)androsta- 5,16-diene), also known as TOK-001, shows an IC₅₀ value of 300 nM in an assay system using intact CYP17expressing *Escherichia coli* in comparison with abiraterone using the same assay system had an IC₅₀ value of 800 nM (Handratta et al., 2005; Vasaitis et al., 2008). However, the inhibitory effect of VN/124-1 is not strictly specific for CYP17 because it was found this compound has multiple targets in androgen signaling pathways (Chan et al., 1996; Schayowitz et al., 2008; Vasaitis et al., 2008). For example, VN/124-1 can act as a ligand antagonist for wild type and a mutant AR (in LNCaP cell) for the binding of a synthetic androgen R1881 as well as in transactivation assay with an ARE linked luciferase reporter and has similar anti-AR potency compared to bicalutamide in LNCaP cells (Chan et al., 1996; Danila et al., 2010). VN/124-1 also reduces AR protein levels. Interestingly, VN/124-1 can inhibit proliferation of CRPC cell lines exhibiting overexpression levels of AR and no longer responding to antiandrogen bicalutamide. In preclinical tests, VN/124-1 at a dose of 0.13 mmol/Kg twice daily showed 93.8% reduction of LAPC-4 tumor volume which seemed to be more effective than castration (Chan et al., 1996, Handratta et al., 2005). The results of all these studies together seem to indicate that VN/124-1 acts as an AR antagonist as well as a CYP inhibitor. It would be very useful if the authors demonstrated that serum testosterone levels can be reduced by this compound.

TAK-700 [(1S)-1-(6, 7-dimethoxy-2-naphthyl)-1-(1H-imidazol- 4-yl)-2-methylpropan-1-ol] isanon-steroidalimidazole] is a CYP17 inhibitor with IC₅₀ = 28 nM. This is a selective inhibitor for CYP17 over 11 β -hydroxylase (Vasaitis et al., 2010). In animal studies, TAK-700 showed inhibitory effects on testosterone production and reduction of prostate gland and seminal vesicles in rats. In addition, results of testing on monkeys showed a single TAK-700 oral administration at 1 mg/kg body weight was sufficient to decrease serum testosterone

levels to castration levels after 8 hours of the treatment (Matsunaga, et al., 2004). Clearly, clinical trials in humans will determine if this CYP inhibitor has values to treat CRPC.

9. Conclusions

As discussed above, surgical or medical androgen deprivation appears to be a major force to cause AR overexpression and gain of function with the consequence of developing CRPC. Intriguingly, recent studies found that androgen ablation can induce leukocytes infiltration into treated tumour areas. Particularly, infiltrated B cells may produce lymphotoxin which in turn causes IKK- β activation results in production of cytokines that activate IKK- α and STAT3 in prostate cancer cells (Ammirante et al., 2010). The activation of STAT3 or IKK- α could activate AR and enhance survival of hormone-deprivation prostate cancer (Ammirante et al., 2010; Jin et al., 2008). Mainly due to overexpression and reactivation of the AR, the resistance to the hormonal deprivation treatments may be overcome by developing new, high affinity AR antagonists. Since recent findings indicated that CRPC tumor can gain the ability by up-regulating genes in androgen synthesis pathways to produce sufficient androgens to activate AR, searching and developing more safe and effective inhibitors, in addition to abiraterone, to the key enzymes in the pathways would also be important approaches for CRPC therapy.

Target molecules	Potential drug development
AR including wild type, mutants and variants	AR ligand antagonists (Norris et al., 2009; Tran et al., 2009; Shen et al., 2009) AR NTD antagonists (Joseph et al., 2009; Narizhneva et al., 2009; Sadar, 2011; Sadar et al., 2008) AR anti-sense DAN or RNA or AR ribozymes (Zegarra-Moro et al., 2002; Sonpavde & Hutson, 2006)
AR co-activators	Anti-sense DNA or RNA or protein interaction disrupting agents (Joseph et al., 2009; Sadar, 2011; Charlier et al., 200)
Androgen synthesizing enzymes	Enzyme inhibitors (Attard et al., 2009b; Reid et al., 2010; Vasaitis et al., 2010)
Androgen independent AR activating molecules (e.g., EGF, interleukin 6, etc.)	Signaling pathway inhibitors (Lee et al., 2002; Wallner et al., 2006; Yu

Table 1. Drug development in Targeting AR and AR related molecules in CRPC

Although it has been shown that the expression of the constitutively active AR variants may be increased by androgen deprivation and overexpressed in CRPC tissues, whether these AR isoforms can become prevalent in CRPC without the presence of wild type AR is currently unknown and should be an interesting topic for further investigation. However, if this will be the case, more proactive strategy for generating new types of non-ligand AR antagonists will be needed, because the oncogenic activities of this type of AR variants will no longer be inhibited by conventional antagonists. Also, AR in microenvironmental stromal areas that surround CRPC cells would be a useful target site. Clearly, nucleotides like

antisense molecules being suggested as drugs for targeting AR will have to overcome numerous obstacles in order to become effective and safe for CRPC treatment. Of course, further understanding AR activities in CRPC will help develop better ways to treat the advanced prostate cancer including CRPC. Finally, many laboratories have attempted to develop drugs targeting AR and/or AR signaling related molecules as listed in Table 1 for CRPC treatment, many of these potential drugs face site specificity, toxicity, efficacy, and delivery challenges/problems.

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Prostate Cancer: Current and Emerging Therapies

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1. Introduction

Prostate cancer (PC) is the second most prevalent cause of death in men in the USA and Europe. The dependence of PC on androgens has been recognized for more than 7 decades. Medical and surgical androgen deprivation therapy (ADT) has been a standard palliative therapy for metastatic PC. However, an estimated 217,730 new cases and 32,050 PC related deaths in the USA alone in 2010 despite ADT, make the need for finding new targets and novel therapies an absolute priority [1]. Despite medical treatment, the vast majority of patients with metastatic prostate cancer inevitably progress and die from their disease. While initially majority of metastatic prostate cancers rely on the availability of androgens for growth and survival, in their final stages of disease, these patients eventually progress clinically under androgen-deprived conditions. Under the selective pressure of drug treatment, prostate cancer cells are then able to acquire molecular changes that allow them to survive androgen-deprived conditions, gain a selective growth advantage, and finally, result in progression of disease. Our knowledge about this disease is increasing. However, the cellular and molecular events that are necessary to cause progression of prostate cancer from an androgen-dependent (AD) to an androgen-independent (AI) state of disease are not completely understood.

With a 9% response rate, chemotherapy was once thought to play a clinically insignificant role in metastatic and castration resistant prostate cancer (CRPC) [2]. More recently, however, a role has emerged for systemic chemotherapy after the demonstration of a small but significant survival benefit for taxane-based chemotherapy in the two landmark studies, TAX-327 and SWOG-9916 [3, 4]. Since median survival for patients with metastatic CRPC is still only about 18 months, there is plenty room for further improvement. Moreover, there is a strong need for second and third-line regimen for patients progressing after docetaxel, and these patients should be enrolled into clinical trials.

2. Novel biomarkers

PC is a highly curable disease if diagnosed at an early stage and 5-year relative survival rates based on Surveillance, Epidemiology, End Results (SEER) database's cancer statistics

were 100.0% for both localized and regional disease, and 30.6% for distant metastatic disease [5]. Given the enormous importance of early detection, selection of biomarkers for early diagnosis and monitoring the treatment are absolutely essential. Traditionally, serum prostate specific antigen (PSA) has been used as biomarker. However, in about 10% of patients, whose tumors are associated with low serum prostate PSA, a decline in PSA cannot be used as an indicator of response. Several studies also suggest that serum PSA level does not reflect PSA levels in the tumor tissue or the growth of tumor [6]. Therefore, there is an urgent need to find out new biomarkers that may be more useful in diagnosis of PC.

2.1 Fluoro-dihydrotestosterone (FDHT)

FDHT is a biomarker of androgen receptor expression in human prostate cancer, and has been particularly useful in the setting of advanced prostate cancer, when the patient has castrate levels of circulating testosterone in the blood. Two small prospective studies have shown the feasibility of using FDHT scan with excellent imaging characteristics and a rapid uptake in the tumor at metastatic sites expressing androgen receptor with acceptable dosimetry [7, 8]. This scan is currently incorporated and compared to fluoro-deoxyglucose (FDG) positron emission tomography (PET) in a phase I/II study of CRPC that are being treated with chemotherapy [9]. This study uses PET scans, which is a type of imaging test that uses a radiotracer, to see whether these scans may be better able to find places in the body where prostate cancer cells may have spread. Initial reports presented at the 2009 ASCO annual meeting showed a > 50% decline in the standardized uptake value (SUV_{max}) on FDHT PET observed in 11 out of 12 of patients (92%) at 4 and 12 weeks, while 6 patients (50%) had a decreased SUV_{max} on FDG PET [10].

2.2 Circulating tumor cells

Circulating tumor cells (CTCs) are epithelial cells that shed from tumors. The CTC count is based on a test that works by using fluorescence labeled antibodies against epithelial cell adhesion molecules combined to microscopic iron particles, called ferrofluid [11]. These antibody/ferrofluid combinations attach very specifically to CTCs. Powerful magnets then “pull” the CTCs out of the blood sample and they are then stained with additional biomolecules and chemicals so that they can be positively identified as CTCs [12]. This system, approved by the US Food and Drug Administration (FDA) is commercially available as CellSearch™ for monitoring of metastatic prostate cancer, metastatic breast and metastatic colorectal cancer patients [13]. In a prospective study, De Bono and colleagues [14] reported that CRPC patients with ≥ 5 CTCs per 7.5 mL of blood prior to chemotherapy had a significantly shorter median survival compared to those with < 5 CTCs (10 *vs.* 21 months). Also, changes in number of CTCs following chemotherapy correlated with prognosis. Patients who had < 5 CTCs at baseline and at their last assessment had a median survival of more than 26 months, while those who had ≥ 5 at baseline but then had < 5 at their last assessment had a median survival of 21 months. In contrast, those with < 5 CTCs at an early assessment who had ≥ 5 at their last assessment had a median survival of 9 months, and those who had ≥ 5 CTCs at all assessments had a median survival of only 7 months. Two recent prospective studies have also validated that increased levels of circulating tumor cells predict worse outcomes in patients with metastatic CRPC [14]. Thus, CTC number, analyzed as a continuous variable, has a potential to be used to monitor disease status and might be useful as an intermediate endpoint of survival in clinical trials.

2.3 Clusterin

Clusterin is a stress-induced cyto-protective chaperone protein expressed in virtually all human tissues. Clusterin over-expression is demonstrated in various human malignancies including prostate, breast and colon cancers [15, 16]. It has been shown that in prostate cancer, clusterin levels are low in hormone-naïve tissue, but increase significantly after hormone therapy [17]. Clusterin levels have also been correlated with preoperative PSA value and also the pathological grade on both biopsy and radical prostatectomy specimens. Further, clusterin expression has also been reported to be a possible predictor for biochemical recurrence following radical prostatectomy [18]. In a recent phase II clinical trial, serum levels of clusterin was used a biomarker of response and was reported to be significantly reduced following treatment with OGX-011, an antisense oligonucleotide against clusterin [19]. All these data suggest that serum clusterin level could be used as a potential diagnostic and prognostic indicator and also a marker of response to treatment in CRPC with metastases.

3. Androgen Deprivation Therapy (ADT)

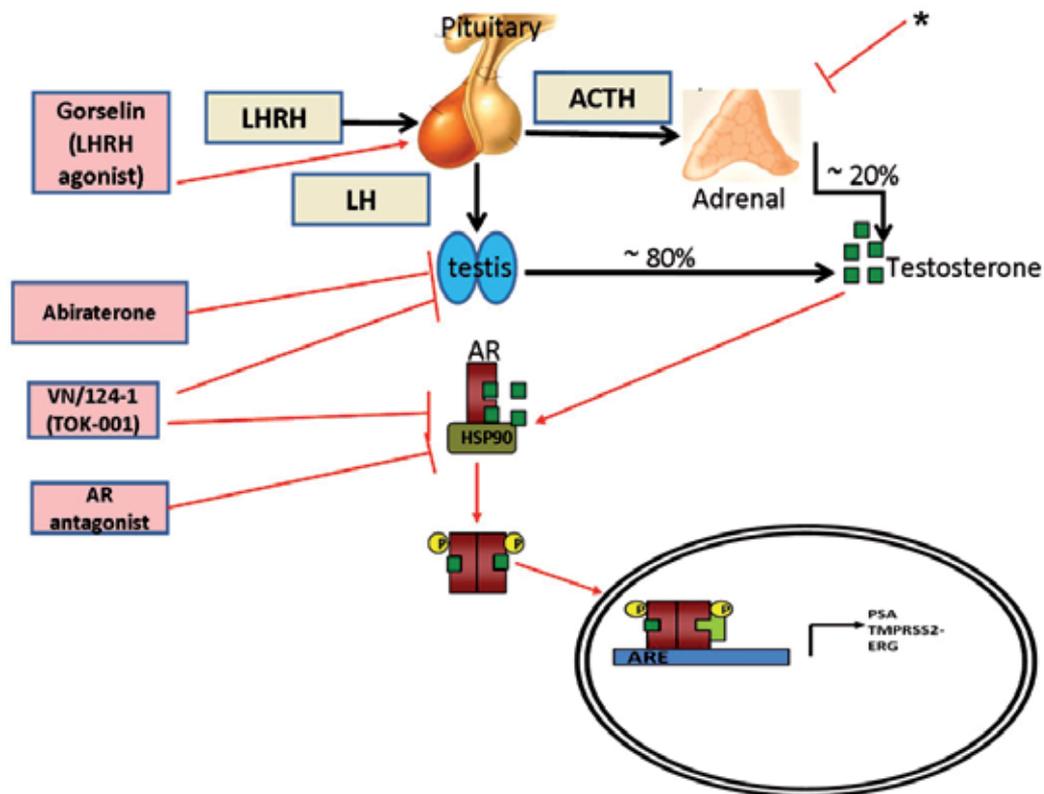
ADT is the cornerstone treatment of advanced prostate cancer. In 1941, Huggins and Hodges first noted the beneficial effects of castration [20]. In addition to its well established role in treating patients with metastatic disease, ADT is sometimes used to treat patients with increasing PSA levels after local treatment, even without radiographic or other evidence of metastatic disease. It is also used as adjunct therapy for men undergoing radiation therapy for high-risk localized disease. Several large-scale phase III studies reported in the 1980s have shown that the suppression of plasma testosterone by medical or surgical castration in men with advanced or metastatic prostate cancer leads to symptom reduction, and a marked clinical response [21].

Several studies have attempted to pharmacologically target androgenic stimulation at different points in the hypothalamus-pituitary-testis-AR pathway. The goal of these drug interventions is to slow disease progression, and to treat the disease. **Surgical castration** completely eliminates testosterone production by the testes, whereas administration of an **LHRH agonist** (medical castration) generates castrate levels of serum testosterone (< 20 or < 50 ng/dL respectively) by having a negative hormonal feedback on the hypothalamus [22]. There was no statistically significant difference in disease free or overall survival for metastatic patients treated with either of the these testosterone lowering treatments [23]. Conventional ADT was associated with a number of adverse effects like hot flashes, loss of libido, decreased quality of life.

AR antagonists and CYP 17 inhibitors are some of the newer ADT therapies. Figure 1 is a schematic representation of agents that target the AR signaling.

3.1 Androgen Receptor (AR) antagonism

There is ample evidence in the literature that prostate cancer growth can be inhibited by blocking the AR. AR antagonists compete with dihydrotestosterone (DHT) for binding to the AR and thus block AR signaling. Despite the significant reduction in circulating testosterone, castration does not affect adrenal androgen production. Therefore, anti-androgens were introduced to directly prevent the binding of testosterone and DHT to the AR. Anti-androgens competitively inhibit ligand binding to the AR and may also prevent ligand-independent AR activation through various pathways, such as inhibiting the recruitment of



*Abiraterone and VN/124-1 (TOK-001) also inhibit the synthesis of adrenal androgens. Gorselin inhibits secretion of LH from the pituitary. Abiraterone and VN/124-1 (TOK-001) inhibit CYP17 enzyme. VN/124-1 (TOK-001) also antagonizes AR.

Fig. 1. Schematic representation of AR regulation in prostate cancer and agents targeting AR signaling.

coactivators or activating corepressors [24]. Anti-androgens are typically classified as steroidal or nonsteroidal based on their respective chemical structures [25]. The major anti-androgens in clinical use worldwide are the nonsteroidal bicalutamide, flutamide and nilutamide and the steroidal cyproterone acetate (CPA) (**Figure 2**). CPA is used in Europe, but is not commercially available in the USA. CPA is one of the least studied anti-androgen. Conversely, bicalutamide is the most extensively studied nonsteroidal anti-androgen [26]. Lowered percentages of hot flashes as compared with castration have been reported with bicalutamide, flutamide and CPA treatment. Patients treated with bicalutamide have reported better preservation of sexual interest compared with LHRH agonist alone [27]. It is also important to note that a meta-analysis of randomized trials comparing CPA and ADT with ADT alone showed a survival decrease in the CPA group [28]. Overall, the nonsteroidal anti-androgens appear to be better tolerated than castration, however it is important for clinicians to explain the tolerability profiles of all treatment options in order to find an individual match for each patient [29]. Agents targeting AR that are in clinical trials are summarized in **Table 1**. As monotherapy with an AR antagonist is not yet a standard treatment for patients with advanced or metastatic prostate cancer, it has been combined

with medical (or surgical) castration, initially in studies conducted in the late 1980s and early 1990s (complete androgen blockade). These clinical trials showed that the combination of surgical or medical castration plus the administration of an AR antagonist resulted in only a limited improvement in disease-specific and overall survival in patients with advanced and/or metastasized prostate cancer compared to those who receive castration only [30].

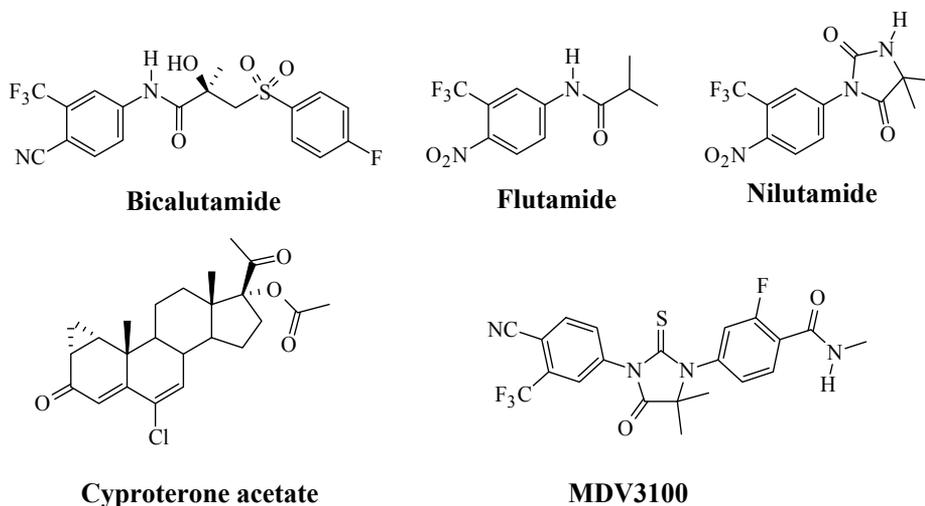


Fig. 2. Structures of currently used anti-androgens and clinical candidate MDV3100.

3.1.1 MDV3100

Following the evidence that AR expression is increased in CRPC, the diarylthiohydantoin MDV3100 (Figure 2) was developed as a second-generation anti-androgen capable of sustained AR antagonism under conditions of AR over-expression. In preclinical evaluation MDV3100 was shown to bind to the AR with a five- to eight-fold higher affinity than bicalutamide [6, 31]. In a Phase I/II study in CRPC, anti-tumor activity of MDV3100 was assessed by time on treatment, PSA, soft tissue and osseous disease and circulating tumor cells (CTC). Doses of up to 600 mg/day were investigated. Out of 114 patients treated with 30–360 mg/day and followed for over 12 weeks, 65 were chemotherapy-naïve and 49 were post chemotherapy. At 12 weeks, reduced PSA levels were seen in both groups, with a 57% (37/65) decline in the naïve group and 45% (22/49) in the post-chemotherapy patients [31, 32]. No progression was noted in 74% (35/47) of patients with evaluable soft tissue lesions and 62% (50/81) of patients with bone lesions. Dose-limiting toxicity was observed at 600mg/day. Fatigue was noted at 360 and 480 mg/day. Hence, the dose was reduced. At concentrations of 60, 150 and 240 mg/day, MDV3100 was well tolerated and no serious adverse events related to the drug were reported. Of the 73 patients, 63 had available CTC counts. A total of 85% of those with favorable pretreatment CTC counts maintained favorable post-treatment CTC counts and 58% of patients treated at 240 mg/day converted from unfavorable to favorable, post-treatment. Bone scans revealed stable disease in 29% (6/21) patients with osseous disease on 240 mg/day. A half-life of 1 week was established and the current reported data suggest a dose-response trend. Ultimately 240 mg/day was selected for the Phase III trials and the results are much anticipated.

Drug	Mechanism of action	Patient characteristics	Phase of development	Clinical trial Registration number
MDV-3100	AR antagonist	Chemotherapy-treated	Phase III	NCT00974311
		Chemotherapy-naïve	Phase III	NCT01212991
ARN-509	AR antagonist	ND	Phase I-II	NCT01171898
AZD3514	AR antagonist	ND	Phase I-II	NCT01162395
Abiraterone acetate	CYP 17 inhibitor	Chemotherapy-treated	Phase III	NCT00638690
		Chemotherapy-naïve	Phase III	NCT00887198
Orteronel (TAK-700)	CYP 17 inhibitor	Chemotherapy treated	Phase III	NCT01193257
VN/124-1 (TOK-001)	AR downregulating agent, CYP 17 inhibitor and AR antagonist	ND	Phase I-II	NCT00959959

Abbreviations: ND = not defined.

Table 1. Agents targeting AR in clinical development for CRPC.

3.2 CYP17 Inhibitors

Blocking the *in situ* production of androgens by inhibition of CYP 17 enzyme is a critical key in the treatment of patients with advanced and/or metastatic prostate cancer. The structures of CYP 17 inhibitors ketoconazole, abiraterone acetate and VN/124-1 (TOK-001) are presented in **Figure 3**.

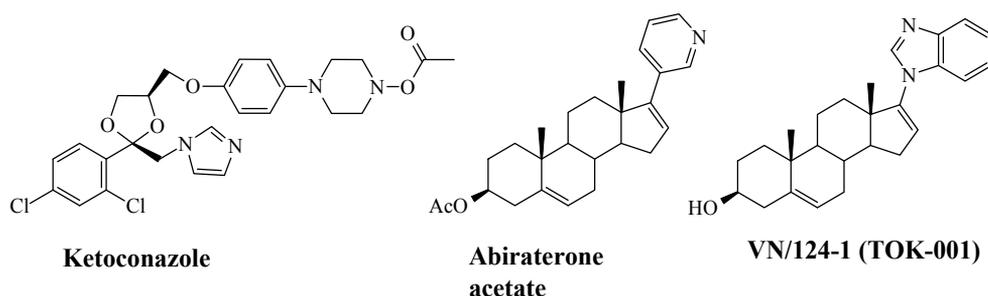


Fig. 3. Inhibitors of CYP17.

3.2.1 Ketoconazole (HDK)

Ketoconazole is a broad spectrum anti-fungal agent that has been extensively used off-label as second-line hormonal therapy for prostate cancer. Ketoconazole inhibits 11- β hydroxylation, cholesterol side chain cleavage to pregnenolone and CYP17 [33]. Two single center trials on the

use of HDK in CRPC found PSA declines >50% in 55% (11/20) [34] and 63% (30/48) of patients [35]. A larger phase III study of HDK therapy in 260 patients with post-ADT metastatic PC on anti-androgen withdrawal (AAWD) demonstrated a PSA decline > 50% in 27% of patients treated with HDK plus AAWD. Overall survival was not different between the treatment groups; however, those patients with a > 50% PSA decline had a median survival of 41 months compared to 13 months for those without a PSA decline. Time to PSA progression in PSA responders was 5.9 *versus* 8.6 months in AAWD alone and AAWD+HDK groups, respectively [36]. Androstenedione, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEAS) levels decreased with HDK therapy. However, there was no change in testosterone level from baseline in either treatment groups.

3.2.2 Abiraterone acetate

Abiraterone, a highly selective irreversible CYP17 inhibitor, was developed as a mechanism-based steroidal inhibitor of CYP17 following observations that nonsteroidal 3-pyridyl esters had improved selectivity for inhibition [37]. Abiraterone has been shown to reduce serum testosterone levels to below a detection threshold of 1 ng/dl [38]. Promising results from clinical trials of abiraterone acetate in CRPC patients have recently been reported. In a phase I trial of abiraterone acetate treatment of both ketoconazole pre-treated and ketoconazole naïve CRPC patients [4], PSA declines of $\geq 50\%$ were seen in 18 (55%) of 33 patients, including nine (47%) of 19 patients with prior ketoconazole therapy and nine (64%) of 14 patients without prior ketoconazole therapy. Significantly, the anti tumor activity was nearly equivalent in both populations. The activity observed in castrate, ketoconazole naïve patients confirms that abiraterone acetate is an active agent, whereas the activity in ketoconazole pre-treated patients implies that a more selective and potent inhibitor of CYP17 may be an improvement beyond ketoconazole, or an additional sequential therapeutic option. The most common adverse events in patients treated with abiraterone acetate were fatigue, hypertension, headache, nausea, and diarrhea.

In addition to chemotherapy-naïve patients, a multi center phase II study evaluated the efficacy of abiraterone in patients with docetaxel-treated CRPC [39]. All patients were treated with 1000 mg/d. Forty seven patients were enrolled, and treatment resulted in observed PSA declines $\geq 50\%$ in 51% (24/47) of patients at least once. Partial responses (by RECIST criteria) were reported in 27% (8/30) patients with measurable disease. Decreases in circulating tumor cell (CTC) counts were also observed [39].

Two phase III clinical trials of abiraterone acetate are now in progress. The first of these trials is designed to evaluate abiraterone + prednisone against a placebo + prednisone in patients with progressive CRPC after docetaxel chemotherapy. This trial has an estimated study completion date of June 2011 [40]. The second study will evaluate abiraterone + prednisone against a placebo + prednisone in CRPC patients prior to chemotherapy. The estimated study completion date is in 2014. Both trials list prior ketoconazole treatment in their exclusion criteria.

3.2.3 VN/124-1 (TOK-001)

VN/124-1 was rationally designed as an inhibitor of androgen biosynthesis via inhibition of CYP17. Utilizing intact CYP17 expressing *Escherichia coli*, VN/124-1 was shown to be a potent inhibitor of the enzyme with an IC_{50} value of 300 nM compared to abiraterone which had an IC_{50} value of 800 nM. The high efficacy of VN/124-1 in several prostate cancer

models is believed to arise from its ability to downregulate the AR as well as competitively block androgen binding. In competitive binding studies against the synthetic androgen [³H] R1881, VN/124-1 was equipotent to bicalutamide in LNCaP cells. Transcriptional activation assays showed VN/124-1 to be a pure AR antagonist of the wild- type AR and the T877A mutation found in LNCaP cells [6]. VN/124-1 inhibited the growth of CRPCs, which had increased AR and were no longer sensitive to bicalutamide [6].

VN/124-1 (0.13 mmol/kg twice daily) caused a 93.8 % reduction ($P = 0.00065$) in the mean final LAPC-4 xenograft volume compared with controls. In another anti-tumor efficacy study, treatment of VN/124-1 (0.13 mmol twice daily) was very effective in preventing the formation of LAPC4 tumors. VN/124-1 (0.13 mmol/kg twice daily) and VN/124-1 (0.13 mmol/kg twice daily) + castration induced regression of LAPC4 tumor xenografts by 26.55 and 60.67 %, respectively [6]. This impressive pre-clinical data led to further clinical development of VN/124-1 by Tokai Pharmaceutical Cambridge, Mass. Tokai Pharmaceuticals initiated ARMOR1 (Androgen Receptor Modulation Optimized for Response 1) phase 1/2 trials in castrate resistant prostate cancer patients on November 5, 2009 [41]. The results of this clinical trial are awaited. The study is expected to be completed by July 2012. The benefits of ADT in selected clinical trials are summarized in **Table 2**.

Source	Outcome	Control Arm (95% CI)	ADT-arm (95% CI)	P value
Bolla et al 1997 [42] and Bolla et al 2002 [43]	Increase in 5-yr survival	62 (52-72)	78 (72-84)	.0002
D'Amico et al 2004 [44]	Increase in 5-yr survival	78 (68-88)	88 (80-95)	.04
Messing et al 1999 [45]	Increase in 10-yr survival	49.0	72.4	.025

Table 2. Benefits of ADT in prostate cancer.

3.3 Resistance to ADT

During the development of CRPC, there is evidence that the testosterone-AR pathway is bypassed, and that prostate cancer cells find alternative ways to continue AR-mediated functions [46]. Concurrently, this renewed and continued AR activation leads to renewed cell proliferation, unsustained growth, and eventually causes the prostate cancer host to have biochemical and clinical progression of disease. Although CRPC is androgen independent, it remains dependent on a functional AR. Various mechanisms contribute to resistance to ADT. They include AR amplification, AR mutations and hypersensitivity of AR to androgens or other ligands (**Figure 4**).

3.3.1 AR amplification

One of the mechanisms by which a prostate cancer cell might escape and survive the low testosterone conditions and sustain growth is by amplification of the AR gene and by up-regulation of the AR protein [47]. CRPC expresses more AR than benign prostatic tissue and hormone-naïve prostate cancers [48]. As a consequence, even very low levels of intracellular testosterone and/or DHT might cause androgen signaling and AR-regulated transcription [49]. Several studies have reported that during the process of the tumor becoming CRPC, the AR protein has increased stability and it becomes hypersensitive to androgens [50].

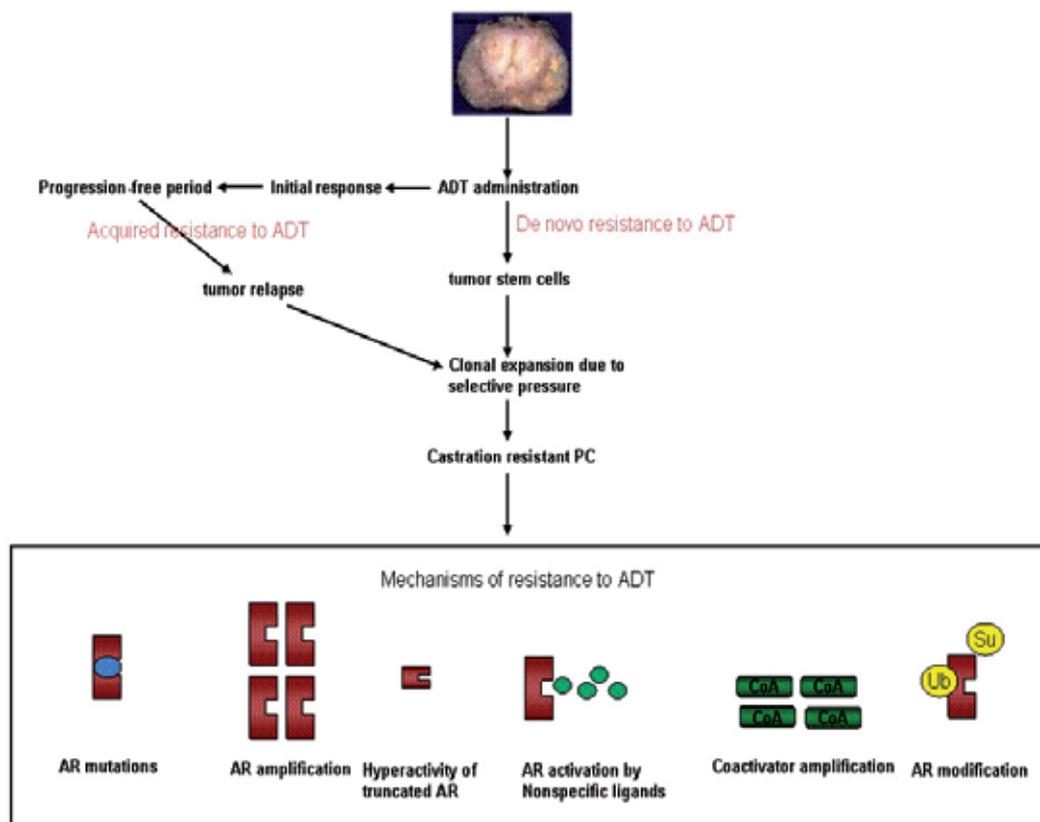


Fig. 4. Mechanisms of development of resistance to androgen deprivation therapy: Chronic ADT therapy leads to development of resistance in prostate cancer (PC) cells by various mechanisms such as activating AR mutations, AR amplification, overactive spliced / truncated AR isoforms, amplification of coactivators and modifications of AR by various physiological processes such as ubiquitylation and sumoylation.

3.3.2 AR mutations

Mutations in AR lead to change in the specificity of ligand binding. It has been reported that the mutated AR might thus be activated by other steroid hormones, such as progesterone, estrogens, adrenal androgens and metabolic by-products of DHT [51]. In other AR mutations, the AR protein might become even more promiscuous, and bind AR antagonists such as cyproterone acetate and flutamide [52, 53]. The withdrawal of flutamide in patients with CRPC, and with this the discontinuation of the activation of the AR, causes a rate of improvement of serum PSA in 30–40% of patients. This effect is now defined as the ‘**anti-androgen withdrawal**’ syndrome [54]. The splice variant AR isoforms, expressing the NH₂-terminal domain and the DNA binding domain only, can be overexpressed in CRPC, are functionally active, promote the expression of AD genes, and might support growth of CRPC [55]. Co-activators can cause conformational changes of the AR and with this, alter the ligand binding domain (LBD) and the specificity of the AR protein [56]. Mutations in co-activator genes and/or changes in the expression of these co-activator proteins have been reported [57].

3.3.3 Hypersensitivity of AR to low levels of androgens

Recent evidence suggests that plasma levels of androgens do not correlate with intraprostatic androgen levels [58]. Also, it has been shown that despite castration levels of plasma testosterone, DHT levels in the prostate itself remain at 15–40% of that at baseline [59]. These low intraprostatic levels of DHT are still sufficient to activate the AR and stimulate the expression of androgen dependent genes [60]. Thus, even decreased levels of intraprostatic DHT might be sufficient to support biological processes that concurrently lead to cell proliferation and a defense against apoptosis. After ADT, Mizokami *et al.* [61] showed that intraprostatic androstenediol levels are similar to those in benign prostate hypertrophic tissue, and are able to activate a mutated AR. Craft *et al.* [62] showed *in vitro* that ADT provides for selective pressure, resulting in an outgrowth of a few AI cells. This clonal expansion of androgen independent cells then further resulted in all the cascades of CRPC. However, most androgen independent prostate cancer cells continue to express the AR, and rely on AR signaling pathways, even in ligand-independent AR activation.

4. Chemotherapy

Mitoxantrone, estramustine, and docetaxel (Figure 5) are the three drugs which are currently approved by the FDA for first-line chemotherapy in CRPC. In the landmark

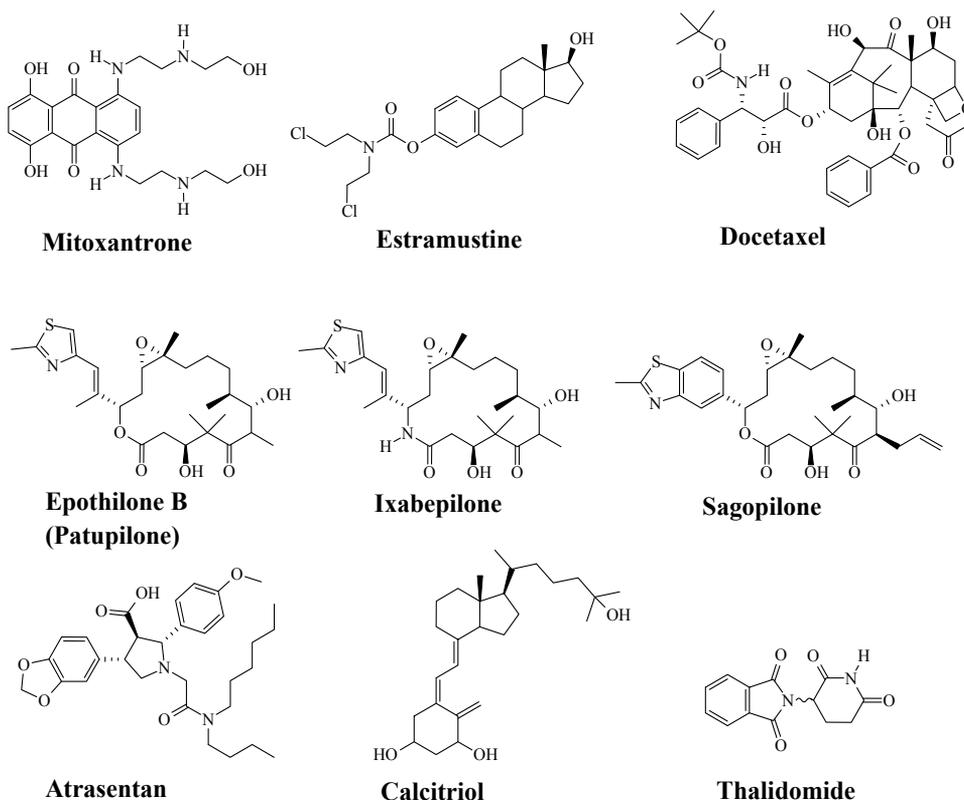


Fig. 5. Structures of chemotherapeutic agents and other types of anti-cancer agents.

TAX-327 trial, 1006 chemotherapy-naïve CRPC patients were randomized to three different treatment arms – docetaxel 30 mg/m² every week, docetaxel 75 mg/m² every three weeks and mitoxantrone 12 mg/m² every three weeks (**Figure 6**). All patients received prednisone 5 mg orally twice a day. Patients receiving docetaxel every three weeks had a significant improvement of survival compared to weekly docetaxel and mitoxantrone (18.9 months *vs.* 16.5 months; $P < 0.009$). PSA response, quality of life and control of pain were also significantly better with docetaxel every three weeks compared to mitoxantrone [3]. An update of the results of TAX-327 trial in 2007 showed a persistence of a survival benefit of docetaxel every three weeks compared to mitoxantrone and no survival benefit with the weekly docetaxel. At three years, survival was 17.2% for docetaxel every three weeks compared to 12.8% with mitoxantrone ($P = 0.005$) [3].

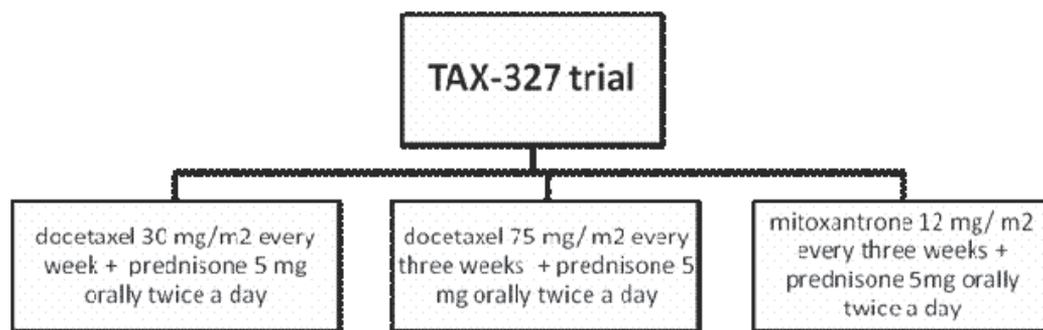


Fig. 6. Schematic flow chart of study design of TAX-327 clinical trial.

The **Southwest Oncology Group (SWOG)-9916 study** also showed survival benefit with Docetaxel. 674 patients with metastatic CRPC were randomized to docetaxel/estramustine and Mitoxantrone/prednisone arms (**Figure 7**). Treatment regimen was 280 mg of estramustine three times daily on days 1 through 5, docetaxel 60 mg/m² on day 2 in the docetaxel arm and 12 mg of mitoxantrone mg/m² on day 1 plus 5 mg of prednisone twice daily in the mitoxantrone arm. Docetaxel was reported to be superior to mitoxantrone with a median survival of 17.5 months *vs.* 15.6 months ($P = 0.02$), median time to progression (6.3 *vs.* 3.2 months; $P < 0.001$) and PSA declines of 50% (50% *vs.* 27%; $P < 0.001$). However, there was no significant objective tumor response difference between the two arms [4]. TAX-327 and SWOG-9916 trials showed a 20–24% reduction in mortality in patients with CRPC docetaxel-based combination chemotherapy.

Although the taxanes provide impressive results against CRPC, their survival benefits remain far from being long lasting. This is primarily due to development of resistance against the taxanes [63]. Several molecular mechanisms account for *de novo* and acquired resistance to taxane-based chemotherapy in prostate cancer. Multidrug resistant phenotype (MDR) is a common cause of *de novo* resistance. Acquired resistance to taxanes can result due to alterations in the molecular target, tubulin. Some of these mutations alter drug binding, while others cause shifts in the equilibrium of the tubulin dimer and microtubule polymer, thereby affecting taxane efficacy [64, 65]. Preclinical studies have shown that overexpression of class III β -tubulin confers *de novo* and acquired resistance to taxanes in several tumor types, as shown in prostate, breast, lung cancer cell lines [66, 67].

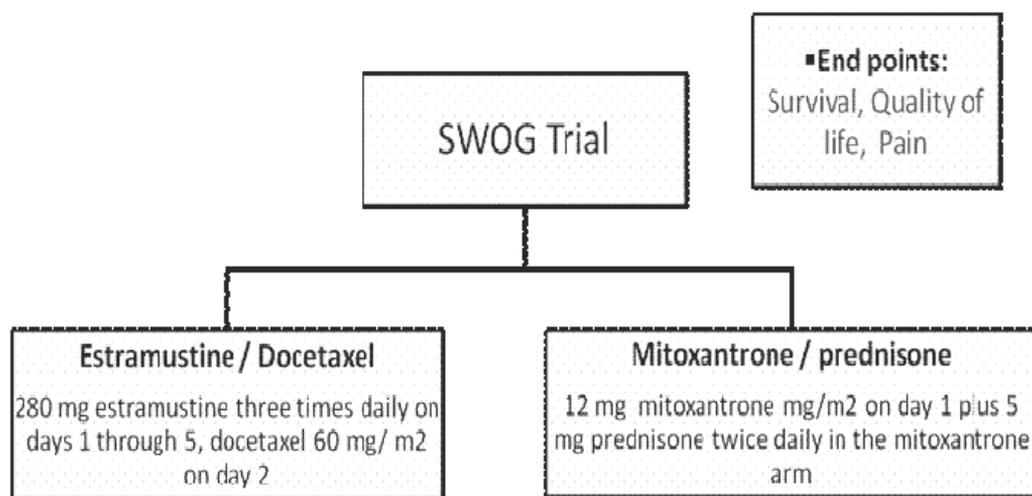


Fig. 7. Schematic flow chart of study design of SWOG-9916 clinical trial.

Drug	Mechanism of action	Primary endpoint	Clinical trial Registration number
Docetaxel/Prednisone every 3 weekly vs 2 weekly (PROSTY)	Taxane (antimitotic, antimicrotubule agent)	TTF	NCT00255606
Docetaxel/Prednisone + Dasatinib	Multi-target Tyrosine Kinase inhibitor	OS	NCT00744497
Docetaxel/Prednisone + Atrasentan	Endothelin A receptor antagonist	PFS	NCT00134056
Docetaxel/Prednisone + ZD4054	Endothelin A receptor antagonist	OS	NCT00626548
Docetaxel/Prednisone + Bevacizumab	VEGF blocking monoclonal antibody	OS	NCT00110214
Docetaxel/Prednisone + Aflibercept (VENICE)	Soluble decoy receptor for VEGF	OS	NCT00519285
ZD4054 (ENTHUSE M0; ENTHUSE M1)	Endothelin A receptor antagonist	OS	NCT00554229 NCT00617669
Abiraterone Acetate + Prednisone	CYP17A1 inhibitor	OS, PFS	NCT00887198

Abbreviations: OS = overall survival; PFS = Progression free survival; and TTF = time to treatment failure.

Table 3. Active phase III trials in first-line chemotherapy for CRPC.

Drug	Mechanism of action	Primary endpoint	Clinical trial Registration number
XRP6258 + Prednisone vs Mitoxantrone + Prednisone	Taxane with a low affinity for P-gp	OS	NCT00417079
Sipuleucel-	T Active cellular immunotherapy	Safety and efficacy	NCT0065442
Abiraterone Acetate + Prednisone	CYP17A1 inhibitor	OS	NCT00638690
MDV3100 (AFFIRM)	AR antagonist	OS	NCT00974311
Ipilimumab	CTLA-4 blocking monoclonal antibody	OS	NCT00861614
Sunitinib + Prednisone	Multitarget Tyrosine Kinase inhibitor	OS	NCT00676650

Abbreviations: OS = overall survival.

Table 4. Active phase III trials in second-line chemotherapy for CRPC.

5. Newer chemotherapy

New chemotherapeutic agents that are less susceptible to the mechanisms that give rise to taxane resistance in CRPC are urgently needed. Of the novel chemotherapeutic agents, the epothilone analog class (Figure 5) is of particular interest.

5.1 Epothilones

The epothilones are microtubule stabilizing agents that initiate apoptosis in cancer cells by disrupting the dynamic characteristics of microtubules [68]. The epothilones (Figure 5) include natural epothilone B (EPO906; patupilone; Novartis, Basel, Switzerland) and several semisynthetic epothilone compounds such as BMS-247550 (ixabepilone; aza-epothilone B; IXEMPRA; Bristol-Myers Squibb, New York, NY) and sagopilone (ZK-EPO; Schering AG, Berlin, Germany). Ixabepilone is the first of these agents to receive FDA approval for use in the treatment of metastatic or locally advanced breast cancer in combination with capecitabine after failure of an anthracycline and a taxane, or as monotherapy after failure of an anthracycline, a taxane, and capecitabine.

The epothilones induce cell cycle arrest at the G2/M phase via tubulin polymerization [69]. However, epothilones and taxanes have important differences in modes of binding and the sites of binding to tubulins [70]; ixabepilone has been shown to affect multiple β -tubulin isoforms. It suppresses the dynamic instability of class III β -tubulin and class II β -tubulin microtubules, whereas taxanes are not known to bind to class III β -tubulin [71, 72]. It has also been shown that the tubulin polymerizing activity of epothilone B is approximately 2- to 10-fold greater than that of the commonly used taxane- paclitaxel [73].

The epothilones appear to be less susceptible to classic tumor resistance mechanisms such as P-gp or MRP efflux, tubulin mutations, and alterations in tubulin isotypes [74, 75]. It has been quite well documented that epothilones are more efficacious in taxane-resistant cell lines and xenografts [76, 77]. There is no evidence of cross-resistance between taxanes and epothilones which is another justification for their potential use to tackle taxane resistance [78].

5.1.1 Clinical activity of epothilones

Epothilones have been tested as first-line (against chemo-naïve tumors), second-line (against tumors previously treated with chemotherapy) or third-line (against tumors previously treated with 2 types of chemotherapy). Some of the important clinical trials are described below.

First-line therapy: In a multi-institutional, randomized, phase II study in chemotherapy-naïve patients, Galsky and colleagues [79] showed that ixabepilone was active in the treatment of CRPC, irrespective of the addition of the well established chemotherapeutic-estramustine. PSA declines of > 50% were reported in 31/45 patients (69%) in the combination arm and 21/44 patients (48%) in the ixabepilone monotherapy arm. Median progression-free survival (PFS) was 5.2 months and 4.4 months in the combination and monotherapy arms, respectively. The most important side effect of ixabepilone was neutropenia.

The Southwest Oncology Group trial SO111 extended these results in a study of 42 patients with metastatic CRPC treated with ixabepilone 40 mg/m² [80]. Fourteen patients (33%) achieved a PSA response (the definition of which required at least stable measurable disease), with the majority (72%) achieving a reduction > 80%. Median PFS was 6 months and median overall survival was 18 months.

In the pilot study reported by Smaletz and colleagues [81] they examined the efficacy of intravenous ixabepilone in combination with oral estramustine (280 mg 3x daily on days 1 to 5) in 13 chemotherapy-naïve patients with CRPC. The reported decline in PSA levels > 50% was in 11 patients (92%), out of which 5 patients achieved reductions in excess of 80%. Among the 7 patients with measurable disease, there was 1 complete response (CR) and 3 partial responses (PRs), and an additional patient achieved disease stabilization. The most common adverse events was neutropenia reported in 4 patients.

Second-line therapy: The utility of ixabepilone as a second-line agent in patients previously treated with a taxane has also been evaluated [78]. A phase II randomized study compared ixabepilone 35 mg/m² every 3 weeks with intravenous mitoxantrone 14 mg/m² every 3 weeks plus prednisone 5 mg twice daily in 82 patients with taxane refractory CRPC [78]. PSA declines > 50% were reported in 17% of patients treated with ixabepilone and 20% of those treated with mitoxantrone plus prednisone. In patients with measurable disease, the objective response rate (ORR) was 7% and 6%, respectively.

To sum up, epothilones represent a very effective option to treat taxane resistant CRPC.

6. Endothelin receptor antagonists

Endothelins are regulators of cell vasomotor tone, and angiogenesis. The endothelins bind to two receptors, endothelin-A and endothelin-B, and play a major role in tumor growth, proliferation, angiogenesis, and bone metastasis [82]. Several studies have shown that patients with metastatic prostate cancer have elevated levels of plasma endothelin-A

compared with patients with localized cancer. Endothelin-A is also thought to promote osteoblastic activity characteristic of bone metastases in prostate cancer [83].

Atrasentan (**Figure 5**) is mainly an endothelin-A receptor antagonist. In a phase II, randomized, double-blind trial on patients with metastatic CRPC, 288 asymptomatic patients received either placebo or once-daily atrasentan, 2.5 or 10 mg [4]. The 10 mg atrasentan group had a longer median TTP (time to progression) (187 *vs.* 137 days for the placebo group, $P = 0.02$). Median time to PSA progression was 155 days for the atrasentan 10 mg group compared with 71 days for the placebo group ($P = 0.002$). Headaches were the main reversible side effect. Encouraging results from this trial led to phase III investigations. In a phase III multicenter trial, 809 men with CRPC were randomized in a 1:1 fashion to atrasentan 10 mg daily *vs.* placebo [4]. The primary endpoints were TTP assessed radiographically and clinically. Atrasentan did not reduce TTP relative to the placebo arm (hazard ratio 0.89, $P = 0.136$). In an exploratory analysis, however, bone alkaline phosphatase and PSA levels were significantly lower in the atrasentan arm ($P < 0.05$). In a second phase III trial, 941 men with PSA-only CRPC were randomized to receive atrasentan 10 mg daily *vs.* placebo [83]. Fewer men treated with atrasentan (227) experienced disease progression compared with placebo (267), and the median survival was longer for the atrasentan group ($P = 0.176$), however, this longer median survival was not statistically significant. PSA doubling time prolongation and a decrease in alkaline phosphatase were seen in the treatment group ($P = 0.031$ and $P = 0.001$, respectively). although atrasentan did not meet the primary endpoint expectations, it did have an impact on molecular markers that indicate disease progression. Hence, Southwest Oncology Group is currently conducting a phase III trial investigating docetaxel with or without atrasentan in men with metastatic CRPC.

7. Antisense oligonucleotides

Antisense oligonucleotides (ASOs) offer a novel approach to regulate genes involved in cancer progression, especially those that are not targetable by drugs [84]. ASOs are single-stranded, chemically modified DNA-like molecules that are 15–25 nucleotides in length. They are designed to be complementary to a selected gene's mRNA and thereby specifically inhibit expression of that gene. It is estimated that any sequence of at least 13 bases in RNA and 17 bases in DNA is represented only once within the human genome. Thus, the specificity involved in the design of ASOs theoretically leads to decreased toxicity. There has been tremendous development in the ASO technology in this decade. However, there are several challenges that need to be addressed such as optimization of ASO's tissue exposure, cellular uptake and demonstration of mechanism of action and antitumour activity.

The clusterin gene encodes a cytoprotective chaperone protein which has been implicated in a number of physiologic processes [85]. During times of stress, it is thought to act as a survival protein and stabilizes conformations of proteins [86]. In prostate cancer, increased clusterin levels are in direct linear relationship with Gleason score [17]. Although clusterin expression is low in most untreated hormone-naïve tissues, levels increase significantly within weeks after neo-adjuvant hormone therapy [87]. Preclinical studies have indicated that clusterin suppresses apoptotic cell death in response to androgen withdrawal and chemotherapy, [88, 89]. OGX-011 (OncoGeneX Technologies, Vancouver, British Columbia, Canada) is a second-generation ASO against the human clusterin mRNA. OGX-011 incorporates 2'-O-methoxyethyl modifications to the four bases on either end of the 21-mer

phosphorothioate backbone [89]. Such modifications maintain the improved tissue pharmacokinetic profile and relaxed dosing regimen but preserve the high affinity for target mRNA and the recruitment of RNase H necessary for target degradation.

In a randomized phase II trial, CRPC patients who relapsed at or within 6 months of first-line docetaxel were treated with custirsen in combination with either docetaxel or mitoxantrone in a second-line setting [90]. In both arms, efficacy was reported but the docetaxel/custirsen arm appeared to be superior to the mitoxantrone/custirsen arm with respect to PSA response (40% vs. 27%), pain response (8/12 vs. 6/12), PFS (7.5 months vs. 4.2 months), and safety. Median survival duration had not been reached in both arms at a median follow-up of 13.3 months.

In another phase II randomized study [91], patients were randomly assigned 1:1 to receive docetaxel/prednisone either with (arm A) or without (arm B) OGX-011 640 mg intravenously weekly. The primary end point was the proportion of patients with a prostate-specific antigen (PSA) decline of $\geq 50\%$ from baseline, with the experimental therapy being considered of interest if the proportion of patients with a PSA decline was more than 60%. Secondary end points were objective response rate, progression-free survival (PFS), overall survival (OS), and changes in serum clusterin. Eighty-two patients were accrued, 41 to each arm. OGX-011 adverse effects included rigors and fevers. After cycle 1, median serum clusterin decreased by 26% in arm A and increased by 0.9% in arm B ($P < .001$). PSA declined by $\geq 50\%$ in 58% of patients in arm A and 54% in arm B. Partial response occurred in 19% and 25% of patients in arms A and B, respectively. Median PFS and OS times were 7.3 months (95% CI, 5.3 to 8.8 months) and 23.8 months (95% CI, 16.2 months to not reached), respectively, in arm A and 6.1 months (95% CI, 3.7 to 8.6 months) and 16.9 months (95% CI, 12.8 to 25.8 months), respectively, in arm B. Baseline factors associated with improved OS on exploratory multivariate analysis were an Eastern Cooperative Oncology Group performance status of 0 (hazard ratio [HR], 0.27; 95% CI, 0.14 to 0.51), presence of bone or lymph node metastases only (HR, 0.45; 95% CI, 0.25 to 0.79), and treatment assignment to OGX-011 (HR, 0.50; 95% CI, 0.29 to 0.87). Two phase III trials in first-line and second-line treatment have been announced recently. Primary end point will be pain palliation (second-line) and OS (first-line). Thus, custirsen is a promising candidate for the second-line treatment of CRPC.

8. Immunotherapy

Suitability of vaccine development in prostate cancer: Prostate cancer has features that are suitable for vaccine development such as the following: the rate of disease progression is slow enough to allow for a month-long immune intervention, and then some latency until it is evident; the organ is biologically “dispensable,” providing a theoretical safety margin. There are a variety of response end points—PSA response, time to PSA progression, time to radiologic progression, time to symptomatic progression, or overall survival.

Theoretical susceptibility of the tumor to immune mediated attack is difficult to quantify. Several studies show that tumors modify the capacity of the immune system to attack it. Several intratumoral features show that there is impaired immune attack in peritumoral regions. These mechanisms include class I Human Leukocyte Antigen (HLA) downregulation (corresponding to decreasing susceptibility to CD8 CTL lysis) [92], PD-1 ligand expression [93]. A more indirect effect may be a consequence of local expression of cytokines including vascular endothelial growth factor (VEGF), interleukin 10 (IL-10), tumor

growth factor beta (TGF- β) that induce a tolerogenic phenotype in antigen presenting cells (APC). Other intratumoral escape mechanisms [94] include indoleamine 2,3-dioxygenase [95] and nitric oxide synthetase [96].

8.1 Sipuleucel-T

In April 2010, sipuleucel-T became the first immunotherapeutic agent to be approved by the U.S. Food and Drug Administration for prostate cancer, based on consistent observed improvements in overall survival. Sipuleucel (Provenge, APC8015) contains mature, autologous antigen-presenting cells (APCs). APCs are obtained from the patient via a standard leukapheresis procedure approximately two days before each scheduled infusion. The patient's APCs are co-cultured with a recombinant fusion protein containing prostatic acid phosphatase (PAP). The activated, antigen-loaded APCs are then infused into the patient, where it can potentially stimulate a T cell response against prostate cancer cells. The process is performed three times over the course of a four-week period. The vaccine has been studied in three phase III clinical trials. The first phase III study, D9901, consisting of 127 men with asymptomatic, metastatic CRPC, compared sipuleucel-T every two weeks for three cycles with placebo in a 2:1 ratio [97]. The final three-year follow-up of the D9901 phase III study showed a median survival benefit of 4.5 months and a threefold improvement in survival at 36 months for patients who were randomized to receive Provenge [97]. In another similar phase III trial, D9902, 98 men with asymptomatic, metastatic CRPC demonstrated a 20% improvement in OS for patients randomized to sipuleucel-T. In both studies, the vaccine was well tolerated, and the most common adverse events were fever and chills. The third phase III trial, D9902B, also known as the IMPACT trial (Immunotherapy for Prostate Adenocarcinoma Treatment) was a randomized, double-blind, placebo-controlled study comparing Provenge with placebo in 512 men with CRPC randomized in 2:1 ratio. The results were presented at the 2009 American Urological Association Annual Meeting. The median overall survival favored the vaccine arm with a 4.1-month increase in overall survival for patients treated with sipuleucel-T (25.8 *vs.* 21.7 months; $P = 0.032$). Also, 31.7 percent of sipuleucel-T patients were alive at three years as compared to 23.0% of placebo patients. The 36-month overall survival was 33% in the sipuleucel-T group and 20% in the placebo group [97]. Sipuleucel-T is the first active immunotherapy to demonstrate an improvement in overall survival for advanced prostate cancer. Given the short duration of the therapy (one month) and its favorable benefit-to-risk ratio, sipuleucel-T provides an attractive new option for the management of advanced prostate cancer. The FDA approval was granted to sipuleucel when confirmatory IMPACT trial found a 22.5% improvement in mortality risk compared to placebo (median survival: 25.8 months *vs.* 21.7 months) [98]. Treatment with sipuleucel-T was well tolerated; the most common complications included mild-to-moderate chills, pyrexia, and headaches, which were transient.

8.2 GVAX

Another immunotherapy in development is GVAX (Cell Genesys, San Francisco, California, USA). Unlike Provenge, GVAX is a cell-based gene-transduced multiantigen vaccine. It was developed using two human prostate cancer cell lines, LNCaP and PC-3. The cells in these vaccines are modified to produce granulocyte macrophage-colony stimulating factor (GM-CSF)-stimulating APC [3, 99]. GVAX was developed with a hypothesis that combining

GM-CSF with the prostate cancer-specific antigens would promote synergy and, thus, a stronger cytotoxic response against prostate cancer cells. GM-CSF has already shown modest activity in advanced prostate cancer [3]. After the vaccine is administered, GVAX is recognized as foreign and engulfed by the APC. Subsequently, APC carry these cells to lymph nodes that are recognized as foreign, stimulating antibody production with activation of CD4+ and CD8+ cells. The first phase III trial (VITAL-1) compared GVAX with Docetaxel and prednisone for 6 months [100]. The second phase III trial (VITAL-2) compared GVAX/Docetaxel with Docetaxel/prednisone. VITAL-2 was terminated in August 2008 because of excessive deaths in the GVAX arm [101]. VITAL-1, which completed accrual, was terminated because of futility analysis indicating that there was less than 30% chance of achieving a survival benefit. Thus, the future development of GVAX remains uncertain.

8.3 Gene therapy

Prostate-specific antigen-expressing poxvirus vaccine (PROSTAVAC) is a form of immunotherapy using poxvirus that has been genetically engineered to carry a human PSA gene and has been transformed into the PROSTAVAC vaccine, stimulating the cytotoxic T cells to attack prostate cancer cells. Several phase I trials have demonstrated activity with this vaccine, and it is fairly well tolerated [102]. A phase II trial demonstrated 45.3% of men with CRPC free of PSA progression at 19.1 months, and 78.1% demonstrated clinical PFS. The analysis of antibody titers revealed no significant increases in anti-PSA antibody; however, it did demonstrate an increase in PSA-reactive T cells [103]. Although promising, these results need to be verified in larger phase III randomized trials.

9. Calcitriol

Calcitriol (1,25-dihydroxycholecalciferol) (**Figure 5**) is the hormonal form of vitamin D3. In unphysiologic concentrations, calcitriol has shown antitumor activity in several *in vitro* and *in vivo* models [104]. Furthermore, its antitumoral activity is synergistic in combination with other cytotoxic agents. After a successful phase II trial with an improvement in OS of up to 24.5 months in the experimental arm with docetaxel and calcitriol, a phase III trial was initiated (ASCENT 2) [4]. In a weekly setting, the combination of docetaxel with calcitriol was compared with docetaxel alone. But this trial was abruptly closed due to a higher death rate in the calcitriol arm. Analysis of clinical data that could explain the causes of deaths have not been reported. Due to these findings and the missing analysis of the ASCENT 2 trial, calcitriol cannot be recommended in CRPC after docetaxel failure.

10. Thalidomide

Thalidomide (**Figure 5**), designed in the 1950s of the 20th century, was used as a sedative and antiemetic against sickness in the first trimester of gestation. Unfortunately, it was accountable for more than 10,000 congenital abnormalities and thus it was withdrawn from the market. It has been shown to inhibit angiogenesis-induced by fibroblast growth factor (FGF) and VEGF. Furthermore, it has immunomodulatory functions. Due to the fact that angiogenesis is an important step in metastasis of any cancer, several trials with use of thalidomide were performed. As a single agent it showed modest PSA responses in a range between 15% and 18% [94, 105]. In a multidrug combination (docetaxel/ estramustine

/thalidomide), however, a PSA decline of 90% (18/20) was observed, one of the highest response rates ever seen in such trials [106]. In a phase I trial, only 2/13 (15%) docetaxel pretreated patients showed PSA declines > 50% when receiving thalidomide in combination with oral daily cyclophosphamide [107]. Another phase I/II trial similar to the study mentioned above was performed in pretreated CRPC. Paclitaxel was used in place of docetaxel. 14 of 38 patients had previous taxane therapy; 9 of these 14 patients (64%) had PSA declines > 50% [108]. In another phase II trial, 39 pretreated CRPC patients, most of whom had prior docetaxel (35/39), received thalidomide and daily oral dexamethasone; 26% (10/39) had PSA declines and no signs of radiologic progression [109]. Currently, there is one active trial in pretreated CRPC (thalidomide + doxorubicin). Briefly, we have some evidence that thalidomide has modest effects in taxane pretreated CRPC. However, the clinical data suggests that its effects can be enhanced when it is combined with other cytotoxic agents.

11. Conclusions

The multifaceted problem of CRPC needs a multidisciplinary approach. Many aspects of the disease need to be taken into account when deciding on treatment. Relatively few therapy options exist for patients with prostate cancer that has become resistant to ADT and has metastasized to distant sites. Survival of such patients is poor, with a median survival time of 20 months from the time of initiation of standard docetaxel-based chemotherapy. Over the last decade, our understanding of the pathogenesis of prostate cancer, including the molecular basis of androgen resistance and other regulatory pathways, has been advancing. This advancement has further led to more novel agents that specifically target these molecular pathways in the treatment of CRPC. When prostate cancer progresses following ADT, there are currently few treatment options with only docetaxel shown to prolong life as indicated by TAX-327 and SWOG studies. The introduction of docetaxel for the treatment of CRPC came along with advances in OS and quality of life. Nevertheless, referring to a prolongation of survival of approximately 3 months in a phase III trial, its overall benefit constitutes only a small step in this challenging field.

Approaches in fundamental research are providing us with understanding of more and more the mechanisms of carcinogenesis. As a result of this advancement, the targeted drugs take a major place in the treatment of several cancer entities. The use of a targeted drug as a single-agent often demonstrated only weak or no efficacy. The problem in their use is that tumor cells exhibit plasticity in signaling pathways. Plasticity means that inhibition of one pathway may lead to up-regulation of parallel pathways or that inhibition of an upstream pathway is unable to down-regulate an overactive and uncoupled downstream pathway. Recently, several promising approaches yielded disappointing results in the phase III setting (GVAX, calcitriol); nonetheless, expectations for other agents (Abiraterone, VN/124-1 (TOK-001), Atrasentan, Provenge) still remain high. These agents will need to demonstrate survival benefit for approval. Due to the rapid progress of this field it is beyond the scope of this review to cover all compounds under investigation. However, we have focused on several broad therapeutic categories and selected targets with significant biologic rationale and a reasonable likelihood of success in this review. We sincerely hope that this chapter will add immensely to our knowledge about the current and emerging therapies to fight prostate cancer.

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13. Disclosure of potential conflicts of interest

Professor. Vincent C. O. Njar is a co-inventor on patents and patent applications covering VN/124-1 (TOK-001) and related compounds and serves as consultant for Tokai pharmaceuticals Inc. No writing assistance was utilized in the production of this manuscript.

14. Note added in proof

Abiraterone acetate (ZYTIGA™) was recently (April 28, 2011) approved by the US Food and Drug Administration (FDA) for the treatment of men with metastatic castration-resistant prostate cancer who have received prior chemotherapy containing docetaxel.

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Adjuvant Therapy for Early Breast Cancer

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1. Introduction

Breast cancer is the most common cause of cancer mortality among women worldwide (Key et al., 2001). There is a steady increase in the incidence of breast cancer during reproductive years with a slower rate after the age of 50 (Key et al., 2001; Collaborative Group on Hormonal Factors in Breast Cancer, 1997). Cancer of the breast is a heterogeneous disease with variable response to therapy. The incidence of breast cancer among women in Europe and North America is about 2.7% by age 55, 6.3% by the age of 60, 5.0% by age 65, and about 7.7% by age 75 (Collaborative Group on Hormonal Factors in Breast Cancer, 1997). This incidence is lower in developing countries and in Japan. Risk factors for developing breast cancer include family history, early menarche, nulliparity, oral contraceptives use, late age of first pregnancy, late menopause, hormone replacement therapy, alcohol, obesity, exposure to radiation and genetics including mutations in any of *BRCA1*, *BRCA2*, *P53*, *PTEN*, and *ATM* genes. (Kelsey & Gammon, 1991; Key et al., 2001; Nittin, 1996; Børresen-Dale et al., 2010; Peto et al., 1999; Goldhirsch et al., 2009). Breast-feeding and moderate exercise have been reported to have a protective effect in some studies (Layde et al., 1989; Friedenreich et al., 1998). Tamoxifen and Raloxifen may be used in patients with increased risk for chemoprevention of breast cancer (Key et al., 2001; Visvanathan et al., 2009). A recent study have demonstrated that Exemestane use in postmenopausal women with moderately increased risk for breast cancer have significantly reduced invasive breast cancers after a median follow up of 3 years with no serious toxic effects and only minimal changes in health-related quality of life. Observational and metanalysis studies have shown some beneficial effect of Aspirin and other NSAID use in the prevention of occurrence as well as recurrence of breast cancer (Takkouche et al., 2008; Holmes et al., 2010). Diagnosis of breast cancer is considered early when the disease is detected in the breast only (T1-T3), or in the breast and locoregional lymph nodes (N1) and all detected disease can be removed surgically without spread to distant organs (e.g. bone, liver etc.).

2. Biology of breast cancer

2.1 Molecular pathology

Breast Cancer is a genetic disease. Mammary epithelial cells transform into malignant cells through a complex mechanism that involves a multistage process of sequential events of initiation, promotion, and progression at the genetic and epigenetic levels (Nittin, 1996). Premalignant lesions that increase the risk of malignant transformation include atypical

ductal hyperplasia and hyperplastic alveolar nodules (Nittin, 1996; Dupont & Page, 1985). Tamoxifen use in patients with atypical ductal hyperplasia results in a 75% risk reduction of developing invasive breast cancer (Fisher et al, 2005). Reproductive hormones are thought to influence breast cancer risk through effects on cell proliferation and DNA damage, as well as promotion of breast cancer growth. Estrogen receptor (ER) or progesterone receptor (PR)-positive and Epidermal growth factor 2, *HER-2/neu*, overexpressing tumors account for approximately 75% and 20% of all breast cancer cases, respectively. Half of the *HER-2/neu* overexpressing breast cancers also expressing ER and, or PR receptors. About 15% of breast cancers lack expression of these three proteins and are named as triple-negative breast cancer. The hormone receptors status is used for histopathological classification of breast cancer as well as prediction of response to specific targeted therapeutic agents. Ki-67 is a marker of proliferation that is used in determining prognosis and identification of high-risk patients who may benefit from the addition of chemotherapy. Ki67-labelling index is considered low when it is < 15%, intermediate 15-30% and high when > 30%.

In the normal breast, there are three distinct types of epithelial cells: luminal or glandular cells, basal or myoepithelial cells, and stem cells. There are seven major breast cancer molecular subtypes based on patterns of gene expression and hierarchical clustering. These subtypes are: Luminal A, Luminal B, Luminal C, HER-2-enriched, Basal-like, Claudin low and Normal Breast-like group and they relate loosely to histologic and phenotypic properties and clinical outcomes.

Luminal breast cancer is called as such because of its similarity to expression of luminal breast epithelium. Luminal A tumors have the best prognosis and they make up to 40% of all breast cancers with high expression of ER-related genes, low expression of the *HER-2* cluster of genes, and low expression of proliferation-related genes (Hu et al. 2006; Voduc et al., 2010; Kennecke et al., 2010). These cells have high expression of cytokeratins 8, 18 (Perou et al., 2000). The luminal B tumors are less common and have a lower expression of ER-related genes, variable expression of Her-2 clusters and higher expression of proliferation-related genes (Voduc et al., 2010). Molecularly, a third type known as Luminal C is distinguished from luminal subtypes A and B by its high expression of a novel set of genes with unknown function, which is a feature they share with the basal-like and HER-2 subtypes (Sorlie et al., 2001). The luminal subtype B and C seem to have a worse relapse free survival and overall survival when compared to luminal A (Sorlie et al., 2001).

The *HER-2* enriched subtype is characterized by high expression of *HER-2* and proliferation genes and low expression of luminal clusters. These tumors are usually *HER-2* positive and ER/PR-negative. In the pre-*HER-2* targeted therapy era, these tumors were associated with a poorer prognosis and a higher rate locoregional as well as metastases to the brain, liver, and lung when compared with luminal A tumors. (Voduc et al., 2010; Kennecke et al., 2010)

Basal-like tumors are so called because of their similar expression to basal epithelial cells. They are typically ER/PR and *HER-2/neu* negative (triple negative) due to low expression of the luminal and HER2 gene clusters. There is a strong association between BRCA1 mutation and basal-like breast cancer (Sorlie et al., 2001). These cancers have a wide genomic instability, high expression of the proliferation cluster of genes and they are always of high grade. They also have high expression of the epidermal growth factor receptor (EGFR), p-cadherin, smooth muscle actin, c-kit, cytokeratins 5, 6, 14, and 17 (Maegawa & Tang, 2010; Perou et al 2000; Sorlie et al., 2001). Basal-like tumors are associated with high risk of locoregional relapse and a higher rate of brain, lung and distant nodal metastases with lower rate of liver and bone

metastases (Voduc et al., 2010; Kennecke et al. 2010). Luckily, these cancers are more sensitive to Anthracyclines and Taxanes (Carey et al, 2007; Maegawa & Tang, 2010).

Claudin-low is a relatively new identified subtype of breast cancer that is characterized by the low to absent expression of luminal differentiation markers, high epithelial-to-mesenchymal transition markers, immune response genes and cancer stem cell-like features (Prat et al, 2010). The majority of these tumors are ER/PR and *HER-2/neu* negative (triple negative) invasive ductal carcinomas with a high frequency of metaplastic and medullary differentiation. Their response rate to standard neoadjuvant chemotherapy is intermediate between that of basal-like and luminal tumors (Prat et al, 2010).

Normal breast-like tumors do not fall into any of these specific five types and they show high expression of many genes known to be expressed by adipose tissue and other nonepithelial cell types. These tumors also showed strong expression of basal epithelial genes and low expression of luminal epithelial genes (Sorlie et al., 2001).

Although metastatic breast cancer is beyond the scope of this chapter, it is worth mentioning that discordance in tumor characteristics (i.e., change in receptor status) between a primary breast cancer and sites of recurrence are common and re-biopsy of metastatic disease might be indicated especially if a long time has passed since the first diagnosis.

Other histologic Subtypes of breast cancer include secretory, adenoid cystic, tubular, medullary and lobular carcinomas.

2.2 Screening and testing for breast cancer

Breast cancer screening is performed in women without any signs or symptoms for early detection of breast cancer. A thorough history and detailed clinical breast examination are crucial for early detection and management of breast cancer. Symptoms or positive findings on clinical examination include a palpable lump or mass, asymmetric thickening or nodularity, nipple discharge in the absence of a palpable mass, and skin changes such as erythema, scaling, eczema, peau d'orange skin or nipple excoriation. Mammographic screening has resulted in early detection and decreased mortality from breast cancer (Humphrey et al., 2002). Ultrasonography can be a useful screening adjunct to mammography in select group of women who are young with dense breast tissue (Bever, 2008). Breast cancer screening should be personalized and tailored to the patient age and risk factors. Asymptomatic women without any findings on physical examination should be risk stratified. Accordingly, women are either at normal or increased risk. The National Comprehensive Cancer Center (NCCN) guidelines suggest that the following women are at increased risk of breast cancer: (1) women who have previously received therapeutic thoracic or mantle irradiation; (2) women ≥ 35 years old with a 5-year risk of invasive breast carcinoma $\geq 1.7\%$ using the modified Gail model (3) women with a lifetime risk of breast cancer $> 20\%$ based on models largely dependent on family history; (4) women with a strong family history or genetic predisposition; (5) women with lobular carcinoma in situ (LCIS) or atypical hyperplasia; and (6) women with a prior history of breast cancer (www.nccn.org).

Societies recommend yearly clinical breast exam (CBE) and mammograms starting at age 40 and continuing for as long as a woman is in good health. CBE every 1 to 3 years is recommended for women older than 20 and younger than 40. (Humphrey et al., 2002; Smith et al., 2003).

There is no data to support Breast self-exam (BSE) and the United States Preventive Services Task Force (USPSTF) recommends against it. Instruction in BSE has no effect on reducing breast cancer mortality (Thomas et al., 2001).

The concept of breast self-awareness, where women should know how their breasts normally look and feel-like and report any breast changes promptly to their health care provider, has been introduced over the past several years. Periodic, consistent BSE may facilitate breast self-awareness. Premenopausal women may find BSE most informative when performed at the end of menses. *A paradigm shift from SBE to breast self-awareness has been adopted over the past decade.*

Screening with a MRI in addition to mammograms should be considered for women with a lifetime risk of breast cancer >20% based on models largely dependent on family history, women who are 25 years and older with history of thoracic radiation, strong family history or genetic predisposition (i.e. BRCA mutation or first degree relative of BRCA carrier), and women with Lobular Carcinoma in Situ (LCIS). CBE every 6 to 12 months, breast awareness and risk reduction strategies should be considered in the majority of women with increased risk of breast cancer as defined earlier.

The general principles for performing genetic counselling include: (1) there is a personal or family history suggesting genetic cancer susceptibility (2) the test can be adequately interpreted and (3) the results will aid in the diagnosis or influence the medical or surgical management of the patient or family members at hereditary risk of cancer. (Robson, et al., 2010).

Treatment of early breast cancer has evolved significantly over the past two decades. In the following section, we will introduce you to adjuvant (postoperative) systemic therapy for early breast cancer. The main goal of adjuvant therapy is to reduce recurrence rate, control any potentially remaining cancer deposits and improve survival.

3. Adjuvant therapy of early breast cancer

Breast cancer is considered early when the disease is detected in the breast only or in the breast and its locoregional lymph nodes and all the detected disease can be removed surgically (also called operable breast cancer). Surgical management of early breast cancer is beyond the scope of this chapter. Surgical resection is performed for local control and it removes the majority of the macroscopic disease but it doesn't eradicate local and distant microscopic components. This microscopic disease places the patient at high risk of local and systemic relapse. The goal of adjuvant therapy for early breast cancer is to eradicate any hypothetical occult local or distant disease, hence reduce the risk of recurrence and improve overall survival. Adjuvant systemic treatments for early breast cancer include chemotherapy, endocrine manipulation (endocrine therapy and ovarian ablation or suppression) in hormone receptor-positive tumors, and anti HER2 agents, (e.g. trastuzumab) for HER2-positive tumors. ER-positive early breast cancer is usually treated with the combination of chemotherapy followed by endocrine therapy (e.g. tamoxifen, or Aromatase Inhibitors - AIs) in post-menopausal women (www.nccn.org, 2010; Albani, et al., 2009). Endocrine therapy alone may represent an appropriate treatment for a group of patients who do not have high-risk breast cancer or are unlikely to benefit from chemotherapy.

Clusters of gene expression analysis may offer a better insight into breast cancer subtypes and their phenotypic behavior as well as response to therapy (Perou et al., 2000; Sorlie et al., 2001). Commercially available genomic assays like MammaPrint, Oncotype Dx, Theras, Map Quant Dx, and Mammostrat are increasingly used for the prediction of clinical outcome in patients with breast cancer (Sotiriou & Pusztai, 2009). Although commercially available, these assays have not been tested prospectively yet. These assays use a scoring system to classify patients as low, intermediate or high risk for disease

recurrence using a number of different targets to identify prognosis or predict efficacy of adjuvant therapy. Several studies are exploring prospectively the power of these assays (e.g. the TAILORx trial-Trial Assigning Individualized Options for Treatment (Rx), or MINDACT trials). Until the results become available and if successful, our approach for breast cancer treatment remains broad on the basis of expected effects of different interventions in broad categories of patients.

We are witnessing a paradigm shift that will adopt a patient-tailored approach and individualized therapy for early breast cancer based on the specific tumor genetic signature of the particular cancer.

3.1 Adjuvant endocrine therapy

Adjuvant endocrine therapy is recommended for almost all patients whose breast cancers have any detectable estrogen receptors (Goldhirsch et al., 2009). Endocrine therapy includes the use of Selective Estrogen Receptor Modulators (SERMs) and AIs. Optimal endocrine therapy in premenopausal women with early breast cancer remains an area of controversy although tamoxifen has been adopted as a standard therapy for decades and incorporated in treatment guidelines. The benefit of adding ovarian ablation (surgically or chemically with LHRH agonists) to tamoxifen therapy remains an area of investigation. A recent meta-analysis of retrospective data indicates that each of the possible endocrine interventions has an equal role and combinations do not improve long-term outcomes. TEXT, SOFT and PERCHE trials will hopefully shed some additional light and give the treating oncologist more guidance which therapy to choose. Even the duration of tamoxifen use remains somewhat arbitrary. Studies indicate that using tamoxifen beyond 5 years might actually reduce recurrences but some of the long-term side effects might outweigh the benefits.

For *postmenopausal* women, third-generation aromatase inhibitors are the accepted standard to all women with ER-positive cancers. For patients already on tamoxifen, switching to an aromatase inhibitor after 2 or 3 years should be considered in patients who did not experience a recurrent disease (Goldhirsch et al., 2009). There is substantial evidence to suggest that sequential chemoendocrine combination would approximately halve the average annual death rate from breast cancer during the first 15 years after diagnosis. (Early Breast Cancer Trialists Collaborative Group, 1998, 2005). The percentage of hormone receptor positive cells is a strong predictor of response to endocrine therapy both in the adjuvant and the metastatic settings (Regan et al., 2006). About 60% of breast cancers arising in premenopausal women and 80 % of those arising in postmenopausal women are ER or Progesterone receptor positive. Adjuvant endocrine therapy alone is usually advocated for breast cancers that are ER/PR positive, ≤ 2 cm, has minimal peritumoral vascular invasion, $Ki-67 \leq 15\%$ (low proliferation index), node negative, grade I histology, and low multigene assay score (Goldhirsch et al., 2009; Montemurro & Aglietta, 2009). In the following section, we will describe the available hormonal therapies that are approved in the treatment of ER-positive breast cancer.

3.1.1 Tamoxifen

Tamoxifen is (Z)-2-[4-(1,2-diphenylbut-1-enyl) phenoxy]-N, N-dimethylethanamine that is one of the selective estrogen receptor modulators (SERMs) and act as both an antagonist and a partial agonist of the estrogen receptor (Furr & Jordan, 1984). Adjuvant Tamoxifen therapy

for 5 years in women with ER positive breast cancer significantly reduces the annual recurrence, as well as breast cancer mortality in early breast cancer during the period of Tamoxifen use. (Early Breast Cancer Trialists Collaborative Group, 1998). The reduced risk of recurrence that is noticed with Tamoxifen use is solely dependent on the ER and not the PR status. (Early Breast Cancer Trialists Collaborative Group, 2005). The evidence suggests a protective carryover effect in reducing the risk of recurrence over the next few years and up to 15 years from starting Tamoxifen. Risk reduction after 5 years of Tamoxifen therapy is similar for younger and older women; however it is significantly greater for those with node positive disease in comparison to women with node-negative disease. (Early Breast Cancer Trialists Collaborative Group, 1998). The risk of thromboembolic disease and uterine cancer in women who took tamoxifen for 5 years is 0.2% per decade and is considered small in comparison to the absolute 10-year reductions in breast cancer mortality. On the other hand, tamoxifen may have a protective effect against heart disease. Tamoxifen resistance can potentially develop due to exaggerated agonist activity with potential impairment of its antitumor activity. Tamoxifen use for more than 5 years seems to be more effective than 5 years but at a price of much higher side effect profile and the standard recommendation remains for 5 years. (Early Breast Cancer Trialists Collaborative Group, 2005; Fisher et al., 1994)

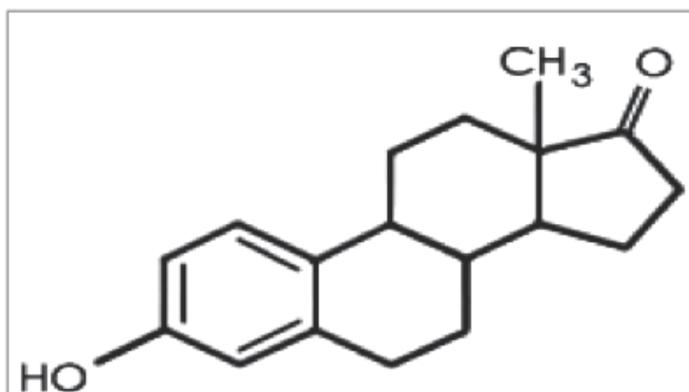


Fig. 1. Estrogen Chemical structure ($C_{18}H_{24}O_2$).

3.1.2 Aromatase inhibitors

In postmenopausal women, estrogen ($C_{18}H_{24}O_2$) is produced in multiple extragonadal sites that include adipose tissues, osteoblasts, chondrocytes, vascular endothelium and smooth muscle cells, adrenal gland and numerous sites in the the brain. (Figures 1 and 2) Breast adipose tissue is not an exception for this synthesis and local production of Estrogen plays an important role in breast cancer microenvironment. These tissues usually produce Estrogen for their local use (as a paracrine or intracrine factor), which results in higher tissue concentration of Estrogen. This Estrogen can also escape metabolism and enter the circulation. Postmenopausal women have a higher level of circulating testosterone ($C_{19}H_{28}O_2$) in comparison to Estradiol. (Simpson, 2003). Extragonadal tissues are dependent on external source of C_{19} androgenic precursors, since these tissues are incapable of converting cholesterol to the C_{19} steroid. Those C_{19} precursors provide a substrate for estrogen biosynthesis in these sites.

Aromatase is a cytochrome P450 enzyme that is involved in the conversion of C_{19} androgens to aromatic C_{18} estrogens (figure 2.), primarily in the ovary, and adrenal gland in females in addition to the testes in males (Santen et al., 2009; Simpson, 2003). Aromatase was recognized as a therapeutic target for the treatment of hormone-dependent breast cancer approximately 40 years ago (Santen et al., 2009). Compounds that inhibit aromatase decrease estrogen levels by affecting a key component of the production pathway, aromatase cytochrome P450. The aromatase cytochrome P450 enzyme is also active in peripheral tissues (fat, muscle, liver, and both epithelial and stromal breast cells).

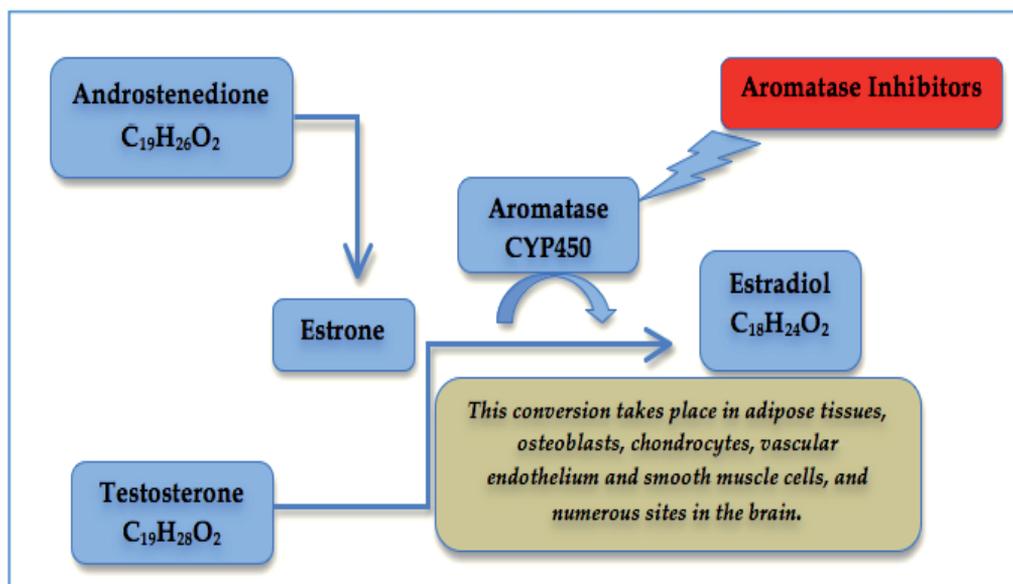


Fig. 2. Mechanism of action of Aromatase Inhibitors.

AIs are the standard of endocrine therapy for postmenopausal women with receptor-positive early, and metastatic Breast Cancer. AIs alone are contraindicated in premenopausal patients because if used alone, they do not suppress estrogen production adequately in women who are still ovulating possibly because of the interruption of the estradiol negative feedback and subsequent rise in luteinizing and follicle-stimulating hormones (Santen et al., 2009). In cases where tamoxifen is contraindicated, AIs may be administered to premenopausal women together with ovarian function suppression. In certain postmenopausal women, e.g. very low risk of recurrence, tamoxifen alone can be considered adequate.

Aminoglutethimide is considered the first generation of AIs. It is a non-selective inhibitor of adrenal steroidogenesis as well as thyroidal organification of iodine. Compared with the antiestrogen tamoxifen, aminoglutethimide had similar clinical efficacy but a higher incidence of adverse events; therefore, tamoxifen became the standard hormonal therapy for breast cancer (Harvey et al., 1982; Lipton et al., 1982; Smith et al., 1982).

More selective AIs that exhibit competitive, reversible, and/or mechanism-based inhibition were developed. The mechanism-based inhibitors produce a long lasting inhibition through their high specificity for the active enzyme site with less toxicity in comparison to with other

competitive inhibitors (Brueggemeier, 1994; Santen et al., 2009). Mechanism-based inhibitors include the steroidal AI, exemestane, and the nonsteroidal AIs, letrozole and anastrozole. Anastrozole and letrozole are reversible inhibitors, whereas exemestane is an irreversible inhibitor.

Several clinical trials evaluating adjuvant therapy in early breast cancer have demonstrated improved disease-free survival (DFS) in postmenopausal women with the use of anastrozole, exemestane, or letrozole in comparison to tamoxifen (Boccardo et al., 2005; Coombes et al., 2007; Cuzick et al., 2010; Goss et al., 2005; Jakesz et al., 2005; Kaufmann et al., 2007; Mamounas et al., 2008).

AIs have been tested in the adjuvant settings in three different scenarios: initial treatment versus tamoxifen; sequential treatment after 2–3 years of tamoxifen to finish the duration of therapy for 5 years; and extended treatment after 5 years of tamoxifen. It is controversial, as of the best timing of AI use in the treatment program after surgery. Neither the optimal timing nor the duration of therapy is well established. Some studies suggest that ER+/PgR-negative patients respond better to upfront strategy of AI. For ER+/PgR+ positive tumors, there is more uncertainty, but initial use of an AI, for say 3 years, may still be better than sequencing it after 2 years of tamoxifen, as this is the period with highest recurrence rates. An upfront strategy may be favored over sequential or extended therapy. Generally speaking, AIs have a more tolerable side effect profile than tamoxifen, with lesser incidence of thromboembolic complications, fewer hot flashes and gynecologic symptoms, but more arthralgias, myalgias and have an increased risk of osteoporosis due to its primary effect in reducing estrogen levels. The combination of Tamoxifen and AIs is not additive and leads to more side effects (Cuzick et al., 2010).

3.2 Ovarian ablation or suppression

Ovarian ablation in premenopausal women is achieved by surgery or irradiation and ovarian suppression is accomplished by treatment with a luteinizing-hormone releasing-hormone agonists. There is evidence to suggest a definite effect of ovarian ablation or suppression both on recurrence and mortality from breast cancer in women < 50 years of age. (Early Breast Cancer Trialists Collaborative Group, 2005). Although tamoxifen plus ovarian function suppression is an accepted standard of endocrine therapies in premenopausal women (Goldhirsch et al., 2009), the addition of LHRH agonist to tamoxifen in this group of patients does not seem to decrease the probability of recurrence or death (Cuzick et al., 2007). In premenopausal women where tamoxifen is contraindicated, AIs with ovarian function suppression are now being tested in 3 randomized prospective clinical trials.

3.3 Chemotherapy

Defining the threshold for the use of cytotoxic chemotherapy is a difficult task: most RCT were performed in patients with early breast cancer who were selected on the basis of their pathological characteristics and anatomical staging, (e.g. large tumors or lymph node (LN) positive early breast cancer). The optimal duration or number of cycles to be given is not well established. Some of the factors used in decision making of chemotherapy use, include tumor size ($pT > 5$ cm), high histologic grade, high proliferation index ($Ki-67 \geq 10$), vascular invasion, low expression of steroid hormone receptors, number of lymph nodes involved (≥ 4) and high multigene assay score. Ongoing research using genomic profiling assays (e.g.

Oncotype, MammaPrint, etc.) will add to the decision making process and identify patients who may or may not benefit from systemic cytotoxic therapy. Chemotherapy is the mainstay of adjuvant treatment of patients with triple-negative disease who are at sufficient risk of relapse to justify its utilization. Studies have showed a 33% risk reduction of recurrence as well as 26% risk reduction in mortality by using any kind of polychemotherapy in women aged 50 to 69 with ER-negative tumors. The respective reductions for patients with ER-positive tumors receiving tamoxifen were 15% for recurrence and 11% for mortality (Early Breast Cancer Trialists Collaborative Group, 2005). Patients with small primary tumors (pT1a pN0 and ER negative) might be spared adjuvant systemic therapy since the probability of recurrence is low and the potential benefit is negligible. In breast tumors that overexpress the HER-2 receptor protein (see section below), treatment with the monoclonal antibody trastuzumab, in addition to cytotoxic regimen provides incremental and significant benefits.

Identifying patients who would benefit from adjuvant chemotherapy in early breast cancer is a subject of debate. To optimize treatment of early breast cancer, it is important to understand the available chemotherapeutic agents and the advantage of choosing one over the other. There is a broad spectrum of chemotherapeutic agents available for the treatment of breast cancer and many regimens are nearly universal today. The first RCT by Bonadonna selected patients with LN positive early breast cancer after mastectomy and patients were randomized to receive chemotherapy or not. Cyclophosphamide is an alkylating agent that is used in combination with two antimetabolites, methotrexate and fluorouracil (CMF), in the treatment of early breast cancer. This combination was introduced into the management of early breast cancer in the mid-1970s and was the standard of care for some time (Bonadonna et al., 1976).

Over the last forty years, the addition of anthracyclines has been adopted as an integral part of most standard regimens. These agents inhibit tumor cell proliferation and gene expression by directly interacting with the DNA leading to the production of free radicals that destroy tumor cells. The most commonly used anthracyclines in breast cancer treatment are Doxorubicin and Epirubicin.

Adding doxorubicin to cyclophosphamide (AC combination) in the treatment of early breast cancer, proved to have equivalent efficacy to, as well as substantial advantages over, CMF in terms of tolerability, ease of administration and duration. Four cycles of AC was found to be equivalent to six cycles of CMF with respect to event-free survival, relapse-free survival (RFS), and overall survival (OS) in breast cancer patients regardless of nodal status, age, or estrogen-receptor (ER) status, but that AC offered the advantages of a shorter treatment course with fewer side effects (Fisher et al., 2001).

Cyclophosphamide, doxorubicin, fluorouracil (CAF) regimen have also shown similar efficacy in terms of OS when compare to CMF (Carpenter et al., 1994; Martin et al., 2003). In the subgroup of high-risk lymph node-negative patients, FAC use is associated with longer DFS and OS in comparison to CMF (Martin et al., 2003). Cyclophosphamide, Epirubicin, Fluorouracil (CEF) regimen has showed improvement in OS when compared to CMF. This benefit is true for women with node-positive disease (Levine et al., 2005; Bonnetterre et al., 2005).

Anthracyclines remain important agents in adjuvant treatment and are indicated for adjuvant therapy regardless of the extent of nodal involvement, hormone receptor status, or HER-2 expression level. Anthracycline containing regimens are more effective at preventing

recurrence and increasing survival than CMF (Cyclophosphamide, Methotrexate and 5-FU) regimens (Early Breast Cancer Trialists Collaborative Group, 2005). This is true for the major subsets of early breast cancer patients including premenopausal (age <50) and postmenopausal (age, 50–69) patients, ER-poor and ER-positive patients, and both node-negative and node-positive patients. There is a positive correlation between delivery of the intended doses of chemotherapy on schedule and better treatment outcomes in breast cancer (Bonadonna et al., 1995; Budman et al., 1998). The optimal dose intensity in combination chemotherapy is a function of both the dose level and schedule. Minimizing the interval between cycles allows delivery of dose-dense chemotherapy (Glück, 2005).

It is believed that breast cancer growth follows Gompertzian kinetics and that shorter intervals between chemotherapy treatments allows dose-dense delivery and may result in a higher log-kill, thus leading to lower relapse rates and longer survival times (Citron et al., 2003; Norton, 1988). The use of growth factors (e.g., G-CSF) has enabled patients to tolerate dose dense chemotherapy by decreasing hematologic toxicities. Dose density and dose intensity are important part of the adjuvant treatment of early breast cancer, especially in women with node positive breast cancer.

Taxanes (docetaxel and paclitaxel) have added further survival benefit in the adjuvant treatment of early breast cancer regardless of hormone receptor status (Nowak et al., 2004; Martin et al., 2005; De Laurentiis et al., 2008). Studies comparing docetaxel, doxorubicin and cyclophosphamide (TAC) versus conventional fluorouracil, doxorubicin, and cyclophosphamide (FAC) in women with node-positive breast cancer showed a survival advantage favoring TAC (Martin et al., 2005, 2010). In addition, women with high-risk node negative disease, TAC was associated with improved rate of disease-free survival in comparison to FAC (Martin, 2010).

Sequential use of FEC followed by docetaxel (FEC-T) produced a significant DFS and OS in women 50-65 years of age with node-positive breast cancer. This regimen has become the standard of care in this age group and is considered a relatively well-tolerated regimen that contains anthracycline and taxane components. Women under the age of 50 years who received this combination did not gain a survival benefit (Roche et al., 2006).

Some studies have suggested that overexpression of HER-2 may correlate with greater sensitivity to anthracyclines. Thus the combination of trastuzumab (monoclonal antibody that inhibits signaling by HER-2 receptor) and an anthracycline-containing regimen for HER-2 positive breast cancer may confer an additional benefit in this disease subset (Paik et al., 2000; Muss et al., 1994).

Capecitabine has recently been tested in the adjuvant setting of early breast cancer. In women with medium- to high-risk early breast cancer, there was no difference in recurrence-free survival between docetaxel, capecitabine, cyclophosphamide, epirubicin (TX-CEX) versus docetaxel, cyclophosphamide, epirubicin, 5-fluorouracil (T-CEF); however, the addition of capecitabine may benefit a specific subgroup of patients with > 3 axillary metastases or triple-negative breast cancers. The use of TX-CEX was associated with higher discontinuation rate due to toxicity (Joensuu et al., 2010). Capecitabine is still being investigated and it has not become as part of the standard therapy of early breast cancer yet.

3.4 Targeted therapy with anti HER 2

Trastuzumab is a humanized hybrid monoclonal antibody that selectively binds to the extracellular domain of HER-2. Its anti tumor function is not well understood but it may

induce apoptosis, and also cause an antibody-dependent cell-mediated cytotoxicity. Trastuzumab has become an important component of breast cancer therapy regimens in metastatic as well as neoadjuvant settings of HER-2 expressing breast cancers (Salmon et al., 2001; Arteaga, 2003; Buzdar et al., 2005; Glück 2009; Dominici et al., 2010). Several large randomized clinical trials of high-risk patients with HER-2-positive early breast cancer have demonstrated that trastuzumab provides additional beneficial effects when used subsequent to anthracycline based chemotherapy and a taxane. One study also identified a non-anthracycline combination that seems to be equally effective without the cardio toxicity that is associated with anthracyclines plus trastuzumab. Several clinical trials have suggested that the addition of trastuzumab to standard chemotherapy may reduce the recurrence rate by approximately 50%. The standard duration of trastuzumab therapy is 1 year, although ongoing clinical trials are testing a shorter and longer duration of therapy.

Several large randomized clinical trials of high-risk patients with HER-2-positive early breast cancer have demonstrated that trastuzumab provides additional beneficial effects when used subsequent to anthracycline-based chemotherapy and a taxane. The National Surgical Adjuvant Breast and Bowel Project protocol B-31 (NSABP-B31) and the North Central Cancer Treatment Group (NCCTG) N9831 adjuvant trials were designed to compare doxorubicin-based chemotherapy followed by paclitaxel (AC→T) with AC→T plus trastuzumab either in sequence or concurrent with paclitaxel. Preliminary efficacy findings from a combined analysis of those trials after a median follow-up of 2.9 years showed more than 50% relative reduction in the risk for recurrence, with significant reductions in risk both in terms of DFS and OS with AC→T plus trastuzumab compared with AC→T (Perez et al., 2007). Doxorubicin-based chemotherapy (AC) followed by paclitaxel plus trastuzumab either in sequence or concurrent with paclitaxel reduces the risk of breast recurrence by half in addition to significant improvement in DFS and OS (Romond et al., 2005).

The trastuzumab Adjuvant (HERA) trial randomly assigned patients with HER-2-positive invasive breast cancer to receive either trastuzumab for 1 or 2 years or observation, with a primary end point of DFS; patients were previously treated with surgery and adjuvant or neoadjuvant chemotherapy (Martine et al, 2005). Unlike the NSABP-B31 and N9831 trials, most patients in the HERA trial did not receive a taxane, and about 30% of patients were node-negative. Median follow-up at 2 year after randomization demonstrated a significant improvement in disease-free survival (DFS) and overall survival (OS) in trastuzumab-treated patients, compared with observation (Smith, I et al., 2007).

The breast cancer international research group trial (BCIRG006) compared AC→T (Docetaxel) with AC→TH (Docetaxel, Trastuzumab) and with TCH (Docetaxel, Carboplatin, Herceptin) in the adjuvant treatment of HER2-amplified early breast cancer. Trastuzumab was found to provide a similar and significant advantage for both DFS and OS when used with either anthracycline-based (AC → TH) or non-anthracycline (TCH) chemotherapy in both high and low-risk patients. TCH seems to have a better side effect profile in comparison of AC→TH (Robert et al., 2007)

The FINHER trial was originally designed to compare treatment-using docetaxel versus vinorelbine in early breast cancer. The patients were randomized to three cycles of docetaxel or vinorelbine followed by three cycles of fluorouracil, epirubicin, and cyclophosphamide. The subset of patients with HER-2-positive cancers was further randomized to either receive or not receive trastuzumab for 9 weeks together with the first three cycles of docetaxel or vinorelbine. Within this subgroup, DFS was significantly better among those who received trastuzumab and there was a trend toward better OS (Joensuu et al., 2006).

The findings of the NSABP-B31, NCCTG-N9831, HERA, BCIRG006 and FINHER trials have established that the addition of trastuzumab to anthracycline-based chemotherapy, either with or without a taxane, may reduce the recurrence rate by approximately 50%. Trastuzumab has become the standard backbone to chemotherapy in treating patients who have HER-2-overexpressing breast cancers.

New compounds that target HER2 are in development; Lapatinib is a dual erbB 1 and 2 tyrosine kinase inhibitor that blocks HER-2 receptor and has an antiproliferative effect. Lapatinib has been approved by the FDA for the use in metastatic breast cancer; these compounds are now under clinical investigation in early breast cancer. Dual HER2 blockade using trastuzumab and lapatinib to overcome resistance is a concept under investigation for treatment of HER2-positive early breast cancer (Abramson & Arteaga, 2011). No data are available in the adjuvant setting at present time. A recent clinical trial in women with HER2-positive primary breast cancer, neoadjuvant lapatinib plus trastuzumab given with paclitaxel was associated with a significant improvement in pathologic CR (pCR) rate versus trastuzumab or lapatinib with paclitaxel alone (Baselga et al., 2010).

3.5 Radiation therapy

Sequencing of radiotherapy in relation to chemotherapy in early breast cancer is a subject of debate and investigation. Synchronous (using CMF based chemotherapy) versus sequential chemotherapy and radiotherapy is feasible but has no advantage in reducing the risk of locoregional recurrence but it shortens the duration of adjuvant therapy.

4. Summary and conclusion

Breast cancer is the most common diagnosed malignancy in the western world and increasingly in the developing countries;. Early or operable breast cancer is a disease that involves the breast only or the breast and its locoregional lymph nodes. Histo-pathological diagnosis, Estrogen, Progesterone and HER-2 receptor status are important markers for prognosis and decision making in choosing the appropriate adjuvant therapy after successful surgical removal of the primary cancer. Modern molecular assays are utilizing the fact that breast cancer is a genetic and heterogeneous disease: these tests have the potential to not only better give the prognosis but also be used as predictive tests to identify effective therapeutic regimen and spare the patient unnecessary and potentially toxic treatment. As standard of practice, estrogen receptor positive cancers should be treated with hormonal therapy; the use of chemotherapy is driven by the risk of recurrence and must be carefully brought into context with its toxicity. HER-2 positive cancers are high-risk cancers regardless of size or ER status and should almost always be treated with trastuzumab in addition to chemotherapy. Anthracyclines and Taxanes containing cytotoxic combinations, as integral components of most regimens, are accepted treatment standards.

Adjuvant systemic therapy has changed the outcome of early breast cancer substantially over the last decades. Newer compounds and better understanding of the available hormonal, targeted and chemotherapeutic agents will further improve our success in treating early breast cancers.

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Part 3

New Modalities of Cancer Therapy

Harnessing the Immune System to Fight Cancer: The Promise of Genetic Cancer Vaccines

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1. Introduction

In spite of significant progress in recent years towards the development of new targeted therapies Cancer is still a largely unmet medical need and the leading cause of death in industrialized countries (Globocan Project, 2008). Cancer is continuously increasing and is associated with a variety of factors, including genetic predisposition, infectious agents, exposure to mutagens, as well as lifestyle factors (Minamoto et al, 1999). Cancer is linked to the occurrence of genetic and epigenetic changes (Heng et al, 2010) and indeed tumour cells harbor hundreds of these modifications as also witnessed by the recent results of genome wide analyses of cancer genomes (Sastre, 2011). This feature of cancer cells implies that they can be recognized as foreign entities and eliminated by our immune system, and is at the basis of the theory of immunosurveillance (Dunn et al, 2004).

Several studies have shown that it is possible to establish clear correlates between the nature, density and location of immune cells within distinct tumour regions and the risk of disease relapse (reviewed in Mleknic et al, 2011). Compelling data have recently led to propose that an immune classification of patients, based on the density and the immune location within the tumour may have a prognostic value even superior to the standard TNM classification (Bindea et al, 2011; Fridman et al, 2011). In recent years a better knowledge of the immune system has led to an evolution of the initial concept of immunosurveillance into a more articulated theory of immunoediting (Schreiber et al, 2011). Cancer immunoediting acts as an intrinsic tumour suppressor mechanism that engages after cellular transformation has occurred and intrinsic tumour suppressor mechanisms have failed. One can envisage the existence of three sequential steps during clinical tumour evolution: elimination, equilibrium, and escape. In the first step, innate and adaptive immunity are capable of destroying transformed cells before they give rise to tumour masses. If this process is maximally efficient, then the host remains tumour free. If, however, cancer cell variants are not destroyed, they can enter into an equilibrium phase, in which their outgrowth is held in check by immunological mechanisms, which are principally due to the activation of IL-12/IFN γ -dependent adaptive immunity, mainly driven by antigen-specific CD8⁺ and CD4⁺

T cells. Equilibrium may still represent the end stage of the process and may restrain outgrowth of occult cancers for the lifetime of the host. However, as a consequence of constant immune selection pressure placed by the host on genetically/epigenetically unstable tumour cells, cancerous cells that are no longer recognized by adaptive immunity may emerge, become insensitive to immune effectors mechanisms and in addition they can induce the creation of an immunosuppressive environment. When tumour cells enter the escape phase in which their growth is no longer blocked by immunity, equilibrium is lost and disease becomes apparent. Re-establishing this equilibrium is the realistic goal of cancer immunotherapy.

In spite of being the object of intensive efforts over the past decades, Cancer Immunotherapy has seen many more clinical failures than successes. However, very recently major breakthroughs have been achieved, and these have led us to believe that this approach may become an established platform for the therapy of cancer within the next decade. One can envisage three distinct avenues for Cancer immunotherapy: a) Adoptive Cell Therapy (ACT); b) systemic immune-modulators; c) therapeutic cancer vaccines. ACT is based upon the possibility to isolate, *in vitro* expand and re-inject in immunodepleted hosts, tumour-specific T cells. This approach has seen its best demonstration in the treatment of patients with advanced metastatic melanoma. Superb clinical results have been obtained with objective response rates of up to 49-72% and disease control in some cases lasting several years (Rosenberg and Dudley, 2009). Although evolution of this approach such as genetic modification of T cells to redirect their effector cell specificity may open up to broader applications (Morgan et al, 2010), this strategy has several limitations that currently limit its wide applicability: it is patient specific, very expensive, requires hospitalization and can only be executed in highly specialized clinical centers. In contrast, systemic immunomodulators such as monoclonal antibodies against CTLA-4 or PD-1/PD-L1, do not suffer the manufacturing and delivery problems shown by ACT. On March 2011, FDA approved Ipilimumab (Yervoy® - BMS) (Culver et al, 2011), a human monoclonal antibody against CTLA-4 for the treatment of metastatic melanoma, based on the results of a randomized, controlled Phase III, where Ipilimumab showed statistically increased overall survival compared with controls (Hodi et al, 2010). Although the clinical development of anti PD-1 antibodies is at an earlier stage as compared to anti CTLA-4, results are highly promising both for efficacy and tolerability (Kline and Gajewski, 2010). Finally cancer vaccines recently gained increased visibility due to the demonstration that Sipuleucel-T, a immune cell vaccine for the treatment of hormone refractory prostate cancer, is capable of increasing overall survival of cancer patients (Kantoff et al, 2010). These results led to FDA approval as Provenge® (Dendreon) in year 2010 (Cheever and Higano, 2011). This recent approval has acted as a strong injection of enthusiasm in an area that has long suffered major setbacks.

In this review we will focus mainly on recent developments for therapeutic cancer vaccines and will not discuss in detail ACT and systemic immunomodulators (Klebanoff et al, 2011). Major emphasis will be given to aspects that are critical to increase vaccine immunogenicity and probability of success in the clinic. We believe these are mainly: a) efficient vaccine delivery systems, b) development of response biomarkers, c) modified clinical endpoints and d) combinatorial treatments with chemotherapy or other agents. In analyzing vaccine delivery systems a greater attention will be given to genetic vaccines which we believe represent the most promising methods to elicit immune responses against a wide variety of tumour antigens

especially when administered in combined immunization protocols (heterologous prime/boost). We invite the reader to other recent excellent reviews for aspects of tumour immunology and cancer immunotherapy that we may have missed in our work (Steer et al, 2010; Klebanoff et al, 2011; Palucka et al, 2011; Vergati et al, 2010; Aldrich et al, 2010).

2. Tumour immunology

Our immune system has the intrinsic capability of recognizing tumour cells as foreign entities and to mount responses capable of impacting upon disease evolution. In this section of the chapter we review the main evidences for this spontaneous response, what are the targets of this response, which are the principal components of the immune system involved and what is curtailing this response leading to tumour escape and lack of control of the immune system over cancer.

2.1 Immunosurveillance and Immunoediting

The key studies that unequivocally demonstrated the role of the immune system in the control of cancer development date back to about a decade ago when mouse models of immunodeficiency on pure genetic backgrounds became available. These studies showed that interferon- γ (IFN- γ) is a key factor responsible for the immunological rejection of transplanted tumour cells (Dighe et al, 1994). Furthermore, mice lacking IFN- γ response (either as a consequence of IFN- γ receptor or STAT1 inactivating mutations) or adaptive immunity as a whole (RAG2 $-/-$ deficient mice) are more susceptible to carcinogen induced or spontaneous tumours (Kaplan et al, 1998; Shankaran et al, 2001, Street et al, 2002). These evidences collectively demonstrated that the immune system can function as an extrinsic tumour suppressor. However, as mentioned in the introduction section, a new emerging concept in cancer immunology is that the immune system is not simply a component that protects the host against tumour development, but rather an agent that shapes tumour quality. In other words, tumours that develop in an immunocompetent organism are the resultant of a selection process imposed by the host and by the type of immune response that the host immune system is capable to mount. This concept was originated by pivotal studies that demonstrated that tumours developing in immunocompetent mice have a different molecular profile, are less immunogenic than tumours developing in immunodeficient hosts and progress more rapidly when implanted into naïve wt recipient mice (Dunn et al 2002).

Although both natural and acquired immunity are required to fully exert this control mechanism, the principal contribution comes from adaptive immunity and in particular from the development of tumour-antigen-specific T cells, mainly CD8⁺, but also CD4⁺. Indeed the fundamental principle of cancer immunology is that tumour cells express antigens (TAAs – tumour associated antigens) that differentiate them from normal cells. The existence of tumour antigens has been abundantly demonstrated both in mouse and human studies (Novellino et al, 2005). In the case of human cancers, identification of tumour antigens was made possible *via* the development of methods that used as probes antibodies and CD8⁺ T cells derived from patients and capable of reacting with the autologous tumours (Sahin et al, 1997; Coulie et al, 1997). In the next section we will describe in more detail the types and nature of TAAs under study.

What is happening in the tumour cells that makes them “invisible” or “poorly visible” to the immune system? Certainly the most common mechanism is believed to be loss of tumour

antigen expression, which can occur in at least three possible ways: a) downmodulation of tumour antigen gene expression consequent mainly to epigenetic changes; b) downregulation of MHC class I protein expression and antigen presentation to the cell surface; c) alterations in tumour cells of the machinery responsible for antigen processing and peptide loading onto MHC molecules. In addition to this, it has to be taken into account that tumour cells develop mechanisms of resistance to apoptosis and to the cytotoxic effects of immunity through, for instance, upregulation of anti-apoptotic BCL-2 proteins or activation of transcription factors such as STAT-3. All these processes are strongly favoured by the genetic/epigenetic instability intrinsic of tumour cells, which in the presence of a continuous selection favors the emergence of “immune stealth” clones.

If we analyze in detail the three phases of immunosurveillance/immunoediting, namely Elimination, Equilibrium and Escape, the phase where we have more direct proof of the activity of the immune system is the Equilibrium phase. This phase can represent a type of tumour dormancy where growth of tumour cells is kept at bay for a long period of time, even for the entire life of an organism. Strong evidence for this phenomenon first came when immunocompetent mice treated with low dose carcinogens such as methylcholantrene, were shown to harbor occult cancers for an extended period of time (Koebel et al, 2007). Intriguingly, when these mice were subjected to treatments that selectively affected adaptive immunity, but not innate immunity, tumours rapidly developed, thus demonstrating that equilibrium is established only when a Tumour Antigen Specific CD8⁺ and CD4⁺ response has occurred. This may explain the clinical findings of aggressive tumour arising in organs from a donor apparently cured from cancer, when transplanted into a patient (MacKie et al, 2003).

Although studies of tumour development in mice served as the main driver for the formulation of the cancer immunosurveillance/immunoediting hypothesis, strong demonstration has also been obtained in humans by three different types of evidence. As mentioned before, the first is the demonstration that cancer patients develop detectable levels of antibodies and T cell responses to tumour antigens (Dougan and Dranoff, 2009). The second one is that patients affected by immunodeficiencies are at higher risk of developing cancers (Dunn et al, 2002). The third and strongest one is that intratumoural infiltration by cells of the immune system correlates with disease evolution. In this respect several studies have shown that the quantity, quality, and spatial distribution of tumour infiltrating lymphocytes correlate with patients survival. In fact, tumour infiltration by IFN- γ producing CD8⁺ and CD4⁺ T cells has been associated with an improved prognosis for patients with several different cancer types, including melanoma (Clemente et al, 1996; van Houdt, 1998), colorectal cancer (Chiba et al, 2004) and ovarian cancer (Nelson, 2008). More recent studies in colorectal cancer have extended these findings and have shown, through a global analysis of the tumour microenvironment from both a morphological standpoint and from a system biology approach, that the nature, functional orientation, density and location of cells of the adaptive immune system within distinct tumour regions influence the risk of relapse (Mlecnik et al, 2011). The same authors have come to the conclusion that the density and the immune cell location within the tumour may have a prognostic value superior to the standard TNM classification, and that tumour spread is statistically dependent upon the extent of the host-immune reaction (Bindea et al, 2011).

2.2 Tumour associated antigens (TAAs)

In the past years, several TAAs have been identified having unique expression patterns or being overexpressed by cancer cells. These antigens, under appropriate conditions, can be

recognized by components of the immune system (Campi et al, 2003; Frenoy et al, 1987; Kawashima et al, 1998). Therefore, many current vaccination strategies are designed to induce antibody as well as cell-mediated immune responses against the antigen of interest. A high number of TAAs has been discovered and evaluated in pre-clinical and clinical studies with different results. A list of well-known TAAs subdivided in four main categories is provided in Table 1. Among the most studied and validated TAAs, vaccinations against CEA (Marshall et al, 2003), HER-2/*neu* (Shumway et al, 2009), TERT (Vonderheide, 2008), EpCAM (Chaudry et al., 2007), survivin (Andersen and Thor, 2002), prostate-specific antigens (Doehn et al., 2008) provided good immunologic results. In light of the increasing interest and potential for cancer immunotherapy, the National Cancer Institute recently conducted an interesting pilot project to prioritize cancer antigens and to develop a priority-ranked list of cancer vaccine target antigens based on predefined and pre-weighted objective criteria (Cheever et al., 2009). **Shared TAAs**

Among the shared TAAs, the following three main groups can be identified: (1) cancer-testis (CT) antigens, (2) differentiation antigens, and (3) widely occurring overexpressed antigens. Among shared tumour-specific antigens, *cancer-testis (CT)* antigens are expressed in histologically different human tumours and, among normal tissues, in spermatocytes/spermatogonia of the testis and occasionally in placenta. CT antigens result from the reactivation of genes which are normally silent in adult tissues but are transcriptionally activated in different tumour histotypes (De Smet et al., 1999). Many CT antigens have been identified and used in clinical trials, although little is known about their specific functions, especially with regard to malignant transformation. This group of TAAs includes MAGE-A1 (Chaux et al., 1999) and NY-ESO-1 (Jager et al., 1998). *Differentiation antigens* are shared between tumours and the normal tissue of origin and found mostly in melanomas and normal melanocytes (Gp100, Melan-A/Mart-1, and Tyrosinase), although they are also found in epithelial tissues and tumours such as prostate tumours (prostate-specific antigen [PSA]). To variable extent, normal tissues can be target of the elicited immunity against shared TAAs. An example is the vitiligo developing as a consequence of the immune response in melanoma patients undergoing immunotherapy. Vaccine-induced T cells recognizing gp100 and tyrosinase are present at the *vitiligo* lesions and normal melanocytes are eliminated by the immune system (Jacobs et al., 2009). Importantly, this effect has been associated to a clinical response. Additionally, expression of several oncofetal antigens appears to be increased in many adult cancer tissues, including carcinoembryonic antigen (CEA), which is highly expressed in colon cancer (Tsang et al., 1995).

TAAs from this group, despite representing self-antigens, have been and still are commonly used in current cancer vaccination trials, often together with CT antigens. Widely occurring, overexpressed TAAs have been detected in different types of tumours as well as in many normal tissues, and their overexpression in tumour cells can reach the threshold for T cell recognition, breaking the immunological tolerance and triggering an anticancer response. Among the most interesting TAAs of this group are the antiapoptotic proteins (survivin) (Schmidt et al., 2003), hTERT (Vonderheide et al., 2008), and tumour suppressor proteins (e.g., p53) (Umano et al, 2001).

Unique tumour antigens. Unique TAAs are products of random somatic point mutations induced by physical or chemical carcinogens and therefore expressed uniquely by individual tumours and not by any normal tissue, representing the only true tumour-specific antigens (Ags) (reviewed in Parmiani et al., 2007). Such Ags characterize each single

neoplasm and were shown to be diverse between tumours induced in the same animal or even in different tissue fragments from the same tumour nodule. A relevant feature of unique Ags is their potential resistance to immunoselection if the mutated protein is crucial to the oncogenic process and thus indispensable for maintaining the neoplastic state. As a consequence, unique Ags should elicit an immune response clinically more effective than that of shared Ags. However, identification of unique tumour antigens for solid human tumours requires sequencing of the whole genome of each individual tumour in order to identify mutated genes and select peptides whose motifs are predicted to be presented by the patient's HLA alleles. Moreover, each tumour bears highly heterogeneous sets of defects in different genes which need to be further verified for their substantial contribution to the tumour development and progression and, consequently, for their relevance as vaccine targets (Fox et al., 2009). An interesting class of potential TAAs is associated with fusions between different proteins. Best example is the Bcr–Abl fusion protein, the driving force in chronic myelogenous leukemia (CML) (Daley et al., 1990). By establishing a causal link between a specific chromosomal lesion and a specific malignancy, BCR–ABL also pioneered cancer therapy: the TK inhibitor, imatinib (Gleevec), was introduced as the first widely used targeted therapeutic (Druker et al., 2001). Similar discoveries led to the characterization of causative fusions in other hematological malignancies. A variety of prostate cancer gene fusions have been identified so far (reviewed in Shah and Chinnaiyan, 2009), characterized by 5'-genomic regulatory elements, most notably the androgen-controlled prostate specific gene, transmembrane protease serine 2 (TMPRSS2), fused to members of the erythroblastosis virus E26 transforming sequence family of transcription factors, most notably ERG, leading to the overexpression of oncogenic transcription factors. This class of potential TAAs is matter of extensive studies and holds promise for personalized vaccine applications.

Viral Antigens. Some viruses, such as human papillomavirus (HPV) and hepatitis B virus (HBV) can induce cancer. As a matter of fact, HBV vaccination in newborns has eradicated hepatocellular carcinoma (HCC) in populations at high risk (McMahon et al, 2011; Blumberg et al., 2010). The high-risk HPV types (e.g., HPV16) are causally related to the development of anogenital lesions, including vulvar intraepithelial neoplasia (VIN), and their subsequent progression to invasive squamous cell carcinoma. The expression of viral antigens (hence non-self proteins) such as HPV E6 and E7 proteins by cancer cells can represent the mechanism through which the tumour becomes visible to the immune system. Recently, promising results have been obtained by vaccination of patients with HPV16 E6/E7 synthetic long-peptide vaccine (Van der Burg and Melief, 2011), providing an important proof of concept for the development of therapeutic cancer vaccines against cervical and anal cancers.

Stromal Antigens. During transformation, reciprocal interactions occur between neoplastic and adjacent normal cells, i.e. fibroblasts, endothelial, and immunocompetent cells. In general, stroma cells contribute 20–50% to the tumour mass, but the stromal compartment may account for up to 90% in several carcinomas. In contrast to cancer cells, tumour stroma cells are genetically more stable so that at least some immune evasion mechanisms of tumours do not apply to these cells. Nevertheless, stroma cells differ from their normal counterparts by upregulation or induction of various antigens (reviewed in Hofmeister et al., 2006). Some of the tumour stroma-associated antigens (TSAAs) are highly selective for the tumour microenvironment. TSAAs are also expressed by a broad spectrum of solid tumours, thus

therapies designed to target tumour stroma are not restricted to a selected tumour type. Cancer-associated fibroblasts (CAFs) are reactive fibroblasts with a phenotype differing from that of quiescent fibroblasts in normal adult tissue. CAFs contribute to the development of cancer by secreting growth promoting factors such as TGF- β , matrix degrading enzymes, and angiogenic factors, e.g. MMPs or vascular endothelial growth factor (VEGF). Endothelial cells have a major part in tumour progression since they are necessary for angiogenesis. Tumour endothelial cells (TECs) express surface receptors and secrete factors that sustain their own growth by an autocrine pathway. Another target cell population for immune intervention present in the tumour microenvironment are tumour-associated macrophages (TAMs, see also paragraph 2.3). Among the proteins explored as promising stromal immunotherapy targets it is worth mentioning Fibroblast Activation Protein a (FAP α , seprase), a surface glycoprotein selectively expressed in reactive stromal fibroblasts of solid tumours, Carbonic Anhydrase IX (CAIX) an important pH regulator, Matrix Metalloproteases (MMP) such as MMP11 (Peruzzi et al., 2009), extracellular angiogenic factors, such as Vascular Endothelial Growth factor (VEGF) and its receptor VEGFR2 and basic Fibroblast Growth Factor (bFGF). Tumour endothelial markers (TEMs), among them TEM1 and TEM8, are overexpressed during tumour angiogenesis and prostate-specific membrane antigen (PSMA) is another endothelial cell surface molecule of particular interest for vascular targeting. In conclusion, ideal target stromal proteins are those selectively induced or upregulated in the tumour *micromilieu*, and confer a growth or survival advantage to the tumour.

Shared Antigens	Features	Type of Tumour	Examples
Cancer Testis (CT) Ags	Expressed only by tumours and testis	Melanoma, lymphoma, bladder, breast, colon, lung	BAGE, GAGE, MAGE, NY-ESO-1
Differentiation Ags	Expressed also by normal cells	Melanoma, prostate, colon, breast	Gp100, MART-1, tyrosinase, CEA, PSA
Overexpressed Ags	Expressed by tumor cells prevalently	Liver, colon, breast, ovary, bladder, prostate, esophagus, lymphoma	p53, Her2/ <i>neu</i> , survivin, hTERT
Unique Antigens	Features	Type of Tumour	Examples
Unique	Expressed by a single tumor	Melanoma, NSCLC, RCC	CD α -actin-m, K-4/m, β -actin-m, Myosin-m
Viral Antigens	Features	Type of Tumour	Examples
Viral	Encoded by genome of oncogenic viruses	HCC, anogenital lesions	HBV, HPV E6 and E7
Stromal Antigens	Features	Type of Tumour	Examples
Fibroblast TSAA	Expressed by CAFs	Ubiquitous	FAP α , CAIX, MMP11
Endothelial TSAA	Expressed by TECs	Ubiquitous	VEGF, VEGFR2, TEMs, PSMA

Table 1. Classification and examples of TAAs and TSAAs.

2.3 Immune suppression mechanisms

A strong and persistent immune response against cancer is necessary but not sufficient to controlling tumour growth in the escape phase. For example, while robust T cell responses generated by vaccinations against HPV are capable of successfully controlling pre-malignant intraepithelial neoplasias (Welters et al, 2010), in clinical trials of tumour vaccines against large, invasive malignancies the effective generation of tumour antigen-specific T cells is not predictive of clinical efficacy (Radoja et al, 2001). Although this discrepancy may be due in part to differences in the affinity/avidity of effector T cells developing against self *vs* exogenous antigens, the principal cause is believed to be the establishment of an immunosuppressive state within the tumour microenvironment (reviewed by Gajewski et al, 2006). This immunosuppression is not due to a single mechanism but to the concerted action of several processes. In first instance the presence of regulatory T cells (Tregs) and Myeloid-derived suppressor cells (MDSCs), which play a direct inhibitory role on host-protective antitumour responses.

Tregs are CD4⁺ T cells which constitutively express CD25 and the transcription factor FoxP3 (Nishikawa and Sakaguchi, 2010). It is unclear what proportion of intratumoural Tregs react with specific tumour antigens (Wang et al, 2005), or instead are recruited through the recognition of shared self-antigens co-expressed by tumour cells (Darrasse-Jeze et al, 2009). At any rate, their inhibitory function is exerted *via* the production of immunosuppressive cytokines such as IL-10 and TGF β , the expression of negative co-stimulatory receptors such as CTLA-4 and PD-1, and the expression of IDO. IDO (indoleamine 2,3-dioxygenase) is an enzyme responsible for a rate-limiting step in tryptophan catabolism and is strongly induced in the tumour environment by IFN- γ . The immunosuppressive effect of IDO expression is due both to reduction of local levels of tryptophan and to the generation of cytotoxic catabolites kynurenins, which affect T cell activity and dendritic cell survival (Soliman et al, 2010). Several studies have shown that in several cancer types the presence of regulatory CD4⁺CD25⁺ T cells in tumours inversely correlates with disease outcome (Woo et al, 2001; Curiel et al, 2004)

MDSCs or Tumour Associated Macrophages (TAMs) are a heterogenous group of myeloid progenitor cells and immature myeloid cells that inhibit lymphocyte function by inducing Tregs, producing TGF β , depleting essential aminoacids as tryptophan, arginine and cysteine, and inducing down-regulation of L selectin on T cells (Ostrand-Rosenberg, 2010; Lindenberg et al, 2011). T cells must have an L-selectin phenotype to home to lymphnodes and inflammatory sites where they encounter antigens and are activated. TAMs therefore, perturb T cell trafficking and inhibit T cell activation. Furthermore, immunosuppression appears to be enhanced by active angiogenesis and angiogenic cytokines like VEGF (Johnson et al, 2007), also through a possible direct effect on dendritic cells.

Recent studies have shown that a symbiotic relationship exists between tumour cells and TAMs, in which tumour cells attract TAMs and sustain their survival, with TAMs responding to microenvironmental factors in tumours such as hypoxia by producing important mitogens, growth factors and enzymes that stimulate tumour growth angiogenesis (Bingle et al, 2002). Actually it seems that in response to different stimuli, TAMs differentiate into subsets capable of stimulating different pro-tumourigenic functions. For example in areas of invasion TAMs promote cancer cell motility, in perivascular areas they promote metastasis, and in avascular and perinecrotic areas hypoxic TAMs stimulate angiogenesis (Lewis and Pollard, 2006). Finally in a very recent study it has been shown that

macrophage infiltration in tumours is able to affect chemotherapy (De Nardo et al, 2011). Indeed these authors have shown that TAM depletion in highly infiltrated tumours increased the antitumour efficacy of paclitaxel, and this was at least in part due to their suppression of the antitumour functions of cytotoxic T cells. This study therefore confirms the high complexity of the immune cell interactions in tumours (DeNardo et al, 2010) and shows that cross-talk between TAMs and cytotoxic T cells impairs effective tumour eradication by immune mechanisms.

Unraveling the mechanisms at the basis of immunosuppression in the tumour microenvironment has led to the definition of novel targets for therapeutic intervention. Agents directed against these new targets, such as for example IDO or PD-1, may act in concert with cancer vaccines to enhance their efficacy, in particular in conditions of advanced tumour development. We believe that the clinical efficacy of anti CTLA-4 antibody Ipilimumab is in part linked to the inhibition of immunosuppressive processes. Indeed recent studies have demonstrated that maximal anti-tumour effects of CTLA-4 blockade are due to the concomitant blockade not only of effector T cells, but also of Tregs (Peggs et al, 2009). Also, it cannot be excluded that at least in part the clinical efficacy of the anti-VEGF antibody Bevacizumab is due to inhibition of the immunosuppressive function of VEGF (Chouaib et al, 2010).

3. Types of cancer vaccines

Different technologies have been employed to develop cancer immunotherapies. These include passive immunotherapy, based on the adoptive transfer of *ex-vivo* activated immune cells, immunomodulators (including cytokines) or tumour specific antibodies; and active immunotherapy, aimed at activating the patient's own immune system via the administration of a therapeutic vaccine. To date, active cancer immunotherapy trials have included therapeutic vaccination with recombinant viral vectors encoding TAAs, recombinant proteins with appropriate adjuvants, antigen-loaded Dendritic Cells (DCs), DNA encoding tumour-associated antigens, heat shock proteins and synthetic peptides (see next paragraphs). However, apart from melanoma, in which impressive clinical responses have been observed in a small proportion of patients, the recent success of Sipuleucel-T (see paragraph 3.1.2.1) and the promise of PROSTVAC-VF (see paragraph 3.2.1), most results have been disappointing. Therefore, the continuous development of novel vaccine strategies and technologies is needed to improve recognition, immune response, effector functions, and trafficking of T cells induced by vaccination. These goals may be achieved by the concurrent administration of novel immunotherapeutics with an immunopotentiating profile (see section 4.3).

3.1 Cell-based vaccines

A first category of cancer vaccines under evaluation is based on delivery of cells. As pointed out before, we will not discuss in this chapter ACT but only Whole Tumour Cell Vaccines and Dendritic Cells vaccines.

3.1.1 Whole cell vaccines

Autologous tumour cells are an obvious source of TAAs for vaccination purposes, since, by definition, all relevant candidate TAAs should be contained within them. In early clinical

trials, individualized tailor-made vaccines prepared from whole tumour cells were associated with limited activity, presumably due to the already biased nature of the host immune response to specific TAAs (Vermorken et al. 1999; Jocham et al., 2004). However, due to the mechanism of immunologic tolerance, this approach has resulted in poor immunogenicity and different categories of adjuvants have been evaluated in the past years (de Gruijl et al., 2008).

Perhaps the most explored approach in the clinic is GVAX (Cell Genesys). Autologous tumour cells, transduced with GM-CSF were shown to induce tumour-specific immunity and durable anti-tumour responses in a number of trials. The efficacy of GVAX depends on the cross-presentation of vaccine-derived TAAs to specific cytotoxic T lymphocytes (CTLs) *in vivo* (Hege et al., 2006). This process of cross-priming is facilitated by the activation of Dendritic Cells (DC), by GM-CSF. This finding led to the realization that allogeneic cells would also present a viable source of TAAs, which would be taken up by DCs and then presented in the context of appropriate MHC alleles to autologous CTLs. Advantages of the use of allogeneic cells are obvious: (1) through the use of antigenically well-defined cell lines one has access to a sustained and virtually limitless source of material, (2) the use of cell lines allows for a highly standardized and large-scale production of vaccine, (3) the use of a single batch of allo-vaccines for all vaccinees, independent of HLA haplotype, eliminates variability in the quality and composition of the vaccines and facilitates reliable comparative analysis of clinical outcome. Eliminating the need for the continuous production of tailor-made individual vaccines simplifies the logistics, reduces the laboriousness of vaccine production and distribution, and increases its cost-effectiveness.

3.1.2 Dendritic cell vaccines

Dendritic Cells (DCs) collect antigens from various tissues and carry them to secondary lymphoid organs to ultimately activate antigen-specific T cells. Myeloid DCs and plasmacytoid DCs are the 2 main subsets of DCs (Palucka et al, 2011). Through toll-like receptors (TLRs) 7 and 9, plasmacytoid DCs recognize viral nucleic acids and secrete type I interferon (IFN). Three myeloid DC subsets localize to the skin. Langerhans cells (LCs) are found in the epidermis, while CD1 α +DCs and CD14+DCs are found in the dermis. CD14+DCs produce interleukin (IL)-1 β , IL-6, IL-8, IL-10, IL-12, GM-CSF, membrane cofactor protein-1, and tumour growth factor- β . LCs produce IL-15, which is a growth and maintenance factor for CD8+ T cells and natural killer cells. LCs are more efficient in cross presentation and prime higher avidity T cells with reported greater capacity for cell kill. Although DC biology is complicated, it is clear that these cells are the critical regulators of adaptive T-cell and B-cell responses. These findings have provided the rationale for *ex vivo* antigen loading of DCs for the preparation of vaccines. DCs have been loaded with tumour antigens in the form of peptides, proteins, tumour lysates, and mRNAs. Alternatively, they have been fused with tumour cells or infected with viral vectors encoding tumour-associated antigens (reviewed in Le et al., 2010).

Clinical development of DC-based cancer vaccines has several aspects that make this technology not ideal for application on a large scale. The first aspect is the difficulty to set up standardized procedures for the reliable production of functioning DCs. Currently, it is difficult to demonstrate that each preparation has the same levels of processed and presented antigen, and can induce an equivalent degree of immune response after administration. Quality control in the processing of cellular products is critical to the

integrity of the product. Large amounts of autologous peripheral blood mononuclear cells must be cultured in the presence of several cytokines making their off-the-shelf marketability challenging. There are critical issues not only in ensuring the proper maturation status of the DCs but also in the precise selection of appropriate subsets of DCs required to elicit the desired response. Other aspects include the significant costs of manufacturing the product and the huge amount of labor required to produce a viable product within a short time frame.

3.1.2.1 The Sipuleucel-T (Provenge) experience

Despite the above described technical hurdles, a immune cell-based vaccine, Sipuleucel-T, recently received Food and Drug Administration approval based on a successful phase III trial showing improvement in overall survival (OS) in men with asymptomatic or minimally symptomatic metastatic advanced castrate resistant prostate cancer (CRPC). The key to manufacturing feasibility of Sipuleucel-T is the absence of DC purification in the preparation. The preparation of a Sipuleucel-T product involves a leukapheresis to obtain the peripheral blood of the patient. The leukapheresed specimen is then transferred to the company manufacturing facility. The cell pellet containing DCs (CD54⁺), T lymphocytes (CD3⁺), B lymphocytes (CD19⁺), monocytes (CD14⁺), and natural killer cells (CD56⁺) is exposed to PA2024, an engineered antigen-cytokine fusion protein consisting of Prostate Acidic Protein (PAP) and GM-CSF. GM-CSF facilitates uptake of the fusion protein by DCs and promotes DC stimulation. PAP is the tumour antigen used in this vaccine. The final product is transported to the patient at 4°C and infused intravenously within 8 hours of formulation. Because the product is a mixture of cell types, the precise mechanism of action has not been established. It is not clear if induction of anti-prostate cancer responses involves *in vivo* activation of T cells by the loaded DCs in the preparation. It is also possible that T cells in the preparation are activated by *ex vivo* manipulations and that this therapy actually represents an alternative form of adoptive T-cell therapy. The paucity of available immunologic data to date precludes mechanistic dissection of this drug.

Phase I and II trials of Sipuleucel-T demonstrated T-cell and antibody responses to the antigen (Burch et al., 2004). Immune responses correlated with improved time to progression (TTP). Two sequential phase III placebo-controlled studies were subsequently conducted in patients with metastatic CRPC, with a primary end point of TTP. Integrated data again suggested a survival benefit but failed to show significance for the predetermined clinical end point. In this combined data set, a total of 225 patients were randomized to Sipuleucel-T (n = 147) or placebo (n = 78). There was a 33% reduction in the risk of death (HR 1.50; 95% CI 1.10– 2.05; P = 0.011). There was only a 4.8% PSA response in the combined analysis. Median survival was 23.2 versus 18.9 months and the percentage alive at 36 months was 33% versus 15% in favor of the treatment groups. Cumulative CD54 up-regulation, a measure of product potency, correlated with Overall Survival (OS). As a result of these studies, Dendreon pursued a new study, known as the Immunotherapy for Prostate Adeno Carcinoma Treatment (IMPACT) trial. OS was the primary end point. Five hundred twelve patients were enrolled in this study. Despite absence of clinical response to Sipuleucel-T or effect on TTP, the study met its primary end point of survival benefit. Subjects in the treatment group experienced a statistically significant increase in median survival vs controls (25.8 vs. 21.7 months respectively) and greater OS at 36 months (31.7% vs. 23%). The final analysis after 349 events demonstrated a median OS benefit of 4.1 months (HR 0.759; 95% CI 0.606–0.951; P = 0.017) (www.dendreon.com).

3.1.3 Heat shock proteins-based vaccines

Another interesting approach in cancer vaccine development is the use of heat shock protein (HSP)-peptide complexes, as natural host vector for vaccination (reviewed in di Pietro et al., 2009). Heat shock proteins are intracellular molecules of a family characterized by members of similar molecular mass (such as hsp70 and hsp90) that act as chaperones for a repertoire of peptides, including normal self-peptides and antigenic peptides. During both protein synthesis and breakdown, heat shock protein complexes are released from cells still associated non-covalently with peptides. Release by necrotic cells function as endogenous danger signals as well as a method to cross-present antigens to DCs. In fact, DCs have a specific receptor for heat shock proteins (CD91) and its engagement leads to their maturation. HSPs complexed with antigenic peptides have been shown to efficiently deliver peptides into the MHC class I processing pathway thus generating cellular immune responses. This phenomenon has been demonstrated in mouse and human tumours. In the latter, hsp70-peptide complexes extracted from melanoma cells have been found to contain well-known peptides on the basis of their ability to stimulate antigen-specific CD8⁺ T cells from melanoma patients' peripheral blood mononuclear cells (PBMCs). This observation has led to the purification of HSP-complexes from the tumours of patients and their administration as vaccines. The immunogenicity of tumour-derived HSP-peptide complexes, like the immunogenicity of experimentally induced tumours of mice and rats, has been shown to be individually tumour specific and not tumour type specific. These observations have led to the conclusion that the relevant tumour-antigenic, immunoprotective peptides are derived from unique rather than from shared tumour antigens.

Heat shock proteins explored for clinical immunotherapy may contain a defined antigen (E7 antigen derived from the human papilloma virus, MAGE tumour antigen, etc) or nondefined tumour antigens, which require the individualized production of heat shock proteins from fresh tumour samples. This could be a limitation, since several grams of tumour tissue must be available for the patient to be eligible for the trial. Following a number of trials (<http://www.agenusbio.com/prophage/past-trials.html>) in a range of tumour types (pancreatic cancer, Kidney cancer, Non-Hodgkin's lymphoma, CRC, gastric cancer) the tumour specific HSP-complexes vaccine named HSP peptide complex-96 (HSPPC-96 or Oncophage® or Prophage; Agenus, Lexington, MA, USA) has been approved in Russia as Oncophage® for the adjuvant treatment of kidney cancer patients at intermediate risk for disease recurrence. Currently Agenus is planning a registration Phase III trial for recurrent and newly diagnosed glioblastoma (<http://www.agenusbio.com/prophage/ongoing-trials.html>) in order to obtain drug approval by EMA.

3.1.4 Peptide vaccines

Peptide-based cancer vaccines represent the most popular approach to direct the immune system against malignant cells, since they are usually made of single epitopes, the minimal immunogenic region of an antigen. Peptides can be synthesized in a standardized manner and their cost of production is relatively low. Thus peptide vaccines have been the technology of choice by several groups. Despite the strong rationale, the promising preclinical results and the frequent induction of antigen-specific immune responses in patients, peptide-based cancer vaccines have yielded relatively poor results in the clinical

setting and so far none of advanced clinical trials with peptide vaccines has resulted in statistically significant increase in survival. A particular mention deserve the results of the phase III clinical trial in 676 metastatic melanoma patients, which compared the efficacy of a gp100 peptide vaccine, with that of the fully human anti CTLA-4 antibody ipilimumab, or with the combined agents (Hodi et al, 2010). Ipilimumab when compared to gp100 alone improved median overall survival from 6.4 to 10.1 months (hazard ratio for death, 0.68; $P < 0.001$). More importantly no difference in survival was detected between the Ipilimumab alone vs Ipilimumab plus vaccine groups (median overall survival 10.1 vs 10.0 months, hazard ration with ipilimumab plus gp100, 1.04; $P = 0.76$). Based on these results ipilimumab was recently approved by FDA for the treatment of unresectable stage III and IV melanoma, with the name of Yervoy® (BMS). A possible interpretation for the lack of efficacy of a peptide vaccine in a patient population otherwise responsive to immunotherapy is the necessity to generate a polyclonal immune response directed simultaneously against several MHC class I epitopes. This could not be achieved in the Ipilimumab trial cited above because of the use of a single peptide. In order to overcome this limitation alternative approaches are being undertaken which make use of a combination of immunogenic peptides.

Advances in the engineering of peptides and in our understanding of the molecular mechanisms underlying an effective immune response against tumours have renewed the enthusiasm for peptide-based vaccination regimens in the setting of cancer and a variety of clinical trials are being conducted based on the use of peptides (Aurisicchio and Ciliberto, 2010). In this respect promising results in phase II studies have been obtained by Immatics (www.immatics.com). This technology consists in the vaccination of patients with multiple tumour-associated peptides (TUMAPs) that can be isolated from tumour specimens and identified by mass spectrometry (Dengjel et al, 2006). The most advanced product, IMA901, a combination of several TUMAPs for the treatment of renal cell carcinoma, completed a Europe-wide multi-center Phase II clinical trial and has recently commenced a Phase III trial. Another advanced peptide vaccine is L-BLP25 (Stimuvax) currently under development by MerckSerono. L-BLP25 is a peptide vaccine that targets the exposed core peptide of MUC1, a mucinous glycoprotein which is overexpressed and aberrantly glycosylated in many human malignancies. MUC1 is associated with cellular transformation and can confer resistance to genotoxic agents. In preclinical studies, L-BLP25 induced a cellular immune response characterized by T-cell proliferation in response to MUC1 and production of IFN- γ (reviewed in Gridelli et al., 2009). Phase I and II trials have established the dose and schedule of the vaccine as well as its excellent safety profile. A randomized phase II trial of maintenance L-BLP25 versus best supportive care in patients with stage IIIB/IV non-small cell lung cancer showed a strong survival trend in favor of L-BLP25 (median survival, 30.6 versus 13.3 months) in a subgroup of patients with locoregional stage IIIB disease (Butts et al., 2011). These promising results are being tested in three phase III trials (START, INSPIRE and STRIDE). The START and the INSPIRE studies are Phase III, multi-center, randomized, double-blind, placebo-controlled clinical trial designed to evaluate the efficacy, safety and tolerability of Stimuvax in subjects suffering from unresectable, stage IIIA or IIIB non-small cell lung cancer (NSCLC) who have had a response or stable disease after at least two cycles of platinum-based chemo-radiotherapy. The primary endpoint of the START study is overall survival (OS). STRIDE is a randomized, double-blind, controlled, multi-center Phase III study designed to determine if Stimuvax can extend progression free survival in patients

treated with hormonal therapy who have inoperable, locally advanced, recurrent or metastatic breast cancer. Overall survival, quality of life, tumour response and safety will also be assessed in this study.

3.1.5 Protein vaccines

Isolated recombinant proteins have been successfully employed for antiviral vaccines. However, soluble proteins are poorly immunogenic and require appropriate adjuvants and delivery systems to induce the desirable level and type of immune responses. For optimal performance, antigen delivery vehicles should closely mimic the composition and immunological processing of actual pathogens; they should actively or passively target APCs such as DCs; protect the antigenic protein from spontaneous degradation; direct the nature of the resulting immune response (i.e., cellular versus humoral responses) and, lastly, induce APC maturation by interacting with elements of the innate immune system such as Toll-like receptors (TLRs). Several strategies have been reported including directly conjugating TLR ligands to protein antigens or co-encapsulating immunostimulatory agents and proteins in liposomes or hydrophobic polymeric particles (Beaudette et al., 2009).

The most advanced approach is the one being pursued for the development of MAGE-A3 antigen specific immunotherapy (ASCI). MAGE-A3 ASCI is a therapeutic cancer vaccine directed against tumour antigen MAGE-A3, which is overexpressed in subset of patients affected by various cancers, and is being developed by GSK (Tyagi and Mirakhur, 2009). The vaccine is delivered as highly purified recombinant protein in conjunction with GSK's own proprietary adjuvant System. The most advanced development for the MAGE-A3 vaccine is a Phase III trial called MAGRIT (MAGE-A3 as Adjuvant Non-Small Cell Lung Cancer Immunotherapy), which began in October 2007 aimed at recruiting 2270 patients randomized to ASCI or placebo. The objective of the MAGRIT trial is to investigate the efficacy of MAGE-A3 ASCI in preventing cancer relapse, when administered after tumour resection, in patients with MAGE-A3 positive stages IB, II and IIIA NSCLC and is going to be the largest-ever trial in the adjuvant treatment for NSCLC. Results of the MAGRIT trial are expected in late 2011 and may lead to registration of this product in the coming years.

3.2 Genetic vaccines

Genetic vaccines represent promising methods to elicit immune responses against a wide variety of antigens, including TAAs. A variety of vectors have been utilized in the past, each of them presenting advantages and drawbacks with respect to "classic" protein-based vaccines. The main advantage of genetic vaccines is that they allow a) endogenous expression of the antigen of interest by muscle and/or antigen-presenting cells, which maximize antigen processing through the endogenous pathway and epitope display on MHC class I molecules, and b) easy molecular engineering of the targeted tumour antigen which help to boost significantly self-antigen immunogenicity.

3.2.1 Viral vaccines

Viral infection results in the presentation of virus-specific peptides in association with both MHC class I and MHC class II on the surface of infected cells. Based on this observation, several strategies have been designed to use viruses as immunization vehicles to elicit antigen-specific immune responses. In this approach, the cDNA encoding one or more antigens, is inserted into a viral vector. The resulting recombinant viruses are used as

vaccine, obtaining the *in vivo* expression of the selected antigen(s) and its presentation to the immune system. A variety of gene therapy viral vectors have been adapted to cancer immunotherapy. For vaccination purposes, the ideal viral vector should be safe with respect to disease-causing potential, transmissibility and long-term persistence in the host. It should enable efficient presentation of expressed antigens to the immune system while preferably exhibiting low intrinsic immunogenicity so that it can be administered repeatedly to boost relevant specific immune responses, often necessary to break immune tolerance to self antigens.

Tumour antigen DNA sequences have been inserted into attenuated pox viruses that are unable to replicate in mammalian hosts (such as modified vaccinia Ankara, fowlpox, or canarypox). Vaccinia poxvirus (VV) was demonstrated to be safe and very effective in the induction of potent cellular and humoral immune response in several tumour model systems (Gómez et al. 2011). For Carcinoembryonic Antigen (CEA), VV as well as ALVAC, a variant of the canary poxvirus, have been successfully used in colorectal cancer patients. As an avian virus, ALVAC has an advantage over vaccinia in that it is unable to replicate in human cells and thus has a very favorable safety profile. Combination of vaccinia followed by multiple injection of ALVAC revealed to be efficient in terms of elicited anti-CEA immune response and overall patient survival (Marshall et al., 2005). Another successful story of a vaccine based on this technology is PROSTVAC-VF (Bavarian Nordic, Kvistgård, Denmark). PROSTVAC-VF is a vaccine against Prostate Specific Antigen (PSA) that includes a number of costimulatory molecules. Three well-characterized costimulatory molecules were found to be synergistic when added to the poxvirus system. This triad, which includes B7.1 (CD80), ICAM-1 (CD54), and LFA-3 (CD58), is designated as TRICOM and has been added to both the vaccinia priming vector and the fowlpox boosting vector. With PSA as the encoded antigen, this configuration constitutes PROSTVAC-VF, vaccinia-PSA-TRICOM, and fowlpox-PSA-TRICOM. Interestingly, a randomized, controlled, and double-blinded phase II study was designed and powered for the short-term end point of PFS, and it failed to find an association between treatment arm and progression. However, a strong association between treatment arm and OS was observed (Kantoff et al., 2010). The estimated hazard ratio is 0.56 (95% CI, 0.37 to 0.85), and the observed difference in median survival of 8.5 months suggests a significant therapeutic benefit. PROSTVAC-VF immunotherapy is, therefore, a promising approach, and a larger pivotal phase III trial is being planned. Another Poxvector based vaccine is Trovax, a vector directed against a tumour enriched surface marker named 5T4 (Kim et al, 2010). Clinical trials with Trovax showed good safety profile, immunologic responses to the target antigen and efficacy in relation to a defined biomarker strategy (see section 4.2). TG4010 is an MVA vector developed by Transgene (Strasbourg, France) that incorporates the MUC1 antigen, which is overexpressed in the majority of cancers. A second gene, interleukin-2 is also incorporated as an immune stimulus. The vaccine has been tested in breast, kidney, prostate and lung cancers with encouraging results in phase II. For RCC, thirty-seven patients with progressive, MUC1-positive tumours received TG4010 10^8 pfu/inj weekly for 6 weeks, then every 3 weeks until progression, when TG4010 was continued in combination with interferon- α 2a and interleukin-2. Assessments included clinical response (primary endpoint), safety, time to treatment failure (TTF), OS, and immune response. No objective clinical responses occurred, but median OS was 19.3 months for all patients and 22.4 months for combination therapy

recipients. MUC1-specific CD8⁺ T cell responses were associated with longer survival (Oudard et al., 2011).

Another emerging viral system for vaccination is Adenovirus (Ad). Ads are very efficient vehicles for gene delivery and have been extensively characterized for gene therapy purposes (reviewed in Dharmapuri et al., 2009). Ad gene therapy products have recently been demonstrated to be safe, well-tolerated and capable of successful gene transfer to target cells. The high immunogenicity of E1-deleted first generation Ad recombinants has largely excluded their use for somatic gene therapy but re-directed their development as vaccine carriers. Ad vaccines have been shown to induce the highest degree of B- and CD8⁺ T-cell responses in experimental animals, including rodents, canines, and primates against a variety of immunogens derived from a variety of infectious agents (e.g., viruses, parasites, or bacterial pathogens) and tumour cells, including tumour-associated antigens (TAAs). Most clinical trials with Ad vectors have been conducted in oncology. Among the others, intratumour (IT) injections of Ad containing the wild-type p53 tumour suppressor gene showed clinical efficacy when combined with chemotherapy and led to the clinical development of Advexin® and Gendicine®. Advexin and Gendicine® are recombinant Ad5 vectors with an E1 region that is replaced by a human wild type p53 expression cassette containing a Cytomegalovirus (CMV) or Rous sarcoma virus (RSV) promoter, respectively. In October 2003, the State Food and Drug Administration (SFDA) of China approved Gendicine as the first commercialized gene therapy product in the world. Another example is Onyx-015, the first engineered replication-selective virus to be used in humans. It is an Ad2/Ad5 hybrid with deletions in E1B and E3B region and replicates exclusively in cells with inactive p53, activated p14ARF and late viral mRNA transport. Onyx-015 has been tested in more than 15 clinical trials by direct IT injection (up to 5×10^9 vp) and resulted in transient antitumoural effects (objective response rate 14%).

For development of cancer vaccines, several groups are currently assessing the immunologic and clinical activity of Ad vectors expressing TAAs, such as prostate serum antigen (PSA), HER-2/*neu*, carcinoembryonic antigen (CEA) and telomerase (hTERT) (see www.clinicaltrials.gov). It will be of great interest to verify how local and systemic suppression exerted by the tumour itself as well as pre-existing immunity to Ad will impact the outcome of these studies.

In conclusion, viral vectors appear promising tools for cancer vaccines. From a practical standpoint, viral vectors also meet criteria that enable their large scale industrialization. These include; efficient growth on a cell substrate acceptable to regulatory authorities; total genetic stability with respect to attenuation and presence of the foreign gene(s), scalability to large doses; easy purification of the vector virus away from cellular debris, and stability in the final formulation.

3.2.2 DNA plasmid vaccines

The inoculation of plasmid DNA coding for a protein antigen by means of a simple intramuscular or intradermal injection currently represents an easily performed vaccine approach that is safe for host and relatively inexpensive. DNA delivery vehicles contain a gene expression cassette bearing the coding region of an antigen gene regulated by a promoter usually with constitutive activity (like the cytomegalovirus early enhancer-promoter). Simple injection of naked DNA sequences results in gene expression and the generation of immune responses. A possible mechanism of how DNA immunization works

is the following: the protein antigen is produced by the target cells (usually skeletal myocytes or dermal fibroblasts, depending upon the injection route) that usually lack the co-stimulatory molecules needed as part of the CTL activation process. Subsequently, the antigen is taken up by host APCs, processed, and cross-presented to the immune system in the draining lymph nodes, although direct transfection of rare APCs residing at the injection site has also been demonstrated (Liu, 2011).

In mouse models, DNA vaccines have been successfully used to generate strong cellular immune responses against a wide variety of tumour antigens and to exert a preventive or therapeutic effect on tumour growth (Liu, 2011). However, clinical trials for DNA vaccines have shown that, albeit immune responses can be generated in humans, there is a need for increased potency if this vaccine technology is to be effective. The reasons for the failure of DNA vaccines to induce potent immune responses when scaled up from mice to man have not been fully elucidated. However, it is reasonable to assume that low levels of antigen production, inefficient cellular delivery of DNA plasmids and insufficient stimulation of the innate immune system have roles in low potency of DNA vaccines (Ulmer et al., 2006). In the design of more potent DNA vaccines, clearly regimens, plasmid dose, timing, adjuvants, alternative delivery systems and/or routes of vaccination are being considered. Methods for enhancing DNA plasmid delivery include tattooing, Gene gun, Ultrasound, Laser and DNA electroporation (reviewed in Bolhassani et al., 2011). In particular, here we will focus our attention on DNA electroporation.

3.2.3 DNA electroporation

In vivo electroporation of plasmid DNA (DNA-EP) has emerged as a safe method resulting in greater DNA uptake leading to enhanced protein expression in the treated muscle, and in a concomitant increase in immune responses to the target antigen in a variety of species (Aurisicchio et al., 2007; Peruzzi et al., 2010a). For its properties, DNA-EP is a desirable vaccine technology for cancer vaccines since it is repeatable several times, as required for the maintenance of anti-tumour immunity (Peruzzi et al., 2010b). This approach uses brief electrical pulses that create transient “pores” in the cell membrane, thus allowing large molecules such as DNA or RNA to enter the cell cytoplasm. Immediately following cessation of the electrical field, these pores close and the molecules are trapped in the cytoplasm (Andre et al., 2010). Typically, milli- and microsecond pulses have been used for EP. In addition to the increased permeability of target cells, EP may also enhance immune responses through increased protein expression, secretion of inflammatory chemokines and cytokines, and recruitment of APCs at the EP site (Liu, 2011). As a result, both antigen-specific humoral and cellular immune responses are increased by EP mediated delivery of plasmid DNA in comparison with levels achieved by intramuscular injection of DNA alone. Indeed, the addition of *in vivo* EP has been associated with a consistent enhancement of cell-mediated and humoral immune responses in small and large animals, supporting its use in humans.

Several devices have been developed for DNA-EP. Cytopulse has developed two clinical vaccine delivery systems: DermaVax™ and Easy Vax™. Easy Vax™ primarily targets the epidermis layer of skin and has been used in mass-scale prophylactic virus vaccination. In contrast, Derma Vax™ primarily targets the dermis layer of skin. Clinical trials in progress and planned using DermaVax™ include Prostate cancer (Phase I/II) and Colorectal cancer (Phase I/II). In this study, DNA vaccine was delivered by intradermal electroporation to

treat colorectal cancer (El-porCEA; ID: NCT01064375). The purpose of this study was to evaluate the safety and immunogenicity of a CEA DNA immunization approach in patients with colorectal cancer. Altogether, the electroporation with DNA vaccines has been investigated in several clinical trials for cancer therapy. They include: (1) Intratumoural IL-12 DNA plasmid (pDNA) [ID: NCT00323206, phase I clinical trials in patients with malignant melanoma]; (2) Intratumoural VCL-IM01 (encoding IL-2) [ID: NCT00223899; phase I clinical trials in patients with metastatic melanoma]; (3) Xenogeneic tyrosinase DNA vaccine [ID: NCT00471133, phase I clinical trials in patients with melanoma]; (4) VGX-3100 [ID: NCT00685412, phase I clinical trials for HPV infections], and 5) IM injection prostate-specific membrane antigen (PSMA)/pDOM fusion gene [ID: UK-112, phase I/II clinical trials for prostate cancer] (Bodles-Bakhop et al., 2009). Inovio (Oslo, Norway) has developed electroporators suitable for muscle DNA-EP, such as MedPulser®. Plasmid V930 is DNA vaccine candidate being developed by Merck. This vaccine is designed to target cancers expressing the antigens HER-2/*neu* and/or CEA, which include breast, colorectal, ovarian, and non-small cell lung cancer (ID: NCT00250419). V934 is a DNA plasmid that encodes human Telomerase (hTERT). The biologic is a Merck proprietary, therapeutic DNA vaccine candidate designed to target cancers expressing the antigen hTERT (ID: NCT00753415), including non-small cell lung carcinoma; breast cancer; melanoma; upper GI tract (e.g. esophagus, stomach, gallbladder) in collaboration with Geron Corp. (Menlo Park, CA, USA). Both vaccines are undergoing Phase I studies using MedPulser® DNA Delivery System in combination with Ad vectors.

Attempts to further enhance immune responses elicited by DNA vaccines are focusing on the use of codon optimization in order to enhance expression in eukaryotic cells. In fact, the potency of current gene delivery methods that include plasmid DNA and viral vectors can also be improved through increasing the expression efficiency of the encoded antigens. Elevated percentages of AU in human mRNA have been shown to result in instability, increased turnover, and low expression levels of the encoded proteins. These findings have prompted modification of the target gene coding sequence through reduction of the AT content with the assumption that these modifications could result in improved mRNA stability and increased expression. These changes have been justified by the observation that for highly expressed genes, G or C is generally preferred over A or T. In fact, optimization of the codon usage of the target gene has been shown in a variety of experimental systems to lead to enhanced expression and increased immunogenicity (Aurisicchio et al., 2007; Peruzzi et al., 2010a, b).

Another strategy to enhance the efficacy of DNA vaccine is the development of gene fusions in which antigens are linked to various immunoenhancing elements (reviewed in Stevenson et al., 2004). The enhancement of immune responses is particularly relevant for cancer vaccines because of the limited immunogenicity of tumour antigens and the need to overcome tolerance. Enhancement of immune response to target antigens has been demonstrated in animal models by vectors encoding antigens fused to heat shock protein 70 (HSP70), the Fc portion of IgG1, lysosome-associated membrane protein (LAMP), the universal helper T (Th) epitope from tetanus toxin (DOM) (Facciabene et al., 2006) and Heat labile enterotoxin B from *E. Coli* (Facciabene et al., 2007). A DOM-PSMA fusion DNA vaccine delivered *via* DNA-EP resulted in highest antibody response to DOM in prostate cancer patients (Low et al., 2009), while LTB fusions are currently being evaluated in the clinical trials for CEA and hTERT cited above.

In conclusion, DNA vaccines have several promising features. They are simpler and cheaper to produce. DNA immunization is not associated with anamnestic immune responses against the vector. On the other hand, it appears that efficacy must be improved, especially for cancer vaccines targeting 'self' antigens.

3.3 Heterologous prime/boost

There are emerging evidences that vaccination schedules comprising more than one delivery method against the same antigen(s) (i.e., genetic vectors, genetic vector/protein, genetic vector/peptides, etc.) may be beneficial to overcome the 'therapeutic immunity' threshold and adequately harness the immune system to fight cancer. In particular, genetic vectors, if appropriately combined with each other and with other agents (immunomodulators and/or chemotherapy) hold promise for clinical development (reviewed in Lu et al., 2009).

The sequential administration of DNA and a viral vector in different combinations may result in synergistic immune activation. Preclinical murine and primate models have shown that this heterologous prime-boost regimen induces 10- to 100-fold higher frequencies of T cells than do naked DNA or recombinant viral vectors alone (Ribas et al., 2003). In addition to the enhanced immune response, the therapeutic proof of concept of DNA/viral vector combination has recently been achieved in dogs affected by B cell lymphoma (Peruzzi et al., 2010b). In this study, the best performing vaccination schedule consisted of Ad followed by DNA-EP boosters, concurrently with chemotherapy (see also section 4.1). Another strategy is the sequential administration of two different viral vectors carrying the same tumour antigen gene, which bypasses the limitation of the development of neutralizing antibodies to the viral backbone by boosting with a different vector without shared viral epitopes (see paragraph 3.2.1).

4. Cancer vaccines clinical development

Due to the complexity of tumour immunology, the success of cancer vaccines points to important considerations for clinical development: (1) evaluation of vaccines in predictive preclinical animal models. (2) Biomarker strategies for patient selection. This turns to be a crucial aspect as revealed by the experience of different cancer vaccine products under evaluation (see next paragraph); (3) value of vaccinating at early stages of cancer progression. Advances in early cancer detection methods and the development of efficacious cancer vaccines will allow vaccination of patients with various types of cancer at early stages of disease, when they still have an intact immune system. This may become the most promising strategy for preventing tumour progression. (4) use of heterologous prime-boost technologies (see previous paragraph). (5) combination with other therapies. Some classes of chemotherapy drugs could act as immunomodulators by affecting antigen cross-presentation, inducing a cytokine storm, reducing the number of regulatory T cells and activating homeostatic lymphoid proliferation. (6) autoimmunity as a possible side effect of cancer immunotherapy. In those instances when intense immunotherapy is necessary the incidence of autoimmunity in early or especially in advanced cancer should be evaluated in the short or long term following immunotherapy.

4.1 Pre-clinical models

Cancer immune therapy and its translation to the clinic are strictly dependent on efficient vaccine technology, delivery systems and evaluation in appropriate pre-clinical models. For

cancer vaccines the most widely used models for immunologic and anti-tumoural studies are transgenic rodents expressing the human TAA which show central and/or peripheral tolerance to the antigen of interest (see Fig. 1). The mouse is an excellent and reproducible system: mice from a given strain are inbred, with same MHC-I, allow experimentation on a large number of subjects. The mouse immune system is well-known and all reagents are available. Nevertheless, the translational relevance of cancer vaccines additionally needs a suitable, outbred therapeutic large animal model. In fact, scaling up experimental protocols from rodents to humans is often not a straightforward procedure, and this particularly applies to cancer vaccines, where vaccination technology must be especially effective to overcome a variety of immune suppressive mechanisms.

Nonhuman primates such as macaques are valid models to determine the safety and immunogenicity of candidate vaccines that are being developed for implementation in humans. In fact, the immune response is similar to that expected in humans. In the last two decades numerous immunogenicity studies have been performed in nonhuman primates utilizing pre-clinical candidate vaccines, most of them utilizing recombinant proteins of bacterial or viral origin as immunogen or genetic vectors coding for viral antigens (Shiver et al., 2002; Montgomery et al., 1993; Jeong et al., 2004). This is a reasonable approach when dealing with bacterial or viral diseases, where the organism recognizes the antigen as an exogenous protein and consequently the elicited immune-response is generally strong and effective against the target pathogen. Similarly, other studies involving the use of TAAs have been conducted with human proteins or vectors encoding for human TAAs: in these reports, the elicited immune response is not expected to be fully predictive of the possible outcome in human patients, since the antigen is recognized as a non-self protein. Therefore, the evaluation of the immune response against the 'self' antigen requires cloning of the nonhuman primate ortholog gene and evaluation of a technology able to break immune tolerance (Auriscchio et al., 2007; Fattori et al., 2009). Still, evaluation of antitumour efficacy cannot be performed in nonhuman primates.

Another emerging model in oncology is the dog. The dog is extensively used in drug discovery and development because of its similarities to human anatomy and physiology. Compared with other animal models, dogs naturally develop cancers that share many characteristics with human malignancies (Paoloni et al., 2008). Cancers in pet dogs are characterized by tumour growth over long periods of time in the setting of an intact immune system, interindividual and intra-tumoural heterogeneity, the development of recurrent or resistant disease, and metastasis to relevant distant sites. Thanks to their large population size, cancer rate in pets is sufficient to power clinical trials, including assessment of new drugs. Examples include non-Hodgkin lymphoma, osteosarcoma, melanoma, prostate carcinoma, lung carcinoma, head and neck carcinoma, mammary carcinoma and soft tissue sarcoma, which share similar histological appearance, tumour genetics, biological behavior and response to conventional therapies with human cancers. With the recent release of the canine genome sequence, the dog is now also amenable to comparative genomic and expression analysis, including tumour samples (Uva et al., 2009). However, to date the application of cancer vaccines in dogs has been poorly explored. A xenogeneic DNA-based vaccine strategy for melanoma is the only example in the setting of minimal residual disease in dogs (Bergman et al., 2006) that led to the successful approval and the commercial launch of a veterinary biological (Merial US). Furthermore, recently we have been able to successfully evaluate a heterologous prime-boost Ad-vector /DNA-EP based

vaccine against telomerase in canine subjects affected by B cell lymphoma (Peruzzi et al., 2010b). Unfortunately, the canine immune system is not deeply characterized and there are not many tools available to further evaluate the impact of a vaccination strategies. However, we expect that pet dog nonclinical studies will increasingly gain interest to help a better definition of the safety and activity of new anticancer agents and the identification of relevant biomarkers associated with response or exposure to these drugs.

Animal Models for Cancer Vaccines

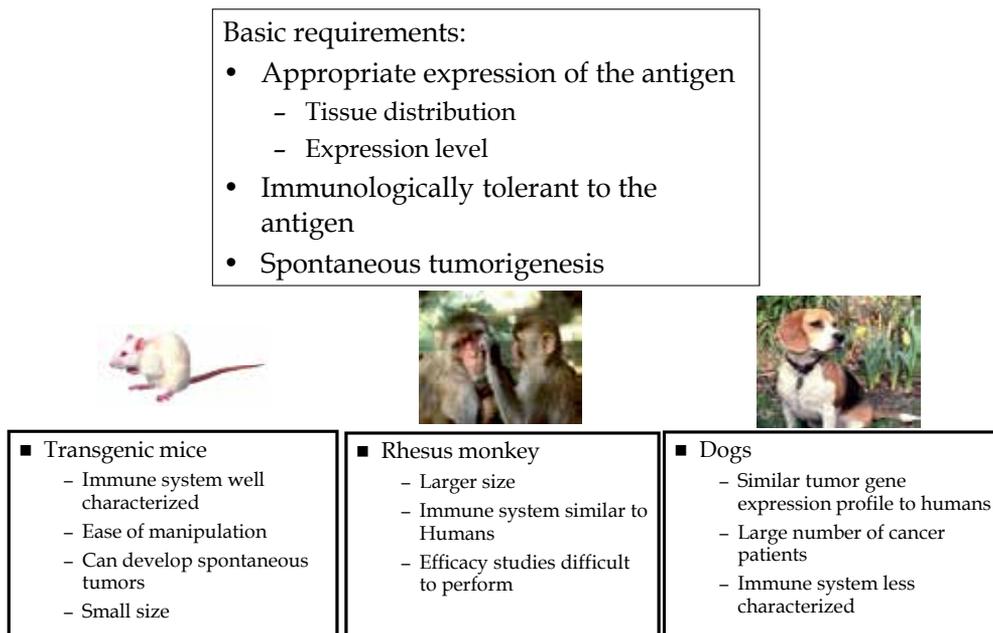


Fig. 1. Animal models for Cancer Vaccines. Advantages and drawbacks of each model are indicated.

4.2 Biomarker strategies

Drug failures in Oncology often originate from a lack of understanding of the biology of the drug, its mechanism of action (MOA), the complexity of patient physiology, and inadequate characterization of patient tumours. Poor understanding of the criteria required for patient selection for the drug may lead to misapprehensions of the drug’s potential for safety and efficacy. It is these misapprehensions that can persist through to late development until the clinical program crashes in a late and costly failure. Clearly, there is an urgent need for detailed information on new anticancer drugs to help make critical development decisions at the earliest possible point, speeding up the development process and enabling valuable time and resources to be placed where they can do the most good.

Molecular biomarkers are widely recognized as being integral to this solution. They provide a set of tools which can provide invaluable information to support two major development concerns:

1. Does the drug perform according to the expected mechanism of action?
2. Which patients will experience benefit in disease management utilizing the drug?

Thus, appropriately selected biomarkers can be used to confirm the MOA, while patient selection biomarkers can be used to guide the selection of the most appropriate patients for therapies. Correct use of biomarkers for patient selection can enrich the treatment population by identifying those most likely to benefit from the treatment. This reduces the risk to the non-responder population and, by allowing earlier assessment of therapeutic efficacy, substantially shrinks the costs of development.

Over the past two decades molecular biomarkers have become established components of clinical research in a way few could have foreseen. Today > 50% of new molecular entities are estimated to have a biomarker element also in development (Carden et al, 2010).

Nowadays the most successful examples of biomarkers for patient selection are HER2 positivity for treating breast cancer patients with Herceptin® and lack of KRAS mutations for treatment of colorectal cancer patients with Erbitux® or Vectibix®. They have completely different histories. The development of Herceptin was guided from its earliest stages by the use of the selection biomarker - HER2 - as an integral part of the original development plan for the product (Pietras et al, 1998). However, in the case of Erbitux® (cetuximab) and Vectibix® (panitumumab) in colorectal cancer, the original hypothesis that the level of epidermal growth factor receptor (EGFR) expression was critical for success of the antibody turned out to be wrong. Only recently the crucial role of KRAS wild type (or non-KRAS mutant carrying) tumour cells has been found to be a necessary element for Erbitux functioning and thus has been introduced into the drug's label (<http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm172905.htm>).

All the considerations above need to be applied also to the development of cancer vaccines and cancer immunotherapeutics in general. It is inconceivable nowadays to imagine that a new immunotherapy will be efficacious when administered to all patients affected by a given cancer pathology. Therefore a rigorous biomarker strategy is absolutely essential to avoid failures in cancer vaccines development (Gajewski et al, 2010; Disis, 2011). As a corollary, we believe that one of the main reasons for the several failures of previous cancer vaccines in phase III is the lack of biomarkers for patients selection, and this in spite of radiological evidences of direct anti-tumour efficacy observed in subsets of patients during precedent Phase II trials. Therefore, several attempts are currently being made to rescue vaccines "failed" in Phase III trials through a rigorous retrospective analysis of data collected in Phase III in order to identify biomarkers that can allow to predict patients who will most benefit from treatment, and then to restart Phase II and Phase III development.

This is the case for example of Trovax (see paragraph 3.2.1). Trovax was developed through a series of Phase I and II trials, until it was brought into a Phase III trial called TRIST (TroVax Renal Immunotherapy Trial) in patients with advanced or metastatic renal cell carcinoma. The study enrolled 733 patients divided in two arms: 1) Trovax + IL2 or IFN α or sunitinib; 2) Placebo + IL2 or IFN α or sunitinib (Amato et al, 2010). The primary predefined endpoint, namely survival (80% power, HR= 0.725; α = 0.05), was not met. However, subsequent analysis showed survival advantage in certain subsets of patients, and this opened up to studies aiming at identifying factors which could maximize benefit. In particular immunological monitoring suggested that 5T4 antibody responses were associated with increased survival (Harrop et al, 2011). However, it was necessary to show that 5T4 antibody responses was not simply linked to the general health status of the patients. This

was possible through the identification of an “immune response surrogate”, capable of predicting antibody responses with a reasonable level of accuracy. This was indeed identified in baseline platelet levels. In fact elevated platelet levels inversely correlate with anti 5T4 antibody responses and therapeutic efficacy. This new biomarker is currently being analyzed in additional ongoing trials and will likely be used to inform future strategies for renewed Phase II/III development of TroVax.

As mentioned in the introduction section, ipilimumab, the fully human monoclonal antibody directed against CTLA-4 has had a luckier developmental fate and was recently approved by FDA for the treatment of metastatic melanoma. A 3.7-month survival benefit was observed in the registration Phase III trial in the ipilimumab arm vs control gp-100 peptide vaccine arm was achieved (hazard ratio 0.68; $P = 0.003$), which met the predefined primary endpoint (Hodi et al, 2010). However if we look only at tumour responses only a minority of patients treated with ipilimumab or with the other anti CTLA-4 antibody under development, tremelimumab, achieve radiographic responses (Sarnaik and Weber, 2009). In the search of biomarkers capable of predicting early efficacy of these two antibodies immunological monitoring has been an integral part of their clinical development. Approaches to immunological monitoring have included: 1) monitoring the frequency of specific populations of cells in peripheral blood or tumour; 2) monitoring changes in expression levels of specific markers on immune cells; 3) quantifying antigen specific immune responses including antibody and CD8⁺ or CD4⁺ T cell responses; 4) monitoring changes in peripheral cytokine levels of cytokines produced by specific immune cell populations. This has led to the identification of several endpoints that may correlate with a variety of clinical parameters (reviewed in Callahan et al, 2010). The most robust correlation to date is with the rate of absolute lymphocyte counts (ALC), which was shown to correlate with clinical benefit (Berman et al, 2009). Also, inducible costimulator (ICOS) a member of the immunoglobulin gene family, seems to be involved. In some studies a correlation between increased frequency of circulating CD4⁺ICOS^{high} T cells and Overall Survival has been shown (Chen et al, 2009; Vonderheide et al, 2010; Charton et al, 2010). Promising biomarkers also appear to be increases in CD54RO and HLA-DR on circulating CD4⁺ and CD8⁺ T cells (Comin-Anduix et al, 2008), poly-epitope antigen specific immune responses (Yuan et al, 2008) and degree of intratumoural Treg infiltration (Ribas et al, 2009). Finally perhaps the most recent but probably most promising biomarker appears to be the change in circulating levels of Th17 as shown in a recent study on 75 patients (Sarnaik et al, 2011), where higher changes in Th-17 inducible frequency was a surrogate marker of freedom from relapse ($p=0.047$). These biomarkers have been so far identified in small retrospective trials, but their validation awaits larger prospective studies. Also, another present limitation of these biomarkers is that they belong more to the category of efficacy biomarkers than to that of stratification biomarkers and therefore, do not look as promising tools to stratify patients that are expected to better respond to therapy.

A clever biomarker strategy has been applied for MAGE-A3 ASCI by GSK (see paragraph 3.1.5). The MAGRIT study applies stringent patient stratification criteria based upon the level of expression of MAGE-A3 in patients' tumours (approximately 40% of NSCLC patients), which are believed to have greater chances of responding to therapy, mirroring the same strategy adopted during Herceptin® development. Furthermore, during the early phases of MAGE-A3 ASCI clinical development a multiple gene signature predictive of response to therapy was derived with an unbiased approach from microarray analysis of RNA extracted from peripheral lymphocytes of treated patients, in the attempt to establish a

correlation between gene expression and disease relapse. The MAGRIT trial will have as an additional objective the validation of this predictive signature in a prospective manner.

4.3 Combination therapies

The greatest potential for cancer vaccines will derive from the possibility to combine these treatments with existing and forthcoming therapeutics in order to create synergies while mitigating side effects (Andersen et al, 2010). Understanding the molecular basis for synergies poses significant scientific challenges, together with the definition of the best protocols for combinations. The establishment and optimization of dosing and scheduling of multiple treatments will require intensive pre-clinical studies as well as the conduct of well designed clinical study protocols with the consequence of significantly increasing development costs. Furthermore, for the combination of experimental drugs the ability to conduct combination studies will require that Companies that are commercially pursuing different drugs and vaccine candidates must come to specific agreements.

4.3.1 Combining vaccines with chemotherapy

It is now clear that chemotherapy, instead of having a general immunodepressant effect, when given in particular combination schedules, can have a potent immunostimulatory effect and may enhance cancer vaccine efficacy. Several pre-clinical studies have been at the basis of what is now called chemoimmunotherapy, a strategy which is structured upon the possibility to enhance cancer vaccine efficacy through well studied combinations with available chemotherapeutic agents. Promising clinical results have been obtained, which are waiting for confirmation in larger randomized trials (Zitvogel et al, 2008).

A leading example is that of cyclophosphamide (CTX), an alkylating agent that has been used for a long time at high dosages as a potent cytotoxic and lymphoablative drug. In recent years careful studies have shown that low doses CTX (also called metronomic CTX) have instead immunostimulatory and antiangiogenic effects, opening up new applications for cancer immunotherapy. By promoting IFN α secretion, CTX influences dendritic cells homeostasis, leading to preferential expansion of CD8 α^+ DCs, i.e. the main subset involved in cross-presentation of cell-derived antigens (Schiavoni et al, 2011; Moschella et al, 2011). Furthermore CTX induces tumour cell death with consequent DCs uptake of tumour apoptotic material, and CD8 $^+$ T-cell cross priming. Finally CTX induces a T-helper 17 (Th17) status, capable of shifting the Treg/Teffector equilibrium in favor of tumour regression (Sistigu et al, 2011). Another drug that is being combined with cancer vaccines with promising results is dacarbazine, due to its known effect in stimulating cytokine production, modulating Treg numbers and favouring homeostatic proliferation of effector T cells (Nisticò et al, 2009).

Chemotherapy-induced cell death can also be qualitatively immunogenic through upregulation of surface calreticulin. This process, called immunogenic apoptosis has been observed with chemotherapeutic agents such as oxaliplatin and anthracyclines and is activated by pre-apoptotic ER stress. Calreticulin, a protein usually residing in the endoplasmic reticulum is translocated onto the plasma membrane surface and triggers cell engulfment by dendritic cells and tumour associated antigens presentation (Zitvogel et al, 2010). Finally, other chemotherapeutic agents such as gemcitabine have been shown to favor depletion of TAMs and may enhance vaccine efficacy through removal of their negative regulation on effector T cells (Suzuki et al, 2007).

4.3.2 Combining vaccines with immunomodulators

From the mechanistic standpoint these are the combinations that should work best. Depending upon their mechanism of action immunomodulators are expected either to increase vaccine immunogenicity by potentiating antigen-specific CD8⁺ and/or CD4⁺ T cell responses, or to increase vaccine effectiveness by impairing one or more of those immunosuppressive mechanisms that operate at the level of tumour microenvironment. It was unfortunate that these expectations were not met in the Phase III trial that led to registration of Ipilimumab. In that 3 arms trial, Ipilimumab alone was as effective at increasing OS as the combination of Ipilimumab plus a peptide gp100 vaccine (Hodi et al, 2010). In other words, adding a therapeutic melanoma vaccine on top of Ipilimumab did not provide additional advantage. Several are the possible explanations of this failure, but we believe, as discussed above (see section 3.1.4) that peptide vaccines, especially those monospecific, i.e. directed against a single epitope, are not potent enough to show efficacy, in particular in large trials like this one, where no patients stratification criteria are being applied.

Nevertheless, we believe that vaccines plus immunomodulators combinations hold a great potential; however, they need to be studied in detail starting from rigorous preclinical studies. Furthermore, success of the same immunomodulators in one combination cannot be automatically extrapolated to another combination, because this may be affected by the combined vaccine/immunomodulators mechanism of action, and by the disease under study. For example we have observed that the same immunomodulators, namely an IMO TLR9 agonist exerts different effects when co-delivered with two genetic vaccines targeting different tumour antigens in two distinct pre-C models. In the BALB/*NeuT* model repeated vaccinations against HER2 with DNA electroporation plus systemic IMO administration proved to be the most effective treatment in the eradication of advanced mammary tumours (Auricchio et al, 2009). In contrast this was not the case when the same systemic IMO was co-delivered together with a genetic telomerase vaccine in an immunocompetent mouse model of melanoma (Conforti et al, 2010). We believe this discrepancy is due to the fact that in the first case the anti-HER2 vaccine acts primarily through the induction of antitumoural antibody responses that are strongly enhanced by systemic IMO in mice (Auricchio et al, 2009). In contrast, the telomerase vaccine mechanism of action is exerted via the induction of antigen-specific cytotoxic CD8⁺ responses (Mennuni et al, 2008) which are not increased by systemic IMO delivery.

Among the most promising approaches to combinations is the one with agents capable to target Tregs (Golovina and Vonderheide, 2010). For example, in a transgenic CEA preclinical model we have observed that administration of an anti CEA vaccine plus an antibody against CD25 strongly enhanced CEA specific CD4⁺ and CD8⁺ antigen-specific immunity and exerted a strong tumour protection (Elia et al, 2007). Indeed a single infusion of daclizumab (Zenapax), a monoclonal antibody against CD25, in patients with metastatic breast cancer is associated with a strong and prolonged decrease of circulating CD25⁺ FoxP3⁺ Tregs. When a peptide vaccine was administered after Zenapax infusion, at the nadir of circulating Tregs, a strong generation of antigen-specific immunity was observed.

In a very recent study, the administration of an agonistic CD40 antibody was shown, when combined with gemcitabine in a small cohort of patients with pancreatic ductal adenocarcinoma to induce tumour regressions (Beatty et al, 2011). Although in theory antibodies against CD40 are believed to act through reversion of immune suppression and induction of antitumour T cell responses, this was shown not to be the case in this trial

and in a relevant mouse model. Surprisingly the antibody seemed to act via a new and unsuspected mechanism of action, which consisted in the stimulation of macrophages which infiltrated the tumours, became tumouricidal and facilitated depletion of tumour stroma.

In conclusion, we believe that combinations of immunomodulators like Zenapax, TLR agonists, anti-CTLA4 antibodies, anti-PD1 antibodies, IDO inhibitors, etc. together with cancer vaccines, may have great potential to increase vaccine effectiveness and to prolong survival, but careful mechanistic studies have to be conducted to identify the best combination and the most appropriate delivery schedule for the two agents.

4.3.3 Combining vaccines with other targeted therapies

The availability of an expanding repertoire of targeted therapies against cancer opens up tremendous possibility for combinations with therapeutic cancer vaccines. This is still a largely unexplored area. However we believe that, in parallel with the clinical progress and the increasing number of FDA and EMA approved vaccines, this area will be the object of extensive investigations. Based on the mechanism of action for example anti-angiogenic agents are expected to act synergically with cancer vaccines. The same concept can be applied to combinations with anti-apoptotic agents targeting Bcl-2 members. Finally we have to be aware that some cancer targeted therapies may exert a negative effect on immune responses. This is for example the case of sorafenib, which has been shown in a preclinical model to significantly affect the immunostimulatory capacity of DCs (Hipp et al, 2008), or of HDAC inhibitors which are able to increase the suppressive functions of Tregs (Akimova et al, 2010)

4.3.4 Adverse effects of cancer Immunotherapy

Cancer Immunotherapy has been initially advocated as being very specific for cancer cells and to have fewer side effects than conventional therapies. This concept is confirmed by reports from cancer vaccines clinical trials of cases of patients experiencing complete responses in the absence of any serious adverse event (Suso et al, 2011). An even more significant example is the very benign toxicity profile of Sipuleucel-T (Plosker, 2011). It has to be pointed out however, when examining large trials, that vaccine-related adverse events, albeit rare and usually mild, are being observed. For example in a recent meta-analysis of 500 cases of advanced cancer patients treated with therapeutic peptide vaccines, 6 severe adverse events (SAEs) were related to the vaccine itself (Yoshida et al, 2011). They consisted mainly in local skin reactions or cellulitis around the injection sites. In some cases, more systemic effects such as edemas of the head and neck regions, colitis, rectal bleeding and bladder-vaginal fistulae were reported.

The occurrence of autimmunity is particularly evident in the case of therapies with systemic immunomodulators more than with cancer vaccines. Indeed, Immune-related adverse events (IRAEs) are being commonly observed in patients after CTLA-4 blockade and most likely reflect the drug mechanism of action and corresponding effects on the immune system (Weber, 2007). Immunotoxicities resulting from Ipilimumab treatment can range from relatively minor conditions, such as skin depigmentation, to severe toxicities against crucial organ systems, such as liver, heart and lung. In the Ipilimumab registration trial Grade 3 or 4 IRAEs occurred in 10 to 15% of patients treated and seven deaths were associated with IRAEs (Hodi et al, 2010). Treatment-related toxicity correlates with better

responses in some cases, and it is likely that serious adverse events from immune-mediated reactions will increase in frequency and severity as immunotherapeutic approaches become more effective (Amost et al, 2011). Hence, scientists and physicians should be on guard for SAEs associated with augmented immune responses and strategies will have to be developed to avoid or circumvent these side effects.

The use of viral vectors in past gene-therapy trials has been shown to cause the occurrence of leukemogenesis (Dunbar, 2007). This phenomenon has been linked to the use of retroviral vectors and is due to their integration into the host genome and the activation of adjacent proto-oncogenes. It is, therefore, important to carefully analyze whether genetic vaccines that make use of either naked DNA or viral vectors may raise similar issues. It has to be pointed out, however, that genetic vaccines bear two significant differences when compared to gene therapy with retroviral vectors. In first instance DNA, also following electroporation (Wang et al, 2004), as well as Adenoviral (Jager and Ehrhardt, 2007) or Pox vectors used for cancer vaccines have a very low or null chromosomal integration respectively in the host genome. The second aspect is that vaccines are inoculated at peripheral sites in the body such as dermal tissue or skeletal muscles which are mainly composed of terminally differentiated and mitotic quiescent cells. At any rate, Regulatory Agencies require the inclusion of genome integration and genotox studies for any new genetic vaccine as part of the documentation to be included in IND filings.

5. Clinical endpoints

Cancer Vaccines and Cancer Immunotherapy in general act via a gradual build up of immune responses in the body that eventually are expected to affect cancer growth and propagation. The realization that the kinetics of this process are relatively slow as compared to the more immediate effects of chemotherapy have led to the conclusion that the conventional clinical trial endpoints cannot be applied as such also to Cancer Immunotherapy trials, and that there was a need for the establishment of new and specific criteria. Several initiatives in this direction were started over the past years and were coordinated by the Cancer Immunotherapy Consortium of the Cancer Research Institute (CIC-CRI) in collaboration with the International Society for Biological Therapy of Cancer in USA and with the Association for Cancer Immunotherapy (C-IMT) in Europe. They led to the issuance in year 2009 of a guidance document by FDA (see next paragraph) whose principles are summarized below (for a detailed description, please refer to Hoos et al, 2010).

Essentially three novel endpoint considerations, which require extensive validation by prospective assessment in clinical studies, were formulated: 1) Harmonize assays directed to assess cellular immune response to tumour antigens in order to minimize assay variability among clinical sites. The goal is to obtain a reproducible biomarker that eventually will allow to establish more precise correlations between immune response and clinical efficacy; 2) Adopt new criteria for antitumour response which are adapted from the standard Response Evaluation Criteria in Solid Tumours (RECIST) criteria; 3) Use different statistical methods for trial design and assessment of survival outcomes.

The Cancer Vaccine Clinical Trial Working group first proposed that immunoassays should be performed at least at three different time points, one baseline and two follow up. At least two assays should be used in parallel to provide relevant data to inform go/nogo decision for further development. Furthermore, the cutoff values for an immune response should be established prospectively both to define a positive vs negative response and to define the

proportion of patients needed to conclude for a positive outcome. With respect to assay harmonization it was soon realized that several assays are being used (ELISPOT, IFN- γ intracellular staining, HLA-peptide multimer-staining, etc) with principles and procedures different in different laboratories. This hampers data reproducibility and comparisons among studies. Immunomonitoring proficiency panels were launched to address these issues for individual assays. These panels have worked by accrual of patients' samples and preparation of peripheral blood mononuclear cells, which were then shipped and tested across multiple laboratories, using their respective protocols. Results were then centrally analyzed. The ELISPOT panel has been the longest running panel, and its results have led to initial ELISPOT harmonization guidelines (Janetzki et al, 2007), which are directed to address key variables across different laboratories that influence assay outcome, but do not impose assay standardizations (Hoos et al, 2010).

Investigators rely on RECIST criteria to assess clinical activity of anticancer agents (Eisenhauer et al, 2009). These criteria nicely capture the effects of chemotherapeutic agents and measure tumour shrinkage. These criteria are used to distinguish Progressive Disease (PD) vs Stable Disease (SD), Partial Response (PR) or Complete Response (CR) and inform about trial continuation or discontinuation of experimental new therapies. However, it has become evident with time that RECIST criteria do not offer a complete description of the response to immunotherapeutic agents and need to be adjusted. This is due to the fact that the dynamics of antitumour effects of immunotherapeutic agents are in general much slower than chemotherapies and that in some cases patients with a stable disease, or a progressive disease at early time points, experience tumour regression at a later time. The Cancer Vaccine Clinical Trial Working Group addressed this issue, concluded that the appearance of measurable clinical activity for immunotherapies may take longer than for cytotoxic therapies (also after conventional progressive disease has been declared) and that application of standard RECIST criteria, may lead to inappropriate trial discontinuation (Hoos et al, 2007). By analyzing data from several different immunotherapy trials on a large number of patients a set of four distinct patterns were detected: immediate response, durable stable disease, response after tumour increase, and response in the presence of new lesions. While the first two patterns are included in conventional RECIST criteria, the other two are not. Therefore, in order to capture all patterns observed, the so called immune related Response Criteria (irRC) were formulated (Wolchok et al, 2009); irPD (immune-related Progressive Disease), irSD (immune-related Stable Disease), irPR (immune-related Partial Response), irCR (immune-related Complete Response) using the same thresholds to distinguish between categories as in the standard RECIST criteria. However, there are two substantial differences: a) Progressive Disease is declared not simply upon the appearance of new tumour lesions but upon the measure of total tumour burden according to a precise formula (Hoos et al, 2010); b) that measure should be confirmed at least at two consecutive time points. Using irRC the appearance of new tumour lesions alone does not constitute therefore irPD if they do not add to the total tumour burden measured at the initiation of the treatment by at least 25%, and if they are not confirmed at the subsequent time point. These new criteria are meaningful because they have received extensive validation in clinical trials with ipilimumab and have shown to correlate with favorable patients survival. However, their prospective evaluation in new trials is required to confirm their clinical utility.

Finally, at variance with chemotherapy, where early clinical effects are possible, immunotherapies often show delayed clinical effects. This is evident when analyzing

Kaplan-Meier survival curves of Provenge trials (Kantoff et al, 2010), where delayed separation of survival curves between active treatment vs control is observed. If this delayed separation is a general phenomenon for immunotherapeutic agents, then the statistical power to differentiate the curves is reduced. Therefore new statistical paradigms need to be established which take this into account in order to avoid miscalculations in the number of patients to be accrued in Phase III registration trials, and in the number of events required to calculate Hazard Ratio and Confidence Interval. It is highly recommended in this case that the quantification to compute the required events comes from previous randomized well designed Phase II trials.

In conclusion to this section we anticipate that the application of these new clinical endpoints is going to positively enhance the probability of success of cancer vaccines, allow faster and more informed GO/NOGO decisions in early clinical development and to prioritize agents that have the best profile to show statistically meaningful survival benefit.

6. Regulatory perspectives

Sipuleucel-T (Provenge®, Dendreon) is the only therapeutic cancer vaccine approved by FDA. However several promising vaccines such as M-Vax, (AVAX Technologies, Inc.) OncoVax (Vaccinogen), TroVax (Oxford Biomedica), ASCI MAGE-A3 (GSK), Oncophage® (Agenus) are in late stage development and are preparing for regulatory review in the United States, Europe, Canada, or other international regions (www.MarketResearch.com). Many of the products with potential approval status over the coming years are already in Phase III development, have orphan drug status, SPA status, or Fast Track status. As a signal of a new open attitude towards cancer vaccines, the FDA has recently issued new draft clinical trial guidelines for makers of therapeutic cancer vaccines intended to treat patients with existing disease (<http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm182443.htm>). Most notably, the draft guidance, in line with the new criteria described in section 5, advises that time-to-tumor-progression may not be an appropriate endpoint for cancer vaccines and that immune response launched against the tumour may take longer than the time it takes for it to progress. As the mechanism of action for most cancer vaccines is thought to be mediated through amplifying a native T-cell response, especially cytotoxic T cells, regulators explained that development of a cancer vaccine can present different considerations for clinical trial design than development of a traditional cytotoxic drug or biological product for the treatment of cancer. Consequently, developers of cancer vaccines are now encouraged to move forward with new products, although they need to weigh the advantages and disadvantages of testing their agents in patients with metastatic diseases vs. patients with no evidence of residual disease or minimal burden of disease.

7. Future directions

We believe that therapeutic cancer vaccines have a bright future and that within the next ten years they will become an established therapeutic modality for cancer, in a manner similar to what have now become monoclonal antibodies. This success will strictly depend upon the respect of the four major principles listed below (see also Fig 2);

The “Key” to successful cancer vaccine development

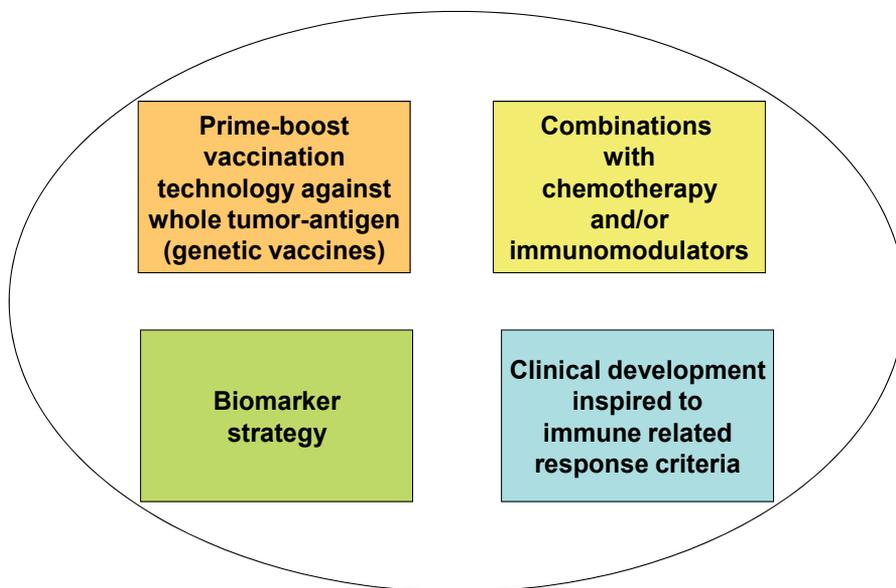


Fig. 2. Components and requirements for successful cancer vaccines development.

1. Use of a well established vaccination technology capable of inducing strong multi-epitope antigen-specific T and B cell responses, while using reproducible and easily scalable technologies. In this respect we believe that the most promising platforms for vaccination are those based on the use of genetic vectors, primarily when used in heterologous prime-boost combinations;
2. Appropriate combinations of vaccines with chemotherapy and/or with immunomodulators;
3. Development of an articulated biomarker strategy, which allows in parallel with clinical development to reproducibly quantify antigen-specific T cell responses as a pharmacodynamic measure of vaccine immunogenicity, and to pre-select the best responders to treatment;
4. A development paradigm that takes into account the evolving scenario and that is constantly inspired to the improved endpoints for cancer immunotherapy trials

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NKG2D-Based Cancer Immunotherapy

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1. Introduction

NKG2D (nature killer group 2, member D) is a C-type lectin-like activating receptor expressed by all human nature killer (NK) cells, most NKT cells, subsets of $\gamma\delta$ T cells, and CD8 T cells. In mouse, NKG2D is also expressed by all NK cells and subsets of splenic $\gamma\delta$ T cells and NKT cells, but only expressed by activated mouse CD8 T cells and activated mouse macrophages. NKG2D is located on a syntenic region of human chromosome 12 and on mouse chromosome 6, clustered with other NKG2 family members (Glienke et al., 1998; Ho et al., 1998) (**Figure 1**). NKG2D serves as an invariant immune activating receptor upon engagement by ligands expressed on target cells, transformed or viral infected cells. Engagement of NKG2D by its ligands can activate NK cell and co-stimulate CD8 and $\gamma\delta$ T cells (Bauer et al., 1999; Groh et al., 2001; Wu et al., 2002). The activation signals transmitted by NKG2D can override inhibitory signals transmitted by other NK receptors. NKG2D is therefore referred as the master activating receptor for NK cells to sense cells under abnormal physiological stress. The ligands for NKG2D are not commonly present in normal tissues but can be induced under abnormal physiological condition, such as cellular transformation or viral infection. The expression pattern of NKG2D ligands in tumor cells has been extensively studied. Emerging experimental evidence have indicated that NKG2D-mediated immunity can be very effective for tumor clearance by activating NK cells, and in some cases CD8 T cells. However, it is widely accepted that NKG2D function is subverted in cancer patients, due to mechanisms of tumor immunoediting and immune suppressive effect of tumor microenvironment (**Figure 2**). Thus, inventions are in need to overcome tumor immune evasion of NKG2D immunity as an effective cancer treatment. In this chapter, we will review the basic understandings of NKG2D function in anti-tumor immunity and the challenges and advances in NKG2D-based cancer treatment.

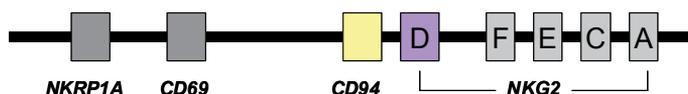


Fig. 1. The NKG2 family gene cluster. Except NKG2D, all other members of the NKG2 family form a heterodimeric complex with CD94. Different from other members, NKG2D forms a homodimer on cell surface.

2. NKG2D

2.1 Molecular structure and expression

NKG2D is a type II transmembrane glycoprotein, containing C-type lectin-like domains, similar to other known NKG2 family (Eagle and Trowsdale, 2007). Although physically clustered with other NKG2 family members, NKG2D only displays 20-30% sequence homology with other members of the NKG2 family. NKG2D is highly conserved between species. For instance, human NKG2D and mouse NKG2D share 70% amino acid identity (Raulet, 2003). NKG2D was originally identified as a key activating receptor of NK cells. Subsequently NKG2D is identified on all human CD8 T cells, NKT cells, subsets of $\gamma\delta$ T cells. In murine, NKG2D was expressed by activated and memory CD8 T cells, a proportion (25%) of splenic $\gamma\delta$ T cells, and activated macrophages (Diefenbach et al., 2000; Mistry and O'Callaghan, 2007).

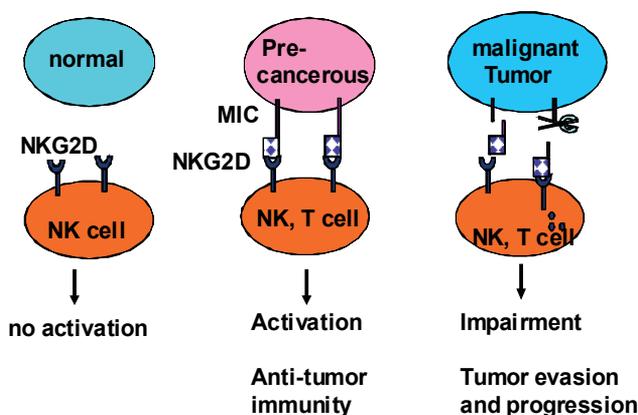


Fig. 2. Tumor cells have developed strategies to evade NKG2D immunity. The ligand of NKG2D is generally absent in normal tissues. In pre-cancerous tissues, NKG2D ligand is induced to stimulate NKG2D immunity in NK and T cells and prevents tumorigenesis. In malignant tissues, NKG2D function is impaired which allows tumor evade to immunity.

2.2 Signaling

The NKG2D molecule contains two β -sheets, two α -helices, four disulfate bonds, and a β -strand (Mistry and O'Callaghan, 2007). NKG2D forms homodimers on the cell membrane (Raulet, 2003). In both human and mouse lymphocytes, stable surface expression of NKG2D requires a complex formation of NKG2D homodimer with a Tyr-X-X-Met (YXXM) adaptor signaling molecule DAP10 at the cell membrane (Ogasawara and Lanier, 2005). Activated mouse NK cells also express a splice variant NKG2D-S, which is 13 aa shorter than normal NKG2D and signals through either DAP10 or the immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor molecule DAP12 (Long, 2002). Upon ligand engagement of NKG2D, DAP 10 is phosphorylated by src-family kinases (Figure 3), which permits the recruitment of the PI3K subunit p85 and the signaling intermediate Grb2-Vav 1 to fully activate NK cell cytotoxic pathways. In activated mouse NK cells, NKG2D-s may also independently signal through ITAM which, after phosphorylation, recruits ZAP70 (zeta-chain-associated protein kinase 70) and Syk (spleen tyrosine kinase). In NK cells, NKG2D-initiated activation signals can bypass signals transmitted through inhibitory receptors,

presumably because SHP phosphatases which are usually recruited by activation of NK inhibitory receptors do not participate NKG2D signaling (Watzl, 2003). Because of this trait, NKG2D is also regarded as the “Master” activation receptor of NK cells. Activation signal provided by NKG2D can override inhibitory signals provided by NKG2D inhibitory receptors.

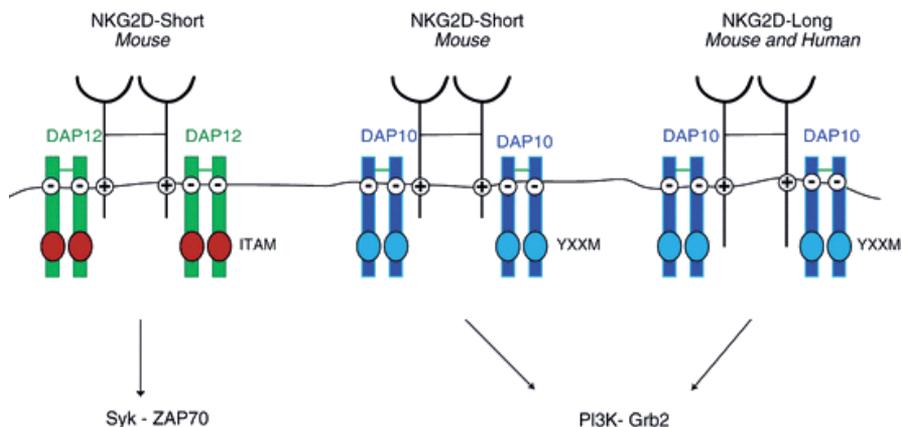


Fig. 3. NKG2D signalling pathways. Mouse NKG2D associates with both DAP10 and DAP12, whereas human NKG2D associates with DAP10 only. Adopted from Champsaur and Lanier, 2010.

3. NKG2D ligands

Multiple genes encode ligands for NKG2D have been identified in human and mice (Table 1). In human, expression of NKG2D is mostly restricted to tumor or certain viral infected cells and rarely identified in normal tissues. The expression pattern of NKG2D ligand in mouse tissues is not well understood. Nonetheless, the regulation of the NKG2D ligand expression is a delicate matter. Inappropriate expression of NKG2D ligands in normal tissues may induce autoimmune diseases, while failure to sustain surface ligand expression in transformed tissues would favor disease development and progression.

3.1 NKG2D ligands in human

Two families of NKG2D ligands are identified in humans: the MHC class I chain related family molecules A (MICA) and B (MICB) and the family of HCMV (human cytomegalovirus) UL16-binding proteins 1-6 (ULBPs 1-6) (Bahram *et al.*, 2005). All these molecules are distant HLA class I homologues but not associated with β -2 microglobulin nor have roles in antigen presentation (Eagle and Trowsdale, 2007). Although highly conserved within each family, members of the MIC family share little sequence or structural similarity with those of the ULBP family. The expression pattern of the MIC and ULBPs are also dissimilar.

3.1.1 Tumor-associated expression of MIC family NKG2D ligand

MIC genes are located within the MHC class I region of chromosome 6 (Bahram *et al.*, 2005). Seven MIC loci exist, but only two loci encode translated genes (MICA and MICB) (Eagle

and Trowsdale, 2007). Although MICA and MICB transcripts are widely found in normal human tissues (Schrambach et al., 2007), MICA and MICB protein are predominantly found in epithelial originated tumors, rarely expressed in normal tissue with an exception to intestinal epithelium, possibly due to the contact of these cells with intestinal microbes. MICA and MICB share over 80% amino acid identity. Both MICA and MICB are highly polymorphic. There are 51 identified MICA alleles and 23 identified MICB alleles (Bahram et al., 2005; Viny et al., 2010). To some degree, this diversity may provide protection against rapidly evolving cancers (Eagle and Trowsdale, 2007). The MIC(A/B) molecule is consisted of three extracellular domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$), a trans-membrane region, and a cytoplasmic tail (Bahram et al., 1994; Bahram et al., 2005).

Name	Alternate Name	Cell Surface Attachment	NKG2D Affinity (K _D)
Human			
MICA	PERB11.1	Transmembrane	1 μ M
MICB	PERB11.2	Transmembrane	0.8 μ M
ULBP1	RAET1I	GPI anchor	1.1 μ M
ULBP2	RAET1H	GPI anchor or not	ND
ULBP3	RAET1N	GPI anchor	ND
ULBP4	RAET1E,LETAL	Transmembrane	ND
ULBP5	RAET1G	Transmembrane or GPI anchor	ND
ULBP6	RAET1L	GPI anchor	ND
Mice			
Rae-1 α	Raet 1a	GPI anchor	690nM
Rae-1 β	Raet 1b	GPI anchor	345nM
Rae-1 γ	Raet 1c	GPI anchor	586nM
Rae-1 δ	Raet 1d	GPI anchor	726nM
Rae-1 ϵ	Raet e	GPI anchor	20n M
H60-a	n/a	Transmembrane	26nM
H60-b	n/a	Transmembrane	310nM
H60-c	n/a	GPI anchor	8.7 μ M
MULT1	n/a	Transmembrane	6 nM

Table 1. NKG2D ligands in human and mouse

3.1.2 Tumor-associated expression of ULBP family NKG2D ligand

The ULBPs were named for their ability to bind to the human cytomegalovirus UL16. protein Six members of human ULBP gene family are identified to encode functional proteins. ULBPs 1-3 and 6 are glycosylphosphatidylinositol (GPI)-linked proteins, whereas ULBPs 4 and 5 are type I transmembrane proteins (Mistry and O'Callaghan, 2007) (Figure 4). Unlike the MICs family, the ULBP family lack the $\alpha 3$ domain and only have the MHC class I-like $\alpha 1$ and $\alpha 2$ domains (Mistry and O'Callaghan, 2007). The expression pattern of ULBP family members are not well defined. ULBP transcripts appear widely expressed in humans (Cosman et al., 2001; Radosavljevic et al., 2002), not restricted to transformed tissues.

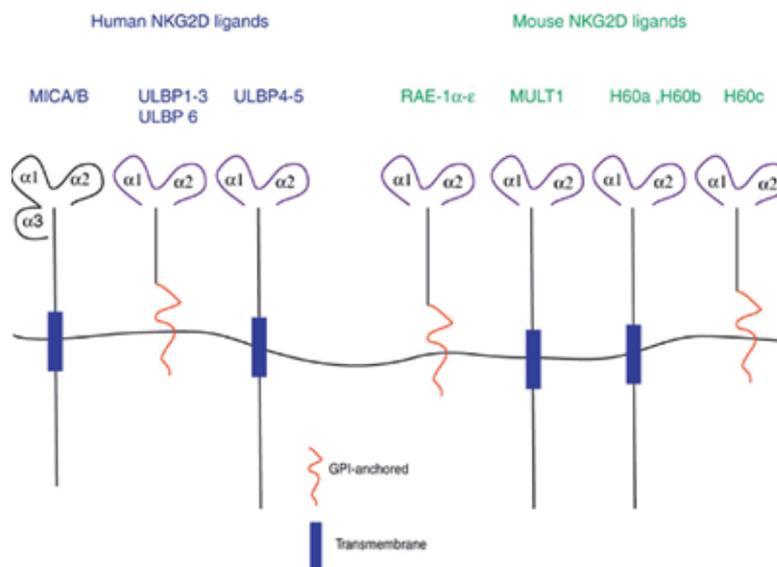


Fig. 4. Structure of NKG2D ligands in human and mice. MICA and MICB are the only known ligands containing three extracellular domains. All others (human and mouse) lack the $\alpha3$ domain and are either transmembrane or GPI-anchored. Adopted from Champsaur and Lanier, 2010.

3.2 NKG2D ligands in mice

No homologue to human MIC protein was identified in mice. The identified mouse NKG2D ligands include family members of: the MHC I-related family members of retinoic acid early transcript RAE-1(α , β , γ , δ , and ϵ) and H60 (a, b, c), and the murine ULBP-like transcript 1 (MULT1) (Cerwenka et al., 2000; Diefenbach et al., 2003; O'Callaghan et al., 2001; Takada et al., 2008). All of these ligands only have the MHC class I-like $\alpha1$ and $\alpha2$ extracellular domains. The prototype member of Rae-1 gene family was first discovered as retinoic acid (RA) early inducible cDNA clone-1 (Rae-1), which was rapidly induced on F9 teratocarcinoma cells in response to treatment with retinoic acid (Chalupny et al., 2003; Nomura et al., 1994). Presently, there are five known members of the Rae-1 family, named Rae-1 α , Rae-1 β , Rae-1 γ , Rae-1 δ , and Rae-1 ϵ , which are differentially expressed in various mouse strains but highly related to each other (>85% identity). The H60 family comprises three members. H60a, the first ligand of the family was initially identified as a minor histocompatibility antigen by immunizing C57BL/6 mice with MHC-identical BALB.B cells (Malarkannan et al., 1998). Two novel members of H60 family were identified, and named as H60b and H60c (Takada et al., 2008). MULT1 is the unique member of the ULBP-like family of mouse NKG2D ligands and was found by database searching for mouse sequences with similarities to human ULBP (Carayannopoulos et al., 2002).

In mice, NKG2D ligand expression in primary tumorigenesis has not been extensively analyzed. Transcripts of mouse NKG2D ligand was found to be expressed in a broad range of normal tissues. H60a mRNA was found in multiple tissues, including the spleen, cardiac, skeletal muscle, thymus, and skin, whereas H60b mRNA is limited to cardiac and skeletal muscles (Zhang et al. 2010). The most recent addition to the H60 family, H60c, is transcribed

largely in the skin (Takada et al., 2008; Whang et al., 2009). H60a is productively expressed in BALB/c mice but not in C57BL/6 mice, whereas H60b and H60c transcripts are detected in both C57BL/6 and BALB/c mouse. MULT1 mRNA is found in the heart, thymus, lung, and kidney across most mice strains (Carayannopoulos et al., 2002; Takada et al., 2008). However, the expression level of NKG2D ligand on normal tissues seem to be below the threshold of inducing activate immune response to cause tissue injury.

3.3 Regulation of the NKG2D ligand expression

As NKG2D serves as the master activating receptor on NK cells, expression of NKG2D ligand NKG2D be delicately regulated in a pathological condition to protect normal tissue integrity and yet maintain the alertness to diseases. The regulation is acheived at multiple levels of regulatory mechanisms, each of which will be discussed below.

3.3.1 Transcriptional regulation

The known mechanisms which regulate the NKG2D ligand transcription are mainly cellular stress, DNA damage, TLR stimulation, and cytokine exposure. The promoter region of the MICA and MICB contains contain sequences that are highly homologous to the heat shock elements of HSP70 (Venkataraman et al., 2007), a stress induced gene. Viral oncoproteins, such as adenoviral E1A protein, or cellular stress-response related products can bind to the promoter region of MICA and/or MICB to induce or upregulate its expression (Venkataraman et al., 2007). Treatment of hepatocellular carcinoma cells with RA was shown to induce the expression of MICA and MICB (Jinushi *et al.*, 2003b). The transcription factor AP-1, which is involved in tumorigenesis and cellular stress responses, was found to regulate Rae-1 transcription through the JunB subunit (Nausch *et al.*, 2006).

The DNA damage response pathway is involved in maintaining the integrity of the genome. The PI3K-related protein kinases ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3-related) sense DNA lesions, specifically double-strand breaks and stalled DNA replication, respectively. This sensing results in cell cycle arrest and DNA repair or cell apoptosis if the DNA damage is too extensive to be repaired. This pathway has been shown to be constitutively active in human cancer cells (Bartkova *et al.*, 2005; Gasser and Raulet, 2006; Gorgoulis *et al.*, 2005). Both mouse and human cells upregulate NKG2D ligands expression following treatment with DNA-damaging agents. This effect was dependent on ATR function, as inhibitors of ATR and ATM kinases can prevent ligand upregulation in a dose-dependent fashion.

TLR signaling also results in NKG2D ligand transcription in multiple mechanisms (Eissmann *et al.*, 2010). Treatment of peritoneal macrophages with TLR agonists *in vitro* and injection of LPS *in vivo* both resulted in Rae-1 upregulation on peritoneal macrophages (Hamerman *et al.*, 2004). TLR agonists increased the transcription of Raet1 genes, but not MULT1 or H60, in a Myd88-dependent fashion. TLR agonists have a similar effect on human cells (Kloss *et al.*, 2008; Nedvetzki *et al.*, 2007). TLR signaling also results in NKG2D ligand expression on DCs.

Cytokines can differentially affect NKG2D ligand expression in different cell types and environments. In humans, IFN- α leads to the expression of MICA on DCs (Jinushi *et al.*, 2003a). IFN- α and IFN- γ treatment can down-regulate H60 expression on mouse sarcoma cells(Bui *et al.*, 2006). Treatment of human melanoma cells with IFN- γ can decrease mRNA

levels of MICA in STAT-1 dependent fashion (Schwinn *et al.*, 2009). Transforming growth factor- β (TGF- β) also decreases the transcription of MICA, ULBP2, and ULBP4 on human malignant gliomas (Friese *et al.*, 2004). Macrophages cultured in the presence of IL-10 show elevated expression of MICA and MICB and ULBPs 1-3 (Schulz *et al.*, 2010).

3.3.2 Post-transcriptional regulation

Various mechanisms are responsible for the post-transcriptional regulation of NKG2D ligands. The endogenous cellular microRNAs (miRNAs) that bound to the 3'-UTR (untranslated region) of MICA, MICB and ULBP1 can repress the translation of these ligands (Stern-Ginossar *et al.*, 2008; Himmelreich *et al.*, 2011). Four miRNAs that suppressed MICA expression have been identified (Yadav *et al.*, 2009). In these findings, silencing of Dicer, a key protein in the miRNA processing pathway, leads to the upregulation of MICA and MICB (Tang *et al.*, 2008). However, miRNA-induced upregulation of NKG2D ligands was found to be dependent on the DNA damage sensor ATM, thus suggesting that upregulation of NKG2D ligands in the absence of Dicer might be due to genotoxic stress in addition to the absence of regulatory miRNAs.

3.3.3 Post-translational regulation

Expression of NKG2D ligand can also be regulated post-translationally via various mechanisms. The ubiquitination on the lysines in cytoplasmic tail of MULT1 was shown to mediate its rapid degradation (Nice *et al.*, 2009). Ubiquitination can be reduced in response to heat shock or ultraviolet irradiation through the MARCH family of E3 ligases and thus allow upregulation of NKG2D ligand expression, such as MULT1 in mice and MIC (A/B) in humans (Nice *et al.*, 2010). The presence of multiple lysines in the cytoplasmic tail of H60a, H60b, MICA, MICB, and RAET-1G suggests that this translational control mechanism might be used by other NKG2D ligands. KSHV (Kaposi's sarcoma-associated herpesvirus)-encoded E3 ubiquitin ligase K5 can down-regulate cell surface expression of MICA and MICB (Thomas *et al.*, 2008). The ubiquitination may also redistribute MICA to the plasma membrane, rather than target to degradation as observed with MULT1. The sorting/internalization motif in H60a may confer the regulation mechanism (Samarakoon *et al.*, 2009). Lastly, one of the most commonly described mechanism to regulate surface NKG2D ligand expression in human cancer cells is protease-mediated shedding (Fernandez-Messina *et al.*, 2010; Liu *et al.*, 2010). This level of regulation will be discussed in details in section 6.1.

4. NKG2D in anti-tumor immunity

4.1 Evidence in experimental models

NKG2D-mediated tumor rejection has been demonstrated very effective in experimental animal models. The rejection was mediated primarily by NK cells or through a cooperation of NK cells with CD8 T cells. Overexpression of a high level of mouse NKG2D ligands Rae-1 or H60 in mouse tumor cells of various origin, including the thymoma cell line EL4, the T-cell lymphoma cell line RMA, and the poorly immunogenic and highly metastatic melanoma variant B16-BL6, induced *in vivo* rejection or retarded tumor growth when implanted into syngeneic mice (Cerwenka *et al.*, 2001; Diefenbach *et al.*, 2001). It was also found that the rejection of a small dose of Rae-1 or H60-expressing tumors (e.g. 1×10^4 cells) could be achieved by NK cells or CD8 T cells alone whereas inhibition the growth of large

dose of Rae-1 or H60-expressing tumor cells (e.g. 1×10^6 cell) required a cooperation of NK cells and CD8 T cells (Diefenbach et al., 2001).

The significance of NKG2D in controlling tumor growth was further emphasized by *in vivo* NKG2D neutralization in experimental models. When mice (B6 or balb/c background) were injected with antibody to neutralize NKG2D, these animals showed increased susceptibility to carcinogen MCA-induced fibrosarcoma in comparison to control IgG-treated mice (Smyth et al., 2005). Perhaps the most direct genetic evidence to demonstrate the role of NKG2D in tumor immunity comes from the NKG2D-deficient mice. When TRAMP mice were crossed with NKG2D-deficient mice, the progeny had 4-time increased frequency of developing poorly-differentiated tumors than NKG2D^{WT} counterparts (Guerra et al., 2008).

4.2 Human cancer

Although NKG2D ligands are prevalently expressed in tumors of many types of human malignancies, there is so far no direct evidence to demonstrate the role of NKG2D in controlling tumor growth or progression. Understanding the significance of NKG2D in human cancer progression mainly comes from correlative observation in cancer patients. Massive clinical data demonstrating impaired NKG2D function in cancer patients was mediated by various mechanisms. A number of studies elegantly demonstrating the positive correlation of impaired NKG2D function with cancer disease stages. We are one of the first groups demonstrating that impaired NKG2D-mediated NK cell function correlated with cancer stages in prostate cancer patients (Wu et al., 2004). In this study, circulating NK cells were isolated from prostate cancer patients with various stages of diseases. NKG2D expression and NK cell function were analyzed *in vitro*. The result showed a gradually loss of NKG2D⁺ NK population from patients with low grade to high grade of cancer, with complete loss of NKG2D expression on NK cells from patients with advanced diseases. As an obvious consequence, NKG2D-mediated cytotoxicity of these NK cells against tumor cells was severely subverted. Similar observations were demonstrated in the progression of other types of cancers, such as multiple myeloma and colon cancer (Dobrovina et al., 2003; Jinushi et al., 2008). In gliomas patients, tumor burden was found to be associated with deficiency of NKG2D expression on NK and CD8 T cells (Crane et al. 2010). A number of studies have also described that dysfunction of NKG2D on CD3⁺CD56⁺ NK-like T cells and subsets of $\gamma\delta$ T cells was associated with poor prognosis of certain cancers (Bilgi et al., 2008; Marten et al., 2006; Wang et al., 2008).

5. Tumor immune evasion of NKG2D immunity

5.1 Tumor shedding of NKG2D ligand as the immune evasion mechanism

Expression of NKG2D ligand on tumors should effectively trigger immune response, at least NK cell innate response at the early stage of tumorigenesis, to eradicate tumors in human. However, in many types of established tumors of human malignancy, the NKG2D ligand MIC was highly expressed (Groh et al., 1999). The very paradoxical question is: how can human epithelial tumors develop and persist while the surface MIC molecule should identify them as abnormal and flag them for immune destruction? Clinical studies demonstrated that most of the human malignancies have developed mechanisms to evade NKG2D-mediated anti-tumor immunity. One of the common mechanisms by which human cancers evade NKG2D immunity is shedding of the NKG2D ligand MIC from tumor cell

surface to release a stable soluble form of MIC (sMIC) to the circulation (Groh et al., 2002). This mechanism has been identified in an array of human malignancies, including carcinomas of prostate, breast, lung, colon, kidney, and ovarian, gliomas, neuroblastomas, and melanoma (Groh et al., 2002). Elevated serum levels of sMIC has been shown to be correlative with advanced cancer stages (Dobrovina et al., 2003; Holdenrieder et al., 2006a, b; Jinushi et al., 2008; Rebmann et al., 2007; Tamaki et al., 2010; Tamaki et al., 2009; Tamaki et al., 2008; Wu et al., 2004). Some studies have suggested that serum levels of sMIC may be used as a valid prognosis factor for cancer progression (Tamaki et al., 2010; Tamaki et al., 2009). Tumor-derived sMIC can impose several negative imprints on host immune system. First, shedding can reduce the density of membrane-bound NKG2D ligand, namely MIC on tumor cells and thus reduce the visibility of tumor cells by the immune surveillance. Second, sMIC in the circulation can not only mask NKG2D on effector NK, NKT and T cells, but also induce NKG2D internalization (Champsaur and Lanier, 2010). Third, sMIC may induce the expansion of immune suppressive NKG2D⁺CD4⁺ T cells in the tumor microenvironment (Groh et al., 2003).

5.2 The alternative hypothesis

The hypothesis that tumor-derived sMIC is immune suppressive in cancer patients is widely accepted. Currently, an alternative hypothesis that chronic exposure to membrane-bound ligands also impairs NKG2D function was also proposed, based on several *in vitro* and *in vivo* studies. This alternative hypothesis raised a concern on the effectiveness and strategy on NKG2D-based immune therapy. The *in vitro* study was conducted by co-culturing purified mouse splenic NK cells with RAE-1-overexpressing tumor cells. The investigator found that NKG2D expression was down-regulated (Coudert et al., 2005). It was not clear in this study whether the down-regulation of prolonged *in vitro* culture is due to soluble RAE-1 or membrane-bound RAE-1, as RAE-1 was recently shown to be shed by mouse tumor cells (Champsaur and Lanier, 2010). With a different aspect of limitations, the existing evidence from *in vivo* studies was based on enforced ectopic constitutive expression of NKG2D ligand on normal mouse, not in the context of tissue-specific expression without resembling the feature of NKG2D ligand expression in cancer patients. For example, one transgenic mouse model that was created by expressing human MICA under the constitutive and ubiquitous mouse MHC class I H-2K^b promoter on a C57BL/6 background showed impaired ability of NK cells to reject MICA-transfected RMA tumors in comparison to the wild-type counterparts (Wiemann et al., 2005). In other models, NKG2D ligand RAE-1 ϵ was expressed in normal mice under the constitutive involucrin promoter (inducing squamous epithelium expression) or the ubiquitous chicken β -actin promoter; local and systemic NKG2D downregulation was noted in these mice in comparison to the wild-type counterparts (Oppenheim et al., 2005). Notably, in these transgenic mouse models, NKG2D ligand expression was “ectopic” under the direction of a constitutive or ubiquitous promoter in somatic cells. Given the magnitude of ligand-induced NKG2D signaling on activating NK cell cytotoxicity, down-regulation of NKG2D function may be expected in these transgenic mice in compare to an otherwise wild type counterpart. This would be a self-regulatory mechanism in response to “a suicide machinery” to allow normal embryonic development. Thus, whether the sustained systemic ligand-induced downregulation of NKG2D in these mouse models truly represents the real situation in cancer patients should be carefully evaluated.

5.3 Does chronic exposure to membrane-bound ligand impair NKG2D function?

The alternative hypothesis raised a fatal therapeutic concern whether sustaining NKG2D ligand on tumor cell surface would be beneficial or detrimental for host anti-tumor immunity. To resolve the controversial, we constructed a mutant shedding resistant membrane-restricted NKG2D ligand MICB.A2. We overexpressed the native shedding-sensitive MICB and the mutant MICB.A2 both of which can be recognized by mouse NKG2D (Wu et al., 2009) respectively in a highly tumorigenic mouse prostate tumor cell line TRAMP-C2 and implanted these cell lines into SCID mice. Interestingly, expression of the membrane-restricted MICB.A2 prevented TRAMP-C2 to form tumors *in vivo* whereas expression of native shedding-sensitive MICB did not (Wu et al., 2009). When the mice were injected with purified sMICB prior to tumor inoculation to imitate the expression of shedding-sensitive MICB, expression of MICB.A2 could not prevent TRAMP-C2 tumor formation. This study provided a proof-of-principle that tumor-specific membrane-bound ligand does not impair NKG2D function *in vivo* and that only the soluble ligand derived from the membrane-bound ligand as a result of shedding induces NKG2D dysfunction to promote tumorigenesis. To provide further evidence supporting this notion, we have created double transgenic TRAMP-MICB and TRAMP-MICB.A2 mice where MICB and MICB.A2 was concurrently expressed with the SV40T oncoprotein in the mouse prostate epithelium directed by the prostate-specific probasin promoter. Sustained immunity was generated by enforced expression of membrane-restricted MICB.A2 to allow long-term tumor-free survival of animals; conversely, enforced expression of shedding-sensitive MICB facilitated bound MIC, is immune suppressive to facilitate tumor progression and metastasis (Wu, unpublished). Together, these studies have suggested that stabilizing membrane-bound NKG2D ligand expression may become valuable avenue for tumor immune therapy.

5.4 Modulation of NKG2D function by tumor microenvironment

Other soluble components than soluble NKG2D ligands in the tumor microenvironment have also been described to facilitate tumors evading NKG2D immunity. One of the widely described factors is TGF- β , which can be secreted by regulatory T cells or tumor cells. TGF- β was well demonstrated to down-regulate of NKG2D expression in Glioma patients (Castriconi et al., 2003; Crane et al., ; Friese et al., 2004). In some cases, TGF- β was also found to inhibit the expression of tumor cell surface NKG2D ligand expression at the transcriptional level (Friese et al., 2004). Indoleamine 2,3-dioxygenase (IDO), a tryptophan (Trp) catabolite, is another well studied component in the tumor microenvironment that may negatively regulate NKG2D function. IDO is generally absent or inactive in cells of the immune system, but it can be induced or activated in macrophages and subsets of dendritic-cell (DC) by specific cytokines, in particular IFN- γ . IDO has also been found in various tumors of different histotypes. Elevated IDO activity was found to be correlated with cancer, such as lung, ovarian, breast cancers, and many other types of malignancies (Ino, 2010; Prendergast et al.). There is evidence that IDO can directly down-regulate NKG2D expression *in vitro* in a time and dose-dependent manner (Song et al. 2010).

6. Interventions to harness NKG2D immunity for cancer treatment

Ample evidence demonstrating that NKG2D function is impaired in cancer patients and that NKG2D dysfunction can facilitate cancer progression to advanced diseases. With the understanding of the mechanisms by which NKG2D function was compromised, in this

section, rationales and optimal strategies to harness NKG2D immunity for potential cancer therapy will be discussed.

6.1 Mechanisms of MIC shedding

Studies have been done in many investigators to understand the mechanisms that regulate MIC shedding for potential therapeutic interventions. A diverse group of enzymes have recently been shown to be involved in MIC shedding. Studies from several groups have shown that inhibition of cellular metalloproteinase activity by GM6001 markedly interferes with MIC shedding. Specific metalloproteinases, such as ADAM (a disintegrin and metalloproteinase)-10 and ADAM-17, were found contributing to MICA shedding (Waldhauer et al., 2008) and ADAM-17 protease was found contributing to MICB shedding (Boutet et al., 2009). The type I membrane MMP (MT1-MMP, also called MMP14) also directly regulates MICA shedding independent of ADAMS (Liu et al., 2010). The thiol isomerase ERp5, which catalyzes disulfide bond formation, reduction, and isomerization, was shown to be required for MIC shedding (Kaiser et al., 2007). This was presumably accomplished by chaperoning conformational alterations of surface MIC through disulphide-bond exchange that render MIC susceptible for proteolytic cleavage.

6.2 Targeting proteases to inhibit MIC shedding

ADAM-10 and -17 and the thioreductase ERp5 have been proposed to be potential cancer therapeutic targets for inhibiting MIC shedding. However, these enzymes are not only involved in pathology of diseases, but also involved in many normal physiological functions. For instance, ADAM-17 is required for generation of the active forms of Epidermal Growth Factor Receptor (EGFR) ligands that is essential for the development of epithelial tissues. In addition, although there are many examples of expression or upregulation of ADAMs in both tumor tissues and cell lines, the precise pattern of their expression within tumors is not always clear (Edwards et al., 2008). Furthermore, targeting ADAM-17 has been in clinical trials with a spectrum of inhibitors for over a decade. However, no single ADAM-17 inhibitor has passed a Phase II clinical trial because of high toxicity and non-specific targeting (DasGupta et al., 2009). As to the possibility of targeting ERp5, it has been suggested that disulfide bond exchange with cell surface molecule to enable the shedding may be a general mechanism by which ERp5 modulates cell signaling (Jordan and Gibbins, 2006). In addition, a wide role of ERp5 in cellular function has been implicated, such as in normal platelet activation (Jordan et al., 2005). These studies suggest that there are many facets of these enzymes that need to be understood before embarking with confidence on targeting them for cancer therapy. Therefore, a more specific and feasible target is needed for inhibiting MIC shedding for cancer therapy.

6.3 Targeting MIC shedding regulatory sequences

By Mass-spectrometry analyses, we and others have shown that MIC is cleaved at multiple sites in the near transmembrane region aa 253-289 in tumor cell lines (Kaiser et al., 2007; Waldhauer et al., 2008; Wang et al., 2009), suggesting that targeting the cleavage site(s) for inhibiting MIC shedding is not therapeutically feasible. Using genetic approach, a dispensable six-aa motif in the $\alpha 3$ ectodomain of MIC (A and B) was identified to be critical for regulating MIC shedding (Wang et al., 2009). Mutation in the six-aa motif completely prevented MIC shedding but did not interfere with MIC to be recognized by NKG2D.

Further study revealed that the six-aa motif is required for MIC to form a physical complex with ERp5, a presumable requirement for MIC to be shed. Due to the “non-invasive” feature of the six-aa motif, molecules or antibodies targeting this six-aa shedding regulatory motif to prevent MIC to interact with ERp5 may be a more feasible therapy.

6.4 Neutralizing sMIC

In a clinical trial with a anti-CTLA-4 antibody blockade or vaccines for melanoma therapy, patients who generated anti-MICA antibodies during the therapy showed significantly better clinical outcome than those who did not (Jinushi et al., 2006). The beneficial effect was shown to act through antibody antagonizing sMICA-induced suppression of NK and CD8 T cell anti-tumor responses. Although not being discussed in this study, the effect of anti-MICA antibody in this particular clinical setting may also be due to elimination of sMIC in the serum and thus elimination of immune suppressive NKG2D⁺ CD4 T cells. More, anti-MIC antibody has also been shown to sensitive tumor cells to antigen-specific T cells by enhancing DC cross-priming (Groh et al., 2005). Based on these observations, using anti-MIC monoclonal antibody (mAb) to neutralize circulating sMIC and concomitantly to enhance DC cross-priming has been proposed as a cancer therapy. However, clinical implication using anti-MIC antibody must take into consideration that the antibody will also block the interaction of tumor-cell surface MIC with NKG2D and thus block NKG2D-mediated NK cell anti-tumor function. Thus, when applying this approach, it is critical to understand whether NK cell or T cell play a critical role in a particular stage of a specific cancer type. As an alternative approach, phase I clinical trial using adoptively transferred haploidentical NK cells to scavenge plasma sMIC has shown some effect in neuroblastoma patients (Kloess et al. 2010). If donor NK cells are obtainable, this approach may become an effective therapy for many type of cancers.

6.5 Engineering T cells with chimeric NKG2D

A new and very interesting mechanism to utilize the NKG2D-mediated immunity in tumor therapy is expressing chimeric NKG2D-CD3 ζ (chNKG2D) in T cells for adoptive cell therapy. By fusing NKG2D with the cytoplasmic signaling domain of CD3 ζ chain, NKG2D may induce the anti-tumor activation of T cells independent of TCR signaling, when NKG2D ligand is present on tumor cells. The chNKG2D expressed on NK cells and T cells does not seem to be down-regulated by soluble NKG2D ligand (Zhang et al., 2006; Zhang et al., 2005). This approach had been demonstrated to be very effective in controlling tumor growth in several experimental animal models (Barber et al., 2011; Barber et al., 2008a; Barber et al., 2008b; Zhang et al., 2007). Treatment of mice bearing established ovarian and multiple myeloma with T cells expressing the chNKG2D receptor can lead to long-term, tumor-free survival and induce host memory responses to tumor antigens. This protection is not restricted to the direct effect of chNKG2D-induced activation of T cells upon ligand engagement. Sustained long-term protection against tumors in animal models was found to be achieved through cytokines secreted by the chNKG2D-engineered T cells to induce a proinflammatory environment and re-activate host NK, CD4 and CD8 T cell anti-tumor responses. In ovarian mouse models, adoptive transfer of chNKG2D T cells was found to not only to induce systemic increase in IFN γ , GM-CSF, and perforin but also to eliminate immunosuppressive regulatory CD4 T cells in the tumor microenvironment (Barber and Sentman, 2009; Barber et al., 2008a). Adoptive transferring chNKG2D engineered T cells has

also been shown effective in our tumor models. However, due to the systemic immunoactivation induced by chNKG2D T cells, the long-term safety in clinical application has to be evaluated. chNKG2D-engineered autologous T cells is currently in phase I clinical trial for treating ovarian cancer patients.

7. Conclusion

As emerging evidence demonstrating the significance of sustained NKG2D-NKG2D ligand interaction in anti-tumor responses, in particular solid tumors, it is time to develop therapeutic interventions to harness the NKG2D immunity for anti-tumor therapy. As soluble NKG2D ligands are the culprit for tumor evading NKG2D immunity, interventions to enforce NKG2D-mediated anti-tumor response should be focused on preventing tumor shedding, removal of soluble NKG2D ligand or counteracting the effect of soluble ligand on NKG2D function. More, in the development of tumor vaccines, one should also take into the consideration that across-priming by NKG2D ligand may boost the clinical efficiency of vaccine-induced immune responses. Last but not least, as tumor microenvironment can negatively regulate NKG2D function, co-targeting tumor microenvironment may be necessarily in stratifying NKG2D anti-tumor immunity.

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Xenovaccinotherapy for Cancer

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1. Introduction

Up to date, a systemic treatment of cancer is based mainly on the use of chemotherapy. However, in the majority of cases, chemotherapy is not a radical treatment. In initially identified tumors there already exist cells that are resistant to toxic drug action, due to their biochemical properties. Furthermore, the proportion of such cells is progressively increased throughout the treatment period because they receive selective growth advantages over the cytotoxic drug-susceptible cells. It should also be noted that cytotoxic action of antineoplastic drugs is not selective: the drugs affect not only tumor, but also normal cells. Hence, there may be serious side effects of chemotherapy, which, by themselves, may be life-dangerous and frequently requiring further medical interventions. An appearance of the drugs with selective cytotoxic activity seems improbable in the near future because the vital biochemical processes in tumor and normal cells are similar in their basis.

Nevertheless, tumor cells are distinguished from normal ones by quantitative and qualitative expression on their surfaces of potentially immunogenic structures (antigens). It is generally accepted that the immune responses induced by these structures can cause destruction of tumor cells, and that reactivity of the immune system can define an outcome of disease. All of the tumor-associated antigens (TAA) can be divided into two groups: the first one involves the differentiation antigens that can be expressed in not only tumor, but also normal cells, whereas the second one comprises of the products of mutated or viral genes, which can be expressed exclusively in malignant cells. The vast majority of TAA belongs to the first group. These TAA can be expressed in a variety of tumors, due to commonality in the intracellular mechanisms involved in malignization of various types of cells. There is considerable interest in developing therapeutic vaccines for cancer, as they hold the promise of delaying or preventing cancer recurrence, particularly in early-stage disease patients. However, there exists a general problem with cancer vaccine application, because most of the TAA are represented by self, nonmutated proteins which are poorly immunogenic for the immune system [reviewed in 1]. Hence, overcoming the immune tolerance toward TAA is indeed a key task of cancer immunotherapy. The use of vaccines based on xenogenic TAA seems to be a promising approach to resolving this problem. Since TAA are typically evolutionarily conservative molecules, there is a strong homology between human and animal TAA. On the other hand, small interspecific structural differences of TAA can be advantageously used in constructing cancer vaccines because xenoantigens may potentially represent an "altered self", with sufficient differences from self-antigens to render them immunogenic, but with sufficient similarities to allow reactive T cells to maintain recognition of self [1].

2. Xenovaccinotherapy in animal models

The majority of studies concerning xenogenic vaccines have been carried out on animals with melanoma, the tumor that expresses a whole number of potentially immunogenic antigens. There is compelling evidence that xenogenic melanoma-associated antigens are much more effective in inducing antitumor immune responses in mice than are their murine analogs. For example, multiple immunizations of mice with human glycoproteins gp75 and gp-100 were reported [2-6] to be effective in preventing the growth of the syngeneic melanoma cells expressing the appropriate mouse analogs [6]. Interestingly, the murine gp75 being initially non-immunogenic became immunogenic in mice when it was administered in the form expressed on a membrane of insect cells [7]. This suggests that the membrane-bound xenoantigens that are not related to tumorigenesis are capable of contributing to self TAA-driven immune responses. The related melanosomal antigens appear to differ in immune response patterns which they induce. For example, the DNA vaccination of mice with human tyrosinase-related protein-1 (TRP-1/gp75) induced antibody-mediated responses and autoimmune depigmentation, whereas the DNA vaccination with human TRP-2 resulted in the generation of tumor-specific CD8⁺ cytotoxic T lymphocytes (CTL) and failed to elicit depigmentation [8].

As surgery is essential for any melanoma treatment, experiments have been performed to evaluate antitumor effects of xenovaccination in the postoperative period. It was shown that the postoperative DNA vaccination of mice with human TRP-2 could prevent the development of melanoma metastasis in the lungs [4]. These data suggest that xenovaccinotherapy can be the most effective when applied in addition to surgical treatment.

The polyantigenic vaccination has apparent advantages over the monoantigenic one in achieving clinically relevant antitumor responses, thank to its ability to simultaneously induce immune reactions directed against different TAA. We demonstrated that the survival in the melanoma-bearing mice vaccinated with destroyed human melanoma cells was noticeably superior than that in the control mice immunized with murine tumor cells. Surprisingly, the antitumor effect in this experimental model was associated with the rise of serum interleukin (IL)-4, but not interferon (IFN)- γ [9].

As already mentioned, the differentiation antigens are expressed not only in tumor but also in normal cells. This raises the possibility of obtaining such antigens from normal tissues in which they are highly expressed. Placenta is well known to express a whole range of differentiation antigens, including those shared by different tumors including melanoma [10]. Therefore, placental tissue, could be a suitable source of the xenoantigens applicable for breaking the immunological tolerance to a number of TAA. In fact, the mice that received the soluble proteins derived from the porcine placenta as a vaccine, demonstrated the immune protection from melanoma where both CD4⁺ and CD8⁺ T lymphocytes were involved [10].

The xenovaccination aimed at breaking the tolerance to a melanosomal antigen gp 100 has been applied in the treatment of 34 melanoma dogs [11]. Canine melanoma cells 17CM98 transfected with a DNA fragment encoding human gp 100 were used as a vaccine. With such vaccinotherapy, a complete or partial response was achieved in 17% animals, and disease stabilization with a duration of not shorter than 6 weeks was recorded in 35% vaccinated dogs. The clinical responses correlated with delayed-type hypersensitivity (DTH) skin reactivity to vaccinal antigens but was independent both of the functional activity of

vaccine-specific CTL and of the serum level of antivaccinal antibodies [11]. In this study the animals that responded to vaccination, survived significantly longer compared to those which did not [11]. An objective antitumor effect in certain dogs with the stage IV melanoma could be also achieved by their vaccinating DNA encoding human tyrosinase [12,13]. This effect was immediately related to occurrence of canine tyrosinase-binding antibodies in the sera [14]. No autoimmune complications and serious side effects of the xenovaccinotherapy were noted in the dogs [12,13]. Noteworthy is also that this therapy could be effectively used in combination with surgical treatment [15].

The ability of xenovaccination to break the immunological tolerance to TAA has been demonstrated in a murine model of breast cancer [16]. Protooncogene HER-2/neu is a self-antigen expressed by tumors and nonmalignant epithelial tissues. DNA vaccination of mice with human HER-2/neu was found to overcome the immunological tolerance to that protein and to induce the antibody-mediated, immune responses directed against both cancer and normal breast cells [16]. Yet, vaccinations of mice with a fragment of human HER-2/neu (435-443) induced generation of the CTL able to effectively recognize the syngeneic, HER-2/neu-positive tumor cells. Importantly, the CTL generation in these experiments depended on the age of vaccinated mice [17].

Survivin, a member of the inhibitors of apoptosis, is considered as an ideal vaccinal TAA, due to its broad expression pattern in many types of human malignancies. Dendritic vaccination of mice with human survivin was shown to induce the T-helper 1-mediated, immune responses, which were markedly enhanced by depleting CD25⁺, Foxp3⁺, CD4⁺ regulatory T cells. Noteworthy is that the generation of survivin-specific CTLs lacked in this model [18]. The antitumor effect of administrating human survivin in mice was also reported in the models of lymphoma [19], glioma [20, 21], and pancreatic cancer [19].

A high expression of epidermal growth factor receptor (EGFr) is attributable to different tumors including lung carcinoma and breast cancer [22]. It is likely that EGFr may be involved in autocrine and paracrine stimulation of tumor cell growth. Vaccination of mice with DNA encoding a extracellular part of EGFr was found to break the tolerance to murine EGFr and to induce immune responses directed against EGFr⁺ tumor cells. The antitumor effect observed in this model was mediated both by IgG antibodies and by CTL and associated with elevation of the serum concentration of IFN- γ , as well as of IL-4 [22].

Prostate-specific membrane antigen (PSMA) is a prototypical differentiation antigen expressed on normal and neoplastic prostate epithelial cells, and on the neovasculature of many solid tumors]. Immunizations of mice with human recombinant PSMA or DNA encoding PSMA were shown to induce the production of antibodies able to bind to murine PSMA; an effective vaccination was also demonstrated with administrating the xenoantigens prepared from the tumor prostate [23, 24].

A high expression of mesothelin is attributed to pancreas cancer. Therapeutic vaccination of mice with human mesothelin was found to result in inhibition of pancreatic cancer disseminating. Such a antitumor effect was associated with the rise in serum antimesothelin antibodies, as well as with an increase in the functionality of mesothelin-specific CTL [25].

Glioma membrane proteins (HGP) are typically expressed in the cells of malignant glioma. Therapeutic vaccination of rats with human HGP was demonstrated to result in the glioma growth inhibition that was mediated by the HGP-specific Th1 cells and characterized by a pronounced infiltration of tumor tissues with CD4⁺ and CD8⁺ T cells. In contrast to the human HGP, its murine analog lacked any antitumor activity [26].

Alfa-fetoprotein (AFP) is highly expressed in liver cancer. Vaccination with the recombinant rat, but not mouse AFP was found to provide a significant prolongation of survival in

hepatocarcinoma -bearing mice. The antitumor effect of such vaccination depended on functional activity of both CD4+ and CD8+ T-lymphocytes [27].

Unlike other cancers, the neuroendocrine tumors, such as a gastrinoma, insulinoma, and medullar thyroid carcinoma, do not demonstrate any clear association with expression of defined classes of membrane-bound TAA. Therefore, for generating antitumor immune responses in such cases, the approach has been offered based on breaking the immune tolerance to tumor-derived, soluble products. In a model of thyroid carcinoma it was demonstrated that therapeutic vaccination of mice with the human (but not murine) calcitonin resulted in a significant inhibition of tumor growth. The antitumor effect of this vaccination was associated with infiltrating the tumor by calcitonin-specific CTLs, as well as with a sharp decline in the serum level of calcitonin [28].

Tumor development requires neoangiogenesis. Therefore, the therapeutic approaches aimed at preventing growth of tumor-feeding vessels are in the focus of experimental and clinical studies. Theoretically, breaking the immunological tolerance to molecules involved in angiogenesis could restrain tumor growth. A fibroblast growth factor receptor-1 (FGFr-1) is one of such molecules. Vaccination of mice with recombinant chicken FGFr-1 was reported to be able to overcome the tolerance to endogenous FGFr-1, eliciting production of FGFr-1-specific, IgG autoantibodies [29].

Matrix metalloproteinase-2 (MMP-2) is well known to play an important role in angiogenesis and to promote tumor cell expansion in the body. Immunizations of mice with the LLC or CT26 tumor cells expressing chicken MMP-2 was found to induce antiangiogenic immune responses, resulting in the appearance of MMP-2-binding autoantibodies [30, 31].

Endoglin is a marker of angiogenesis in solid malignancies, including liver cancer. Therapeutic vaccination of mice with an extracellular portion of porcine endoglin was shown to induce immune responses directed against colorectal and lung cancers. The generation of such responses depended on functional activity of CD4+ T-lymphocytes and resulted in the appearance of endoglin-binding autoantibodies [32, 33]. A significant enhancement of antitumor effect was achieved when protein vaccination was combined with DNA vaccination. Such a combined vaccination induced not only the synthesis of endoglin-binding autoantibodies, but also the generation of endoglin-specific CTL [34]. An antitumor effect of DNA vaccination with porcine endoglin was also demonstrated in a murine model of liver cancer. This effect was mediated by both cellular and humoral immune reactions [35].

Tie-2 is an endothelium-specific receptor tyrosine kinase known to play a key role in tumor angiogenesis. Therapeutic vaccination of mice with human Tie-2 was found to be capable of exerting a negative effect on the growth of melanoma and hepatic cancer. This effect was dependent on functional activity of CD4+ T-lymphocytes and mediated by antibodies binding murine Tie-2 [36].

A pronounced antiangiogenic effect can be induced by vaccination with xenogenic whole endothelial cells [37]. This effect may be associated with overall tumor growth inhibition [38].

An antiangiogenic effect can be also achieved by inactivating soluble angiogenic molecules. For example, the vaccination of 9 dogs with spontaneous sarcomas by human endothelial cell growth factor (VEGF) resulted in the production of autoantibodies capable of binding both human and canine VEGF. The antitumor effect was observed in 3 (30%) vaccine-treated dogs. No complications of the vaccinotherapy were noted [39].

It appears that antiangiogenic immunotherapy can be effectively combined with breaking the tolerance to differentiation antigens in order to induce clinically relevant antitumor responses. For example, the administration of DNA encoding tumor endothelial marker 8 (TEM8) was able to enhance the tumor immunity in melanoma mice, induced either by rat neu or by human tyrosinase-related protein 1 (TYRP1/hgp75) [40]

3. Clinical application of xenovaccinotherapy

Prostate cancer, melanoma, colorectal cancer and renal cancer are usually resistant to the standard cytotoxic therapy, including highly toxic combinations. On the other hand, all of these cancers express TAA which are capable of inducing antitumor immune responses. Hence, immunotherapy has to become the mainstay in treating those cancers.

Prostatic acid phosphatase (PAP) is a differentiation antigen expressed by normal and malignant cells of prostate. Patients (n=21) with metastatic prostate cancer were multiply vaccinated with autologous dendritic cells loaded with mouse PAP. Such vaccinations were found to be safe to use, with no serious side effects being observed. An increased T-cell reactivity to murine PAP was observed in all of the vaccine-treated patients. Only 8 of 21 evaluable patients exhibited enhanced immune responses to human PAP. These responses associated with the enhanced production of IFN- γ and tumor necrosis factor (TNF)- α , but not IL-4 [41]

Immunologic effects of DNA vaccination with mouse tyrosinase have been assessed in 18 patients with melanoma, and the generation of tyrosinase-specific CD8+ memory T cells was found in 7 of them. No serious complications of such a treatment were noted [42]

It should be noted that immunizations with one or several tumor-associated, antigenic peptides frequently fail to control overall tumor development, creating favorable conditions for growth of the tumor cell clones lacking vaccinal determinants. Moreover, due to a high lability of cancer genome, there is an antigenic diversity even in tumor cells of the same origin [43]. Since whole tumor cells express a variety of TAA and are able to elicit a broad spectrum of immune responses, they could be more applicable to constructing cancer vaccines, compared to a single or just few antigenic peptides. However, immunizations with unmodified homologous (autologous or allogeneic) tumor cells have demonstrated only limited therapeutic success in cancer patients. There are two major reasons for the low immunogenicity of homologous cell vaccines. Firstly, most of the homologous TAA represents self-antigens, which are not inherently immunogenic. Secondly, antigen-presenting cells do not recognize the homologous tumor cells as potentially pathogenic targets that should be internalized and their antigens processed [43].

From the aforesaid, we favor a xenogenic cell-based vaccine. Because of their structural distinctions from homologous analogs, the xenoantigens are capable of effectively overcoming the immune tolerance to self-antigens, including TAA. Yet, all humans possess natural (preexisting) antibodies, which provide an acute rejection of any non-primate cells and function as a major barrier for the transplantation of animal organs to humans [44]. A significant part of these antibodies represents the Ig G specific to the α -gal epitope that is expressed abundantly on glycoproteins and glycolipids of non-primate mammals and New World monkeys [45]. In our point of view, by the opsonization of xenogenic tumor cells, the natural antibodies could promote internalization of tumor material in antigen-presenting cells via a Fc-receptor-mediated mechanism, and thereby enhance greatly the immunogenic presentation of TAA to tumor-specific T lymphocytes. This proposition is consistent with the published data indicating a critical role of the FcR-receptors in generating an effective

tumor immunity [46], as well as with the findings showing that the rejection of alpha-Gal positive tumor cells can efficiently boost the immune response to TAA present in alpha-Gal negative tumor cells [47].

A xenogenic polyantigenic vaccine (XPV) under study is composed of disrupted murine B16 melanoma and LLC carcinoma cells. The XPV is stored in the form of frozen-dried preparation and is suspended in physiological salt solution immediately before its administration. Since the XPV includes a wide spectrum of melanoma- and carcinoma-associated antigens, our opinion is that it may be applicable for treating different cancers.

The study with xenovaccination was performed in exact accordance with the protocol approved by the Scientific Council and Ethics Committee at the Institute of Clinical Immunology (Novosibirsk, Russia). Informed consent was obtained from every subject who underwent xenovaccinotherapy. Eligibility criteria included histologically proven, measurable disease, no prior immunosuppressive therapy for a minimum of 4 weeks, a good performance status (Karnofsky scale, 70% or more) and adequate marrow, renal and hepatic functions.

An inducing vaccinal course consisted of 10 subcutaneous immunizations (5 at weekly and 5 at fortnight intervals) and took about 3 months. Each vaccinal dose contained 75×10^6 (B16 + LLC) dead cells. Twenty-four hours following each of the first 5 vaccinations, a part of the patients was given subcutaneously a low dose of a non-oxidated recombinant IL-2. Since, when combined with XPV, IL-2 had no any significant effect on XPV-induced long-lasting immunoreactivity, its administration in the above- indicated way was recognized unpractical. Following an inducing course of the treatment each of the patients received supporting vaccinations monthly or less frequently. Throughout follow-up time the trial patients received no any systemic therapy other than immunotherapy.

A total of 152 patients with advanced forms of melanoma, colorectal or renal cancers have completed an inducing vaccinotherapy consisting of 10 immunizations and had adequate follow-up to monitor toxicity, immune responses and survival. No III-IV grade systemic toxicity associated with the vaccine administration was noted. During 24-to-48 h post vaccination only nearly 10% patients exhibited an influenza-like syndrome in the form of a small body temperature rise and musculoskeletal discomfort, which were usually self-limiting. Irritations at the injection sites were developed in the most patients in response to vaccination. Local manifestations usually disappeared within 72 h following vaccine injection. There were no treatment- related hospitalizations or mortalities.

Cell and biochemical blood parameters, as well as renal and hepatic functions, remained within the initial ranges throughout the inducing vaccinotherapy. Also there were no significant changes in subpopulation composition of PMBCs tested by immunofluorescence for expression of CD3, CD4, CD8, CD 20, and CD16 surface markers.

The development of systemic autoimmune disorders could not be excluded initially in XPV-treated patients because of the broad range of different antigens present in XPV. However, XPV-treated patients exhibited no evidence of any systemic autoimmune disorders. Their serum concentrations of a rheumatoid factor, but also of antibodies specific to DNA, cardiolipin, thyroglobulin, microsomal fraction of thyrocytes remained in the initial ranges throughout the inducing vaccinotherapy.

An inducing vaccinotherapy was found to increase detectably the serum concentrations both of IFN- γ and of IL-4. Yet it should be noted that an increase in the IFN- γ level was more common and greatly pronounced in the XPV-treated patients, compared with that in IL-4 level [48,49].

With inducing vaccinations, a remarkable increase in both T cell- and antibody-mediated immunoreactivity to vaccinal antigens was found in the majority of assessable patients. An

important aim of our study was to determine whether or not murine TAA would be capable of contributing to the generation of immune responses specific to human TAA. Indeed, our data clearly indicated that inducing vaccinations were able to significantly enhance T cell-mediated reactivity to human melanoma-associated antigens, while not affecting that to the control alloantigens [50, 51]

In our study an overall survival in the XPV-treated patients (n=51) with metastatic melanoma was evaluated through 7 year follow-up period. The control group was composed retrospectively of the patients who received conventional therapy, and had the initial clinical characteristics similar to those of the trial group. The characteristics of the trial and control patients are presented in Table 1. If it was reasonable and possible, both trial and control patients underwent a cytoreductive palliative surgery. As shown in Figure 1, the median survival in the XPV-treated patients was significantly longer than that in the control patients (14 *vs.* 6 months, respectively; $p < 0.05$). Of note is that almost all of those trial patients, who have survived for 2 years after immunotherapy initiation, further survived as long as 7 years and longer. The overall 7-year survival rate in XPV-treated and control patients was 20% and 0%, respectively. More impressive results were obtained when a long-term overall survival has been analyzed in the stage III melanoma patients (n=48; 26 females and 22 males aged from 22 to 76 years). The control group was composed retrospectively of the clinically comparable patients (n=27; 12 females and 15 males aged from 35 to 77 years). Initially, each of assessable patients had a high risk of disease recurrence. As shown in Figure 2, an overall 6 year survival rate in the trial group was 54%, whereas that in the control group only 25%. It is important to note that a better survival in melanoma patients was associated with their increased DTH to the vaccinal B16 melanoma antigens [49,50].

Characteristic	Trial	Control
Number of patients	51	32
Males/females	25/26 (49%/51%)	10/22 (31%/69%)
Age, years (median, range)	51.8±2.4 (18-72)	48,2±2,3 (24-77)
Site of metastases:		
Lung	16 (31%)	6 (19%)
Liver	10 (20%)	7 (22%)
Lymph node, skin/soft tissue	34 (67%)	26 (81%)
Other organs	8 (15%)	8 (25%)

Table 1. Characteristics of stage IV melanoma patients assessable for survival.

We also evaluated a long-term overall survival in the 35 XPV-treated patients with stage IV colorectal cancer. The control group was composed retrospectively of the patients (n=35) who received conventional therapy. Since the trial patients were very heterogenous in their clinical characteristics, each control patient was randomly selected to be a clinically comparable counterpart of a trial patient, thus control and trial groups were evenly balanced by both prognostic and clinical parameters. The characteristics of colorectal cancer patients are presented in Table 2. As shown in Figure 3, the median survival in the XPV-treated patients was significantly longer when compared with that in the control patients (18 *vs.* 8 months, respectively; $p < 0.05$). An overall 2-year survival rate in the trial and control group was 27% (10 patients) and 3% (1 patient), respectively. However, patients in the trial group almost completely lost their survival advantages as early as at 3.5 years after the immunotherapy initiation.

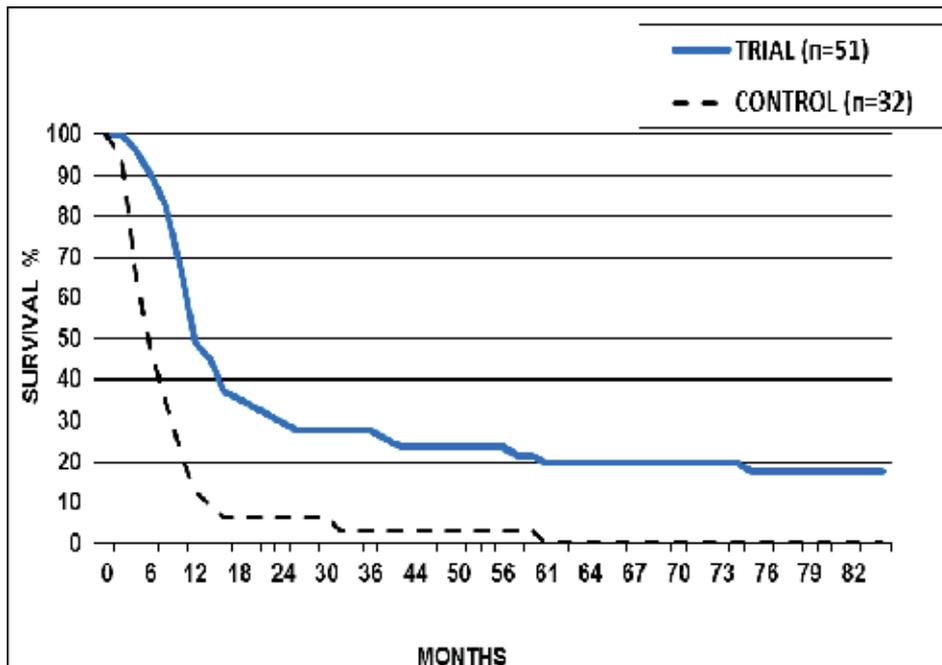


Fig. 1. Survival in the patients with stage IV melanoma. See text for further details.

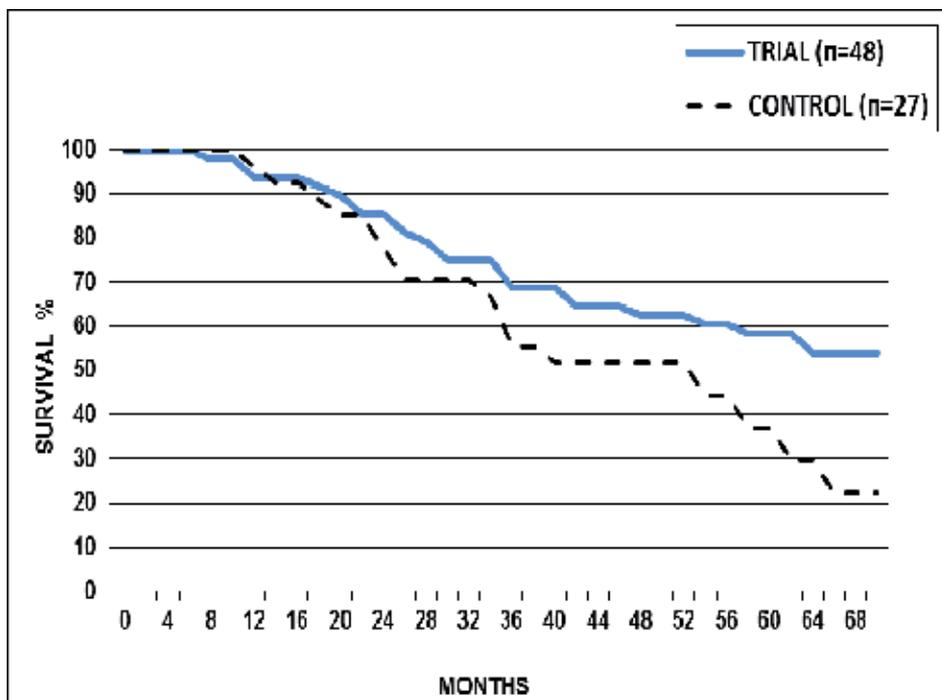


Fig. 2. Survival in the patients with stage III melanoma. See text for further details.

Characteristic	Trial	Control
Number of patients	35	35
Males/females	19/16 (54%/46%)	19/16 (54%/46%)
Age, years (median, range)	61.1 ± 1.4 (38- 79)	55.6 ± 1.7 (30 - 80)
Site of metastases:		
Lung	7 (20%)	6 (17%)
Liver	25 (71%)	19 (54%)
Lymph node,skin/soft tissue	17 (48%)	15 (43%)
Other organs	11 (31%)	8 (23%)

Table 2. Characteristics of stage IV colorectal cancer patients assessable for survival

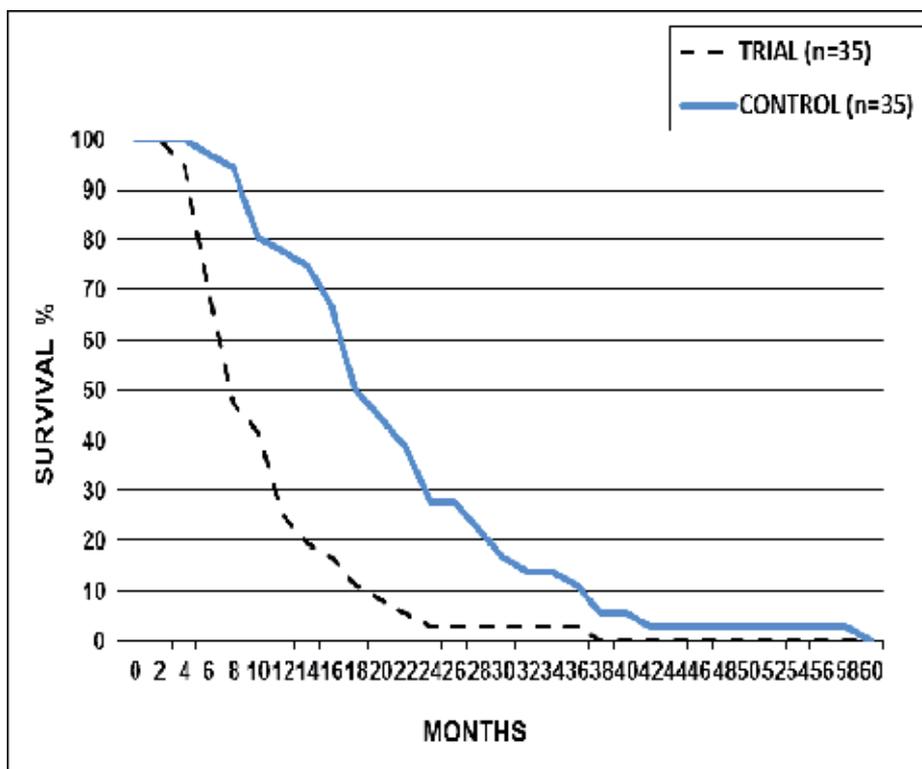


Fig. 3. Survival in the patients with stage IV colorectal cancer. See text for further details.

Figure 4 characterizes a long-term overall survival in the 16 XPV-treated patients (5 females and 11 males aged from 54 to 76 years) with stage IV renal cancer. The control group was composed retrospectively of clinically comparable patients (5 females and 11 males aged from 49 to 77 years) . The median survival in the trial patients was found significantly longer when compared with that in the control patients (20 vs 8 months, respectively; p<0.05). Noteworthy is that patients in the trial group maintained the certain survival benefits from the immunotherapy throughout 5 year follow-up period.

Overall, our results point out that the XPV-based therapy is safe for clinical use, and has no toxicity that is attributable to current standard treatments for cancer. It is also important that

the XPV-treated patients exhibited no evidence of systemic autoimmune disorders, of which a risk of development could significantly limits clinical application of polyantigenic xenovaccination .

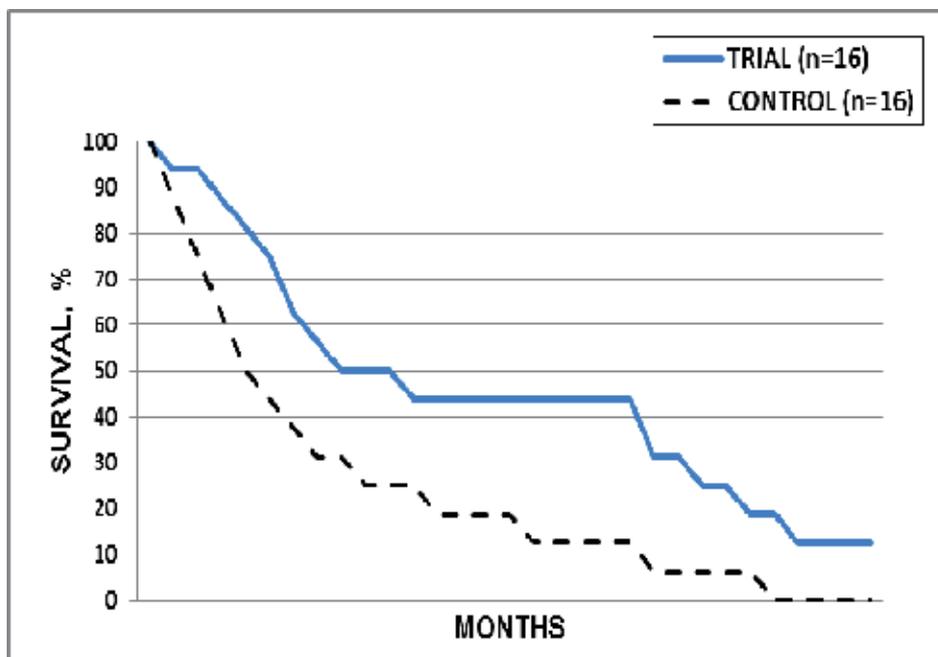


Fig. 4. Survival in the patients with stage IV renal cancer. See text for further details.

It appears that the xenogenic antigens, not only tumor-associated, but also those inherent to normal cells, can be involved in XPV-induced immune responses. As evidenced by both cell- and antibody-mediated reactions [50,51], the immune-mediated sensitization to murine TAA observed in the XPV-treated patients was detectably greater than that to murine spleen cells antigens. This may imply that the antigens associated with tumor cell phenotype might be more significant in generating XPV-driven immune processes than those being only expressed in fully differentiated cells.

Our data demonstrated that the xenovaccinations resulted in serum level elevations of not only of IFN- γ , but also of IL-4, suggesting intensification of both T helper 1- and T helper 2-mediated immune responses in XPV-treated patients [48, 49]. These findings are of great importance in the light of previously reported data that indicate a critical role for cooperating T cell- and antibody-mediated mechanisms in generating tumor cytotoxicity *in vivo* [46].

According to our experience [50, 51], the xenovaccinotherapy can result in generating complete or partial clinical responses in a certain portion of cancer patients. Nevertheless, stabilization of the disease appears to be the most common outcome of effective immunotherapy in advanced cancer patients. The XPV-based therapy is not an exception in this regard. Unlike the cytotoxic chemotherapy, tumor vaccine-based approaches may permit the host to reach a state of balance with the tumor, in which the net result of tumor growth and destruction is zero. That might lead to more significant survival benefits than a rapid destruction and rapid regrowth of the tumor following cytotoxic therapy.

Actually, our results suggest that the polyantigenic xenovaccinotherapy can significantly affect survival in cancer patients. It should be noted that the majority of patients entered into

our investigations were with very advanced (stage IV) disease. It is reasonable to anticipate that, as with other immunotherapies, the XPV-based therapy might be maximally effective when being applied as early as possible following surgical resection of the prime tumor. Consistent with this assertion, the most survival benefits from immunotherapy were noted in the group of stage III melanoma patients when xenovaccinotherapy was initiated before appearing distant metastasis lesions.

4. Conclusion

From the data mentioned above it appears that there are two main ways of using cancer xenovaccinotherapy: the first approach is directed on activation of the immune system against membrane-bound and soluble TAA, and the second one is aimed at overcoming the immune tolerance to the proteins that promote tumor progression. The most antitumor effects are likely to be expected when vaccinal xenogenic TAA elicit both cellular and humoral immune reactions. The present paper is the first demonstration of the positive effect of polyantigenic xenovaccinotherapy on a long-term survival patients with advanced cancers. Although the results are extremely encouraging, they must be interpreted with caution because they are based on a small number of patients with very advanced disease.

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Cancer Vaccine

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1. Introduction

According to the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008; of these, 56% of the cases and 64% of the deaths occurred in the economically developing world (Jemal et al., 2011).

It is now 51 years since Macfarlane Burnet and Peter Medawar won the Nobel Prize in Physiology or Medicine for the discovery of acquired immunological tolerance, and Burnet's 'hypothesis that called for experiment' has driven an enormous amount of progress. A recent advance in anti-cancer therapies has been the use of cancer antigen to develop vaccines. However, immunization with cancer cell-based vaccines has not resulted in significant long-term therapeutic benefits. The search for human tumor antigens as potential targets for cancer immunotherapy has led to the discovery of several molecules expressed mainly or selectively on cancer cells.

Vaccination is an effective medical procedure of clinical oncology setting based on the induction of a long-lasting immunologic memory characterized by mechanisms endowed with high destructive potential and specificity. In the last few decades, identification of tumor-associated antigens (TAA) has prompted the development of different strategies for antitumor vaccination, aimed at inducing specific recognition of TAA in order to elicit a persistent immune memory that may eliminate residual tumor cells and protect recipients from relapses. Current data from trials with cancer vaccine for patients with advanced cancer are however not uniform. Because enormous problems arise from the variability of protocols in the preparation of vaccine, such as dendritic cell-based or peptide vaccine, and the vaccination itself.

Widely occurring, over-expressed TAAs have been detected in different types of tumors as well as in many normal tissues, and their over-expression in tumor cells can reach the threshold for T cell recognition, breaking the immunological tolerance and triggering an anticancer response. Many antigens have been identified and studied as potential targets for vaccine therapy, and several vaccine methods have been investigated to target them. The most well-studied and promising vaccines for the treatment of cancer can be subdivided into three main groups: antigen-specific vaccines, tumor cell vaccines, and dendritic cell vaccines.

Active immunotherapy is aimed either at eliciting a specific host immune response against selected cancer antigens by employing cancer vaccines or at amplifying the existing antitumor immune response by administering nonspecific proinflammatory molecules or adjuvants. Dendritic cells (DCs) are the most potent antigen-presenting cells in vitro and in

vivo. DCs have a central function in the activation of specific effector T cells. On this basis, vaccination strategies with DC were regarded as a promising therapeutic approach even in advanced tumor diseases. DC have always been described as having two distinct functional stages: 1) immature, with high antigen uptake and processing ability, and poor T-cell stimulatory function; 2) mature, with high stimulatory function and poor antigen uptake and processing ability. DC internalize cancer antigens and process their proteins then display them as short peptides on the extracellular surface, in conjunction with major histocompatibility complex (MHC) class I and II molecules. DC then migrates into the corresponding lymph nodes, where it matures and present antigen to naïve T lymphocytes. Helper T cells (CD4⁺) recognize their cognate antigens (MHC class II molecules) located on DCs, whereas CD8⁺ cytotoxic T lymphocytes (CTLs) recognize affected foreign or cancer cells which display the complementary peptide-MHC class I molecule on their cell surfaces. Targeted cell death occurs by perforin/granzyme-induced apoptosis or FAS-L/Fas interaction. Activation of CD4⁺ T cells leads to the secretion of cytokines such as IFN- γ and IL-12, which in turn augment the stimulation of active CD8⁺ T cells. Cancer vaccine aimed at inducing specific recognition of TAA as well as eliciting persistent immune memory T lymphocytes. Programmed death-1 (PD-1) and anti-cytotoxic T lymphocyte-associated protein 4 (CTLA-4) are induced on T cells after a TCR signal, and result in cell cycle arrest and termination of T-cell activation. Blocking by either CTLA-4 or PD-1 monoclonal antibodies can sustain the activation and proliferation of tumor-specific T cells (Hirano et al., 2005; Hodi et al., 2008). Although, to date, no autologous cellular immunotherapy has gained wide use in clinical practice, the first such therapy to show clinical efficacy in a phase 3 study recently gained U.S. Food and Drug Administration (FDA) approval for the treatment of prostate cancer. Sipuleucel-T consists of autologous PBMCs loaded with recombinant human prostatic acid phosphatase (PAP) linked to granulocyte-macrophage colony stimulating factor (PAP-GM-CSF), which has proven to be effective in phase III clinical trials. DC based Vaccine are typically prepared by harvesting large numbers of autologous peripheral blood mononuclear cells (PBMCs) by leukapheresis, then culturing these cells and loading them with antigens ex vivo and injecting them back into the patient. Three general methods have been described concerning DC based vaccine: (1)differentiating DCs from non-proliferating monocyte precursors (so-called “monocyte-derived DCs”); (2)differentiating DCs from proliferating CD34⁺ hematopoietic progenitor cells; or (3)directly isolating DCs or mixed APCs from periphereal blood. Autologous DC can be loaded with a wide assortment of antigen types, including whole tumor cells or cell lysates, or TAA in the form of synthetic peptides, purified or recombinant proteins, RNA, plasmid DNA or non-replicating recombinant viral vectors (Mayordomo et al., 1995; Thurner et al., 1999). Immunogenicity may be enhanced by using antigens combined or fused with other more immunogenic molecules, including xenogeneic proteins such as Keyhole Limped Hemocyanin (KLH) or IL-2, TNF- α , IFN- γ or Toll-like receptor agonist. Adapting single peptide for vaccine is not preferable, because after complete objective response to NY-ESO-1 peptide vaccine, but later recurred with a NY-ESO-1-negative tumor, proving that single-target immunization can result in immune escape tumor variants after initial response (Odunsi et al., 2007). A desirable alternative to vaccines are multiepitope or whole tumor antigen vaccines created using autologous tumor lysate or tumor-derived RNA, which may have universal applicability (Chianese-Bullock et al., 2008; Tsuda et al., 2007). However, the immune responses are often weak, and data on clinical efficacy are limited, as most of these have been small, single arm studies designed only to evaluate safety and immunogenicity. An enormous problem arises from the variability of protocols in the

preparation of DC and in the vaccination itself. A meta-analysis of 56 published peer-reviewed immunotherapy trials of melanoma that used either molecular defined synthetic antigens or whole tumor antigen (4,375 patients) found that only 25.3% of patients vaccinated had objective clinical control (Chi & Dudek, 2011).

A number of studies have found that development of tumor and an unfavorable prognosis for cancer patients were accompanied by accumulation of natural CD4⁺CD25⁺Foxp3⁺ T regulatory cells (Tregs) in peripheral blood, as well as of peripherally induced Tregs in the tumor itself (Wilczynski et al., 2008). Furthermore, depletion of Treg is a critical maneuver to enhance vaccine therapy. Different therapeutic immune strategies have been tested preclinically and are currently in evaluation in early phase I and II trials. DC based vaccine is usually given to peripheral site, whereas Natural Killer (NK)-T cell and LAK are either delivered systematically or into the tumor site. Results from these trials vary, but the overall increased survival and/or clinical benefit obtained so far has been limited. In addition, MHC expression level vary cancer type and stage, it seems difficult to eradicate cancer just administering vaccine. Because CTL induced by vaccine targets MHC expressed cancer cell, whereas NK cell attacks MHC non-expressed cancer cell.

Only three randomized phase 3 clinical trials of DC/APC vaccines for the treatment of cancer have been published. The first study compared subcutaneously administered cytokine-matured, Mo-DCs loaded with a mixture of MHC class II and II-restricted peptide antigens to conventional chemotherapy in patients with stage IV melanoma. Designed to compare clinical response rates as measured by tumor regression, the study showed no statistically significant difference in clinical outcomes between the two treatments. With the FDA-approval of sipuleucel-T, cancer vaccine has become an accepted approach for the treatment of cancer. However, it is not known if the use of dendritic cells or mixed APCs for the active immunotherapy of cancer has an advantage over more conventional vaccine approaches, which are simpler and much less expensive. We usually propose WT1, MUC1, CEA, CA125, HER-2/neu, and PSA as cancer antigens for DC based therapy according to the patient's primary lesion and elevated tumor marker (Sugiyama, 2005; Mukherjee et al., 2000; Nair et al., 1999; Larbcurrentet et al., 2007). It has been reported that WT1 and MUC1 is antigens with high immunogenicity and their-targeted immunotherapy have confirmed its safety and clinical efficacy, although there is few description concerning cancer vaccine adapting WT1 and MUC1 simultaneously to cancer antigen (Ramanathan et al., 2005). Dr Okamoto and his colleagues have already reported that OK-432 generates mature DCs via Toll-like receptor 4 signaling and that OK-432-activated DCs stimulates CD8⁺ T cells to induce antigen-specific CTLs (Ahmed et al., 2004, Itoh et al., 2003; Nakahara et al., 2003; Okamoto et al., 2003, 2004, 2006; Oshikawa et al., 2006). In this analysis efficacy of cancer vaccine, different potential means of DC based vaccination in experimental settings and preliminary data from clinical trial have been examined.

2. Material and method

2.1 Patients, treatment and sampling

This retrospective study was carried out in accordance with the standards of our Institutional Committee for the Protection of Human Subjects. Eligible patients must be those who have failed standard treatment. Informed written consent according to the Declaration of Helsinki was obtained from all patients before giving this therapy, and the

collection of the samples was approved by the Institutional Review Board. From 2007 to 2010, 127 patients with advanced cancer refractory to standard treatment were treated with DC-based immunotherapy (DC vaccine alone or DC vaccine plus NK-T cell therapy) at Kudan Clinic Immune Cell Therapy Center.

Initial patient evaluations included a medical history and physical examination; measurement of performance status, hemoglobin, WBC count, platelet count, blood urea nitrogen, creatinine, alkaline phosphatase, lactate dehydrogenase, AST, ALT, bilirubin, and tumor marker levels; HbA1c; Computed Tomography (CT) scans or Magnetic Resonance Imaging (MRI) of whole body. Patients with evidence of operable tumor were ineligible. To be eligible, patients were required to have an ECOG performance status of less than 3.

Eligible Adequate hematologic, hepatic, and renal function, within the following parameters: WBC count of 2,500/ μ l or greater; platelet count of 100,000/ μ l or greater; hemoglobin value of 10 g/dl or greater; blood urea nitrogen value less than 50 mg/dl; serum bilirubin level less than 5.0 mg/dl; AST level lower than 500 IU.

Autologous DCs (1×10^7 cells) were administered intradermally at 14-day intervals. Tolerable 1 to 5 KE of OK-432 (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan), a streptococcal immunological adjuvant, was administered together with DC vaccine. NK-T cells were simultaneously injected in as many patients at 14-day intervals.

The clinical response was evaluated on the basis of the Response Evaluation Criteria in Solid Tumors (RECIST) Ver1.0 as follows: complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD). Adverse events were evaluated by grading the toxicity according to the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0.

2.2 Preparation of DCs and NK-T cells

PBMCs-rich fraction was obtained from leukapheresis (400 ml \times 13 cycles) using COM.TEC (Fresenius Kabi, Homburg, Germany). The PBMCs were isolated from the heparinized leukapheresis products by Ficoll-Hypaque gradient density centrifugation (Böyum, 1967). These PBMCs were placed into 100 mm plastic tissue-culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) in AIM-V medium (Gibco, Gaithersburg, Md). After 30 min of incubation at 37°C, nonadherent cells were removed, and the adherent cells were cultured in AIM-V containing granulocyte-macrophage colony stimulating factor (GM-CSF, 500 ng/ml; Primmune Inc., Kobe, Japan), and IL-4 (250 ng/ml; R&D Systems Inc., Minneapolis, MN) to generate immature DCs (Okamoto et al., 2004). The population of the adherent cells remaining in the wells was composed of $95.6 \pm 3.3\%$ CD14⁺. After 5 days of the cultivation, the immature DCs were stimulated to be matured with OK-432 (10 μ g/ml) and Prostaglandin E2 (50 ng/ml; Daiichi Fine Chemical Co. LTD., Toyama, Japan) for 24 hrs. It has been reported that Prostaglandin E2 acquires the ability to migrate to the lymph node to DCs (Sato et al., 2003). Peptides (20 μ g/ml) for WT1, Her2 and CEA were pulsed into the DCs at 24 hrs after the treatment with OK-432 and with Prostaglandin E2, while MUC1 long peptide (30 mer) (20 μ g/ml), CA125 protein (500 U/ml) and autologous tumor lysates (50 μ g/ml) were added into the DC culture media at the same time as adding OK-432 and Prostaglandin E2, then incubated for 24 hrs (Kontani et al., 2002; Cannon et al., 2004). To prepare the autologous tumor lysates, tumor masses were obtained by surgical resection exclusion, and were then homogenized. Aliquots of the isolated tumor cells were then lysed by putting them through 10 freeze (in liquid nitrogen) and thaw (in a 37 °C water bath)

cycles. The lysed cells were centrifuged at 14000 g for 5 min, and the supernatants were passed through a 0.22 μm filter (Millipore Corporation, Bedford, MA). The protein contents of the resultant cell-free lysates were determined using DC protein assay kits (Bio-Rad Laboratories, Hercules Aliquots (500 μg /tube) were then cryopreserved at $-135\text{ }^\circ\text{C}$ until use (Nagayama et al., 2003). Surface molecules expressed in the DCs were determined using flow cytometry. The cells defined as the mature DCs were CD14⁺, HLA-DR⁺, HLA-ABC⁺, CD80⁺, CD83⁺, CD86⁺, CD40⁺, and CCR7⁺.

For preparation of NK-T cells, PBMCs were cultured with an immobilized monoclonal anti-CD3 antibody (5 μg /mL OKT3; Jansen Pharmaceutical K.K., Tokyo, Japan) in the presence of recombinant human IL-2 (175 U/mL; CHIRON, Benelux B.V., Amsterdam, Netherlands) and autologous plasma for 14 days. Fresh NK-T cells were prepared every injection as described above; it was composed of more than 85% of $\alpha\beta$ -T cells and about 10% of NK/NKT cells.

2.3 Vaccine quality control

All vaccines were subjected to a quality-control evaluation, which were assessed the total number of live dendritic cell, monocyte-derived dendritic cell characteristics, and percentage of viable cells. For a vaccine to be deemed "adequate," there must have been 4×10^7 viable dendritic cells.

2.4 FACS analysis

The frozen cells were allowed to thaw in a $37\text{ }^\circ\text{C}$ water bath quickly and retrieved from the cryopreservation tubes by rinsing with 0.02% albumin containing Cell Wash™ (eBioscience, San Jose, CA) (FACS buffer). The FACS analysis was performed for cell surface antigen staining. FITC-labeled anti-human CD14, CD40, CD80, HLA-A, B, C, PE-labeled anti-human CD11c, CD83, CD197 (CCR7⁺), HLA-DR and FACS Calibur Flowcytometer were purchased from BD Bioscience, and used for the FACS analysis.

3. Results

3.1 Clinical outcome of patients with DC-based vaccine

Computed tomography scans or MRI was done before and at the end of dendritic cell therapy. Of 127 patients who received DC vaccine, complete responses ($n = 4$; 3.1%), partial responses ($n = 26$; 20.5%), or stable disease were observed in 34 (26.8%) (Table 1).

Although the study was not designed or powered to detect differences between other vaccine treatment groups, it was of interest to compare the response and survival of patients treated with other vaccines.

Most patients received NK-T therapy in combination with the DC vaccination as to induce Th (helper T cell) 1-dominant state for improved CTL response and to attack non-MHC expressed carcinoma cells. The NK-T cells generated according to the methods described in the "Materials and Methods" section secrete IFN- γ and IL-2, and induce helper T cell (Th) 1-dominant state in the cytokine balance of the patients (Chong et al., 1994).

3.2 Adverse events

Therapy was well-tolerated during the treatment and 3 months after last administration. None of the patients experienced adverse events of grade 3 or higher during the treatment period, grade 1 to 2 fevers, grade 1 injected-site reaction consisting of erythema, induration

Cancer type	patient No.	CR (%)	PR (%)	SD (%)	PD (%)	total (%)
esophagus	10	0	20.0	10.0	70.0	100
gastric	24	0	16.7	20.8	62.5	100
colorectal	9	0	22.2	44.4	33.3	100
hepatocellular	8	12.5	25.0	12.5	50.0	100
pancreas	18	5.6	27.8	38.9	27.8	100
lung	14	7.1	21.4	42.9	28.6	100
breast	21	0	9.6	33.3	57.1	100
gynecological	3	0	0	33.3	66.6	100
malignant lymphoma	3	33.3	0	0	66.6	100
prostate	10	0	60.0	20.0	20.0	100
thyroid	3	0	0	0	100	100
malignant melanoma	4	0	0	0	100	100
total (No.)	127	4	26	34	63	100
total (%)		3.1	20.5	26.8	49.6	100

Table 1. Clinical Outcome of Patients treated with DC based vaccine

and tenderness, lasting 24 to 48 hours after injection in 8 patients and resulted in no dose modifications or delays. No signs or symptoms of auto-immune phenomena (eg, arthritis, colitis, inflammation of skin) were observed either during or after therapy.

4. Discussion

Interest in antitumor vaccination arose around 1900 when a series of microbial vaccines by Dr William B. Coley proved to be effective. Boon T and others provided an unambiguous definition of TAA, an important finding the genetic and molecular identification of a large series of TAA (Coulie, 1997; Robbins & Kawakami, 1996).

In many cases, TAA are peptides presented by class I and class II glycoproteins of the MHC. A similar picture is emerging from phase I studies on vaccination of cancer patients. However, clinical responses to the immunotherapy with DC vaccination have only been observed in a minority of patients with solid cancer. Initiation of immune responses requires that professional APC deliver a first signal to T-lymphocytes through the binding of the T-cell receptor by the peptide enclosed in the HLA molecule, that is responsible for the specificity of the immune response, and a second or co-stimulatory signal that is not antigen-specific but it is required for T-cell activation mainly through CD80 (B7-1) and CD86 (B7-2) binding to CD28 receptor, or the CD40:CD40L pathway (Janeway & Bottomly, 1994). Moreover, the capacity of DC to activate NK cells by ligation of the CD40 molecule with its counter-receptor has recently been demonstrated (Cayeux et al., 1999; Kitamura et al., 1999).

Therefore, given the complex network of regulatory signals by professional APC and naïve and memory T lymphocytes occurring in antigen-specific immune responses, it is not surprising that tumor cells may fail to induce efficient immune reactions even when a well known TAA is present. From among the professional APC, DC are the most potent stimulators of T cell responses and play a crucial role in the initiation of primary immune responses (Banchereau & Steinman, 1998). DC have always been described as having two distinct functional stages: 1) immature, with high antigen uptake and processing ability, and poor T-cell stimulatory function; 2) mature, with high stimulatory function and poor antigen uptake and processing ability. Despite several immunotherapeutic approaches having been tested for colon cancer patients, only one study has reported clinical results in a prospective randomized study (Vermorcken et al., 1999). Experimental data and clinical evidence suggest that antitumor vaccines will be a new form of tumor treatment that will be able to be adopted for the management of defined stages of carcinoma, in sequential association with conventional treatments (Sadanaga et al., 2001). Prediction of when the efficacy of antitumor vaccination will be assessed and will become a routine procedure is beyond a simple scientific evaluation. While pre-clinical research has identified several possible targets and strategies for tumor vaccination, the clinical scenario is far more complex. The main cell populations taking part in immunoregulation of tumor growth are presented in Fig. 1.

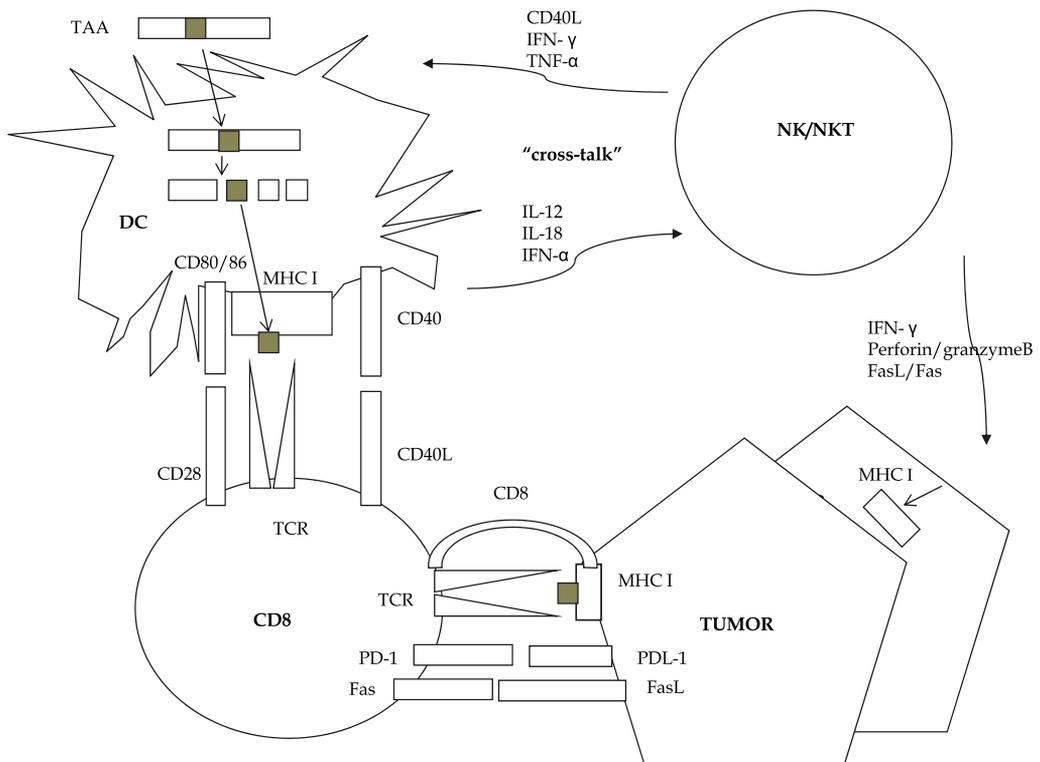


Fig. 1. The main anti-tumor immune cell responses by DC and NK/NKT cell

Most peptide-based vaccines have considered HLA class I restricted peptides only, whereas there is increasing evidence that tumor-specific CD4⁺ T-cells may be important in inducing an effective antitumor immunity. The addition of peptides that bind class II HLA glycoproteins to peptide vaccines could lead to an amplification of the immune response as well as to better clinical effect. The possibility of effectively monitoring the immune response induced acquires critical importance since it may provide a much earlier surrogate end-point, predictive of the clinical outcome. An ideal TAA is a protein that is essential for sustaining the malignant phenotype, and that is not stripped or down modulated by the immune reaction. TAAs were sorted by their anti-tumor potential such as therapeutic function, immunogenicity, oncogenicity, specificity, expression level and % positive cells, and cancer stem cell expression. Among the 75 peptides evaluated by Dr Martin A. Cheever, WT1 was the 1st and MUC1 was the 2nd anti-tumor effect (Cheever et al., 2009). WT1 was originally identified as tumor suppressor gene for Wilm's tumor. WT1 over-expression has been detected in different malignant cell types including gastroenterological carcinoma, gynecological carcinoma, lung carcinoma, prostate, breast carcinoma and hematological malignancy. MUC1 is expressed at high levels over the entire surface of diverse types of carcinoma cells such as gastroenterological carcinoma, gynecological carcinoma, NSCLC, prostate and breast carcinoma. MUC1 transmembrane receptor has revealed a function for this subunit as an oncoprotein that is targeted to the nucleus and regulates gene expression. MUC1-C accelerates the malignant potential by regulating gene transcription, blocking stress-induced apoptosis and necrosis, and attenuating activation of death receptor pathways.

When compared with conventional cancer management, vaccination is a *soft*, non-invasive treatment free from particular distress and iatrogenic side effects. Antitumor vaccines can be expected to have a considerable social impact, but a few large clinical trials enrolling the appropriate patients are now necessary to assess their efficacy. In conclusion, even if cancer vaccines are an old dream, only recently has their design become a rational enterprise (Ehrlich, 1909). There are now many ways of constructing vaccines able to elicit a strong protective immunity. This progress is offering ground for optimism.

Here, we demonstrate that WT1 and/or MUC1 pulsed DC vaccination is feasible, safe, and sufficiently powerful to induce objective clinical and immune responses even in patients with significant tumor burden. Several studies of *ex vivo*, custom-manufactured cancer vaccines using patient-specific idiotypic, idiotypic-pulsed dendritic cells, and tumor lysate-pulsed dendritic cells have also demonstrated objective clinical responses and these prior results prompted our pursuit of the practicable *in situ* approach.

This approach-by its nature-must be studied in patients with clinically evident disease, in contrast to the described randomized studies (Flowers, 2007; Freedman et al., 2009). As compared with other comparably practical vaccines current vaccination has the potential advantage of using more potential TAA encompassing each individuals' relevant tumor antigens. In addition, several recent immune-response studies showing that vaccine-induced T cells peak at day 14 and decline sharply thereafter, have prompted earlier immune-response measurements in an ongoing follow-up study (Deng et al., 2004; Kim et al., 2007; Treanor et al., 2006). There is little or no controversy that patients with Treg-inducing tumors had poorer clinical outcomes after vaccination. This biomarker could be either a specific predictor of response to *in situ* vaccination or a general prognosticator of poor outcomes regardless of therapy. Interestingly, patients with highly Treg-infiltrated tumors have shown favorable

clinical outcomes after standard therapy (Carreras et al., 2006; Tzankov et al., 2008). If Treg induction predicts good response to standard therapy, but a poor response to the in situ vaccine, then it would be a powerful clinical tool for selecting appropriate patients for vaccination. This interesting finding is still preliminary and is being evaluated prospectively in an ongoing follow study (ClinicalTrials.gov-ID: NCT00880581). That the vaccine preparation in current study was optimal was evident from quality-control assessments, because in the study presented here, all vaccines didn't fail to meet quality-control specifications. As prognosis of most advanced carcinoma who failed standard therapy is poor, establishment of effective therapeutic modality for advanced carcinoma is an urgent issue.

Immunotherapy would be implied as one of the important therapeutic modalities against advanced carcinoma and even adjuvant settings because WT1 and MUC1, highly immunogenic target molecules for adaptive anti-tumor immune response, were frequently expressed in most carcinoma tissue (Cheever et al., 2009; Oka et al., 2006). DC-based vaccination has several advantageous aspects for induction and activation of tumor antigen-specific CTLs compared with CTL-epitope peptide-based vaccination (Melief & van der Burg, 2008). Patients with advanced carcinoma who failed standard therapy and met eligible criteria enrolled in current study. Response rate was 23.6%, whereas control ratio was 50.4%. It is a significant tumor control ratio compared with other historical modalities much less no severe adverse event. Is the strongest result from this trial the apparent increase in control ratio? It would be important to understand the mechanisms of immune system underlying the significant increase in cancer control ratio. These results indicate that WT1 and/or MUC1 pulsed DC-based vaccination can elicit significant clinical benefit even for the advanced cancer patients refractory to the standard therapies. Although there was a trend toward treatment being superior to standard treatment only, there was no statistical consideration. However, the study demonstrated that successful active specific immunotherapy with WT1 and/or MUC1 pulsed DC-based vaccine may be dependent on the quality of the vaccine as well as TAAs. These encouraging preliminary results suggest that WT1 and/or MUC1 pulsed DC-based vaccination warrants further study as a novel therapy for patients with advanced carcinoma. The combination of cytotoxic therapy and intratumoral immune stimulation has been studied preclinically for a variety of common tumor types and might also be directly translated to the clinic (Meng et al., 2005; Najar et al., 2008; VanOosten & Griffith, 2007). This trial clearly supports the idea that to be immunologically effective, control of the vaccine preparation and the quality assurance that the vaccine meets specifications are of the highest priority and must be considerations in any future tumor cell vaccine study. A key element in these novel strategies is the identification of suitable patients, the selection being based on detailed immunological and molecular characterization. The most promising finding that emerges from this study is that WT1 and/or MUC1 pulsed DC-based vaccine together with NK-T cell therapy elicit strong anti-tumor response. Progress in the formulation of cancer vaccines will be brought by a more precise knowledge of the requirements for the potent generation of efficient CTL induction and NK cell expansion as well as discovering potent TAA, together with the current ability to closely monitor molecular immune response prediction markers in, will likely provide powerful, individualized vaccines in the near future.

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6. References

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The Management of Small Renal Tumours by Ablative Therapies

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1. Introduction

Renal cell carcinoma (RCC) was the 9th commonest malignancy in Europe in 2008 [1] with an estimated 88400 new cases and 39300 deaths [2] making it the most lethal urological malignancy. Over the last 2 decades there has been a significant increase in the incidence of small renal masses (SRMs) at diagnosis often as an incidental finding as a result of abdominal imaging for the investigation of pain or other abdominal symptoms [3]. This has resulted in a stage migration to smaller and lower stage lesions in asymptomatic patients [4]. Many of these SRMs are slow growing and of low malignant potential although the precise natural history remains unclear [5]. The rate of radiographic growth in most series which have followed renal masses usually for 3 years is between 0 and 0.86 cm/yr with a meta-analysis by Chawla et al reporting an overall median growth rate of 0.28cm/year [6]. Some tumours however will behave more aggressively and at present it is not possible to determine in advance which tumours these are. Nephron sparing surgery (NSS) represents the gold standard for the small renal mass and radical surgery for the T2 and larger tumours.

Although laparoscopic nephron sparing surgery has been demonstrated to be both feasible and oncologically equivalent to open nephron sparing surgery it is widely acknowledged to be technically demanding with a steep learning curve and associated morbidity. This particularly relates to keeping warm ischaemia time to a minimum. In one of the largest series Gill et al reported a median surgical time of 3 hours and a median warm ischemia time of 27.8 minutes [7]. Although robotically assisted partial nephrectomy is gaining acceptance, active surveillance and minimally invasive alternatives including ablative techniques have emerged as alternatives to nephron sparing surgery or radical nephrectomy.

The main ablative techniques in clinical use are cryotherapy and radiofrequency ablation. Cryotherapy is more frequently applied laparoscopically and radiofrequency ablation percutaneously. In some institutions including the Cleveland Clinic selected tumours are preferentially treated with minimally ablative techniques rather than with partial nephrectomy [8]. In addition other emerging techniques that have been described include High Intensity Focused Ultrasound, Microwave thermotherapy, Interstitial Laser ablation and CyberKnife. Whilst there is increasing evidence in support of radiofrequency ablation

and cryotherapy, there is less clear evidence in support of the other minimally invasive techniques at present.

2. Cryotherapy

Tissue destruction by freezing and thawing has been used in a variety of medical problems for over 150 years with minimal clinical significance. However, with the development of vacuum-insulated liquid nitrogen and argon cooled probes, a real breakthrough was made and targeted cryoablation of renal tumours became a reality. Currently cryoablation is performed using an argon gas based system which operates on the Joule- Thompson principle (i.e., rapid cooling of the tip of a probe by highly compressed liquid nitrogen or argon expanding through a restricted orifice to the gaseous state). Using this principle, very low temperatures of -175°C to -190°C can be focused on to kidney tissue to freeze tumours. Based on the same principle helium gas can be used for thawing [9]. Cryoprobes of varying diameters are now available and they produce ice balls of varying shapes [10].

3. Mechanism of cryoablation

Although the exact mechanism of tissue injury resulting from freezing is not completely understood, experimental studies have provided us with a fair knowledge of the mode of action of cryotherapy. The physiological changes of freezing are described as acute and delayed. When the tissues are exposed to temperature of -5°C , ice is formed in the extracellular space. This changes the osmotic gradient and draws water from the intracellular to the extracellular compartment, leading to changes, in the intracellular solute composition, pH and eventually leads to protein denaturation [11,12]. When the temperature reaches to -20°C , ice forms both intra and extracellularly. It is believed that the intracellular ice shears the cell membrane with irreversible cell damage. Complete destruction of normal renal parenchyma occurs at temperatures below -19.4°C [13, 14]. Delayed tissue injury also occurs after cryoablation. This is due to damage to the microvasculature of the target tissue and formation of microthrombi. This phenomenon leads to delayed cell death and is believed to be the significant mechanism of action of cryotherapy [15]. Tissue destruction is achieved better by the combination of freezing and thawing processes. Double freezing, as compared to a single- freeze approach, has been shown to produce large areas of necrosis in animal models with significantly increased cell death [16].

4. Indications and contraindications for renal cryotherapy

The indications for cryotherapy are the same as that for partial nephrectomy. A peripherally situated, enhancing, well- circumscribed tumour which is less than 4cm is the ideal lesion for cryotherapy. In general younger patients are offered partial nephrectomy. In older patients or those with comorbidities such as diabetes, hypertension, congestive cardiac failure cryotherapy is considered. In certain special situations such as tumour less than 4cms in solitary or transplant kidney; hereditary conditions such as Von Hippel- Lindau disease and tuberous sclerosis, cryotherapy may be ideal [17].

The relative contraindications for cryotherapy are young age, tumours greater than 4cms, hilar tumours, intrarenal tumours, and cystic tumours. The only absolute contraindication is untreatable or irreversible coagulopathy [18].

5. Technique of cryotherapy

Most patients are treated with laparoscopic or percutaneous image guided minimally invasive techniques. Open cryotherapy may occasionally be necessary and is also described.

6. Laparoscopic cryotherapy

Tumours that are situated in the anterior aspect of kidney and in the polar region are ideally treated by a transperitoneal laparoscopic technique and the posterior tumours by the retroperitoneal approach. The transperitoneal technique that we use is as follows:

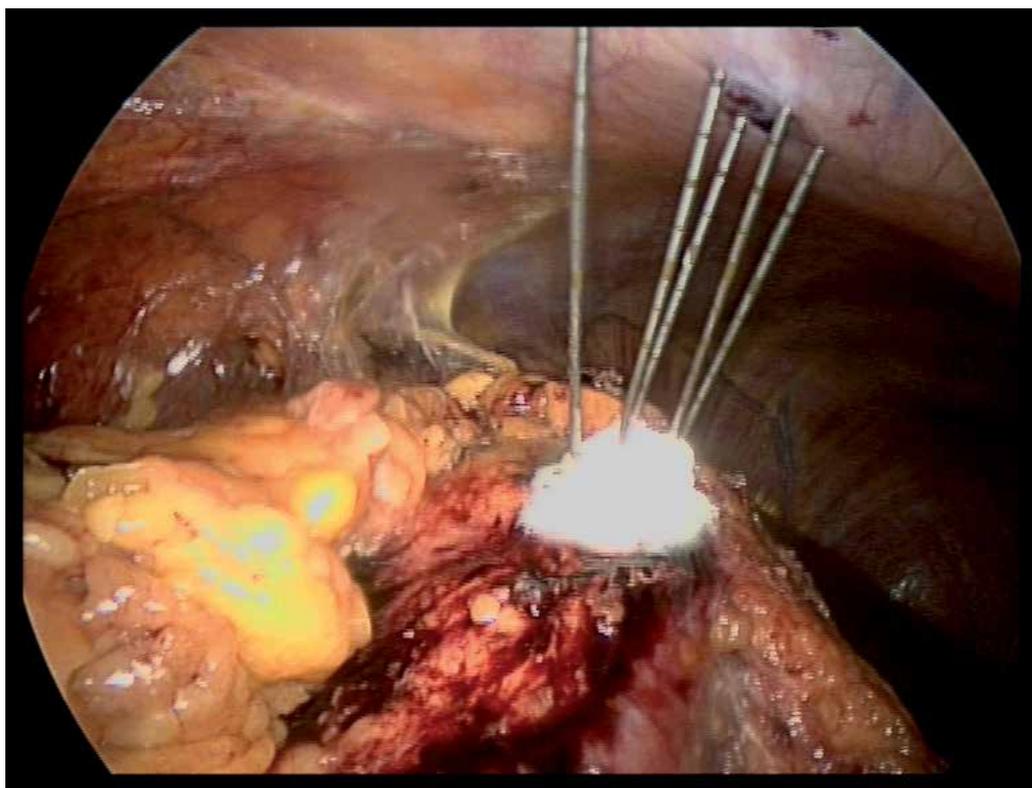


Fig. 1. Showing kidney tumour treated with argon gas cryotherapy- note the spectacular freezing of the tumour.

After placing the ports, the kidney is well mobilised. Gerota's fascia is incised and the perirenal fat is dissected from the surface of the kidney. The exophytic tumour is well seen at this stage and is defined. The position and the depth of the tumour are confirmed and measured using a laparoscopic ultrasound probe. Two to three good core biopsies are taken for histology. Cryoprobes are then placed perpendicularly into the tumour with the tip extending about 5mm beyond. The number of probes placed is determined by the size of the tumour. Two thermosensors are placed one in the centre of the lesion and the other beyond the margin of the tumour to monitor the temperature during cryoablation. The cryoablation starts with a freeze cycle with delivery of the argon gas through the probes. The cryolesion

is readily visible as an ice ball and visible in the ultrasound. Typically the first freeze cycle is for 10 minutes. The target temperature in the centre of the lesion is -40°C and below. This is followed by a thaw cycle with helium gas. Like many other centres we follow a two freeze and two thaw cycle protocol. At the end of the procedure, a passive and an active thaw are performed and then the probes are removed slowly, after the ice has fully melted. Retrieval of the probes should not be attempted if any resistance is noted as it may lead to fracture of the tumour. In this situation continuing passive thaw for some time allows easy removal of the probes (Fig 1)

7. Percutaneous image-guided cryoablation

Most cryoablations are performed under CT and MRI guidance. Tumours situated in the posterior and inferior aspect of the kidneys are ideal for image guided cryoablation. The upper pole tumours are difficult to reach. The procedure of cryotherapy is essentially similar to laparoscopic techniques. The advantages of percutaneous techniques are short hospital stay, ice ball monitoring by cross-sectional imaging, decreased analgesic requirement and lower cost compared to laparoscopy. Currently however, only 25% of tumours are treated by percutaneous image guided cryoablation and the techniques are still evolving [19].

8. Success and follow up

The absence of enhancement in a post contrast CT-scan at three months following cryoablation is considered as a successful procedure. Although no algorithm exists the patients are closely followed with contrast CT-scans at six, nine and twelve months and ideally the tumour should show regression. Periodic long-term follow up should continue till the tumour is regressed completely and then annual surveillance to ensure that there is no recurrence from the margin. The presence of enhancement would indicate incomplete treatment. Rim enhancement only with no increase in size seems to occur commonly in the first few months after cryoablation and this usually settles. This usually is not an indication for biopsies as it is difficult to take and the yield is low [20]. A recent study from the Cleveland clinic concluded that biopsies did not provide sufficient additional information to contrast enhanced MRI or CT findings in the six months post treatment period and hence did not recommend biopsies [21].

9. Results

The accumulating medium and long term data suggests that cryoablation is associated with high efficacy and low morbidity. The oncological control seems to be promising. The recent review by Berger and associates indicated a five and 10-year cancer specific survival of 93% and 81% respectively [22]. A meta-analysis showed that local recurrence (treatment failure) ranges from 4.6% to 5.2% and metastatic progression ranged from 1% to 1.2% [23].

10. Complications

Cryotherapy is a minimally invasive treatment modality which ablates the renal tumour *in situ*. Although, it is reasonably safe it has a few complications. Minor renal laceration and

bleeding from the needle site is common but settles with pressure. Renal fracture and haemorrhage can be avoided by perpendicular placement of the cryoprobes and waiting for complete thawing before removing the probes. The area of skin surrounding the cryoprobes needs to be protected, by warm gauze, to prevent cold injury to the skin. The adjacent internal organs also need protection from cryo injury. Pancreatic injury and ureteropelvic junction stricture has been reported [24]. Data from a retrospective multi centre study for 139 patients revealed that the major and minor complication rates associated with cryoablation were 1.8% and 9.2% respectively. The five major complications were ileus, haemorrhage, conversion to open surgery, scarring with ureteropelvic junction obstruction and urinary leakage. Overall, the most common complication as well as the most common minor complication was pain or paraesthesia at the probe insertion site [25].

11. Conclusions

Renal cryotherapy has proven its safety with good oncological control at medium term with some long term data. Currently the main application of cryotherapy in kidney tumours is for peripheral lesions less than 4cm in patients who would benefit from or require nephron sparing surgery but are not candidates because of comorbidity. Laparoscopic cryotherapy is also on the rise in patients with normal kidneys and relatively normal fitness. The percutaneous approach is constantly evolving and the field is very promising. We have to wait for the long term oncological data but it appears that cryotherapy is here to stay.

12. Radiofrequency ablation

First used in the targeted destruction of hepatic lesions radiofrequency ablation (RFA) has been used since the 1990s. It was first used in the kidney in 1997 [26]. Many of the treatment protocols in RFA are derived from the liver experience.

Although, it can be deployed laparoscopically it is most commonly applied percutaneously by interventional radiologists. The technique involves the insertion of 2 electrodes into the target tissue, in this case a renal tumour and conversion of electrical current between the two electrodes to ultrahigh (radio) frequency. This in turn ablates the intervening tissue with a margin of surrounding normal tissue. The mechanism of tissue destruction is immediate cellular damage and delayed microvascular impairment with the denaturation of proteins and the coagulation of tissue and the disruption of lipid cell membranes. The tissue needs to be heated to a temperature range of 50 to 100°C [27,28]. Over 105°C, the heat distribution and so tissue destruction becomes patchy and unreliable.

Real time imaging of RFA with intra operative USS CT or MRI has proved to be unreliable because of the similarity between normal and ablated tissue and because of the formation of gas bubble artefact. As a result imaging is usually confined to assisting initial probe placement with the RFA being monitored by temperature and or impedance changes.

The advantages of this minimally invasive technique include the ability to apply the energy percutaneously under light sedation, avoiding a general anaesthetic and allowing a more rapid recovery with low morbidity.

The disadvantages of the technique include a lower success rate compared to cryotherapy or nephron sparing surgery. In addition there are only short to medium term results available and the long term efficacy is not established. Also a significant proportion of the reported studies did not establish histological confirmation of a tumour before treatment and so

histological confirmation of successful ablation or cure is problematic. It is difficult to interpret the results if it is not certain that the original lesion was malignant. Furthermore as the natural history of the small renal mass is still not completely clear it may be that a significant proportion of the lesions that are tumours would have followed an indolent course without treatment and that the application of an ablative technique is an over treatment. As with cryotherapy, proximity to large vessels may lead to the draining of ablative energy by conduction that in turn reduces efficacy of the procedure. Another challenge is confirmation of successful ablation or cure. Most series report biopsy of the lesion at 6 months together with serial imaging with CT, usually at 6 monthly intervals. A successful outcome is taken as a lack of contrast enhancement of the lesion on CT. In addition the lesion should show progressive shrinking on subsequent imaging.

13. Results

Early experience has shown promising short and medium term tumour control [29]. McDougal et al [30] have reported on patients with over 4 years follow-up and showed successful ablation of exophytic masses smaller than 5cm in diameter. 13 masses in 11 patients with a mean tumour size 3.2cm were followed over a mean of 4.6 years. 12 (92.3%) of the 13 masses treated showed complete ablation.

Zagoria et al [31] reported a retrospective series of 125 treated tumours in which 116 (93%) were completely ablated with a mean follow up of 13.8 months. All 95 tumours smaller than 3.7cm were completely ablated and 21 (70%) of 30 larger tumours were completely ablated with 9 showing evidence of residual viable tumour on follow-up scans. They reported that with each 1cm increase in tumour diameter over 3.6cm the likelihood of tumour free survival decreased by a factor of 2.19 ($p < 0.001$). There were 8 (8%) complications none of which resulted in long-term morbidity.

Stern reported with a mean follow up of 30 months that RFA had the same outcome as partial nephrectomy for T1a lesions with a disease specific survival of 93.4% [32]. Levinson described over a period of at least 40 months a recurrence free survival of 90.3% in patients undergoing RFA for SRM in a single kidney [33].

There are concerns regarding the reliability of imaging to assess treatment response. Lack of enhancement of an ablated mass on CT does not always correlate with histological confirmation of no viable tumour tissue on biopsy. Weight reported that over 45% of renal tumours that showed no enhancement following RFA demonstrated viable tumour on biopsy at 6 months [34]. Others however have suggested that the presence of tumour on biopsy less than 12 months post treatment may be unreliable and have shown no viable tumour in 20 lesions that were biopsied over 12 months following treatment [35]. Where recurrences or cases of persistent disease do occur they tend to be demonstrated within 12 months of treatment. In one study from several institutions 92.1% of renal tumour recurrences were detectable 12 months or fewer after ablation [36].

One study reported the presence of pathological skip areas where tissue remains that has not been ablated. In this study this was found in seven of nine treated renal tumours [37].

A meta-analysis examining 47 studies comparing RFA and cryoablation for SRM has been published [38]. No differences were detected between the modalities in patient age, tumour size or follow-up. Pre-treatment biopsy was performed more often for lesions treated with cryoablation (82.3%) than with RFA (62.2%) ($p < 0.0001$). There was a significantly higher rate of unknown pathology in lesions treated with RFA (40.4%) compared to those treated with

cryoablation (24.5%) ($p < 0.0001$). Repeat ablation was more frequently performed after RFA (8.5%) than after cryoablation (1.3%) ($p < 0.0001$) and the rate of local tumour progression was significantly higher for RFA (12.9%) than for cryoablation (5.2%) ($p < 0.0001$). Metastasis was reported more frequently for RFA (2.5%) than for cryoablation (1%) ($p = 0.06$).

The authors of this large meta-analysis concluded that ablation of SRMs is a viable strategy based on short term oncological outcomes and that cryoablation results in fewer retreatments and improved local tumour control and may be associated with a lower risk of metastatic progression compared with RFA.

A further meta-analysis by the same authors looked at all studies reporting on the management of SRMs whether by open or laparoscopic partial nephrectomy, RFA, cryoablation or observation [39]. The analysis included 99 studies involving 6471 renal masses. Local recurrence was reported in 2.6% of masses treated with partial nephrectomy, 4.6% of cases treated with cryotherapy and 11.7% of masses treated with RFA.

Morbidity although low includes perinephric haematomas, bile and urinary fistulae, pancreatic pseudocysts and a ureteric stricture [40, 41].

14. Conclusions

Tumours most suitable for RFA are the same as those most suitable for nephron sparing surgery, that is those < 3.5 cm in diameter, peripheral, solid, exophytic lesions and away from the renal hilum and collecting system.

In addition RFA may be recommended for patients with conditions predisposing them to repeated development of renal tumours requiring multiple treatments such as Von Hippel Lindau disease.

A number of series have been reported with short and medium term follow up. The results appear to depend upon the position of the tumour with small exophytic peripheral tumours responding better than central lesions.

Although the majority of incomplete ablations are apparent by 12 months ongoing surveillance imaging much the same as following partial nephrectomy is recommended.

What remains to be seen is whether focal ablation actually alters the natural history of the small renal mass or whether the encouraging short and intermediate results are a function of the favourable nature of the lesion.

15. High Intensity Focused Ultrasound (HIFU)

HIFU is very attractive as a completely non-invasive treatment option. The focused ultrasound beam has a thermal and a cavitation effect. The thermal effect is caused by the absorption of ultrasonic sound waves by the tissues resulting in protein denaturation and coagulative necrosis [42]. The cavitation effect is caused by bubble implosion leading to mechanical disruption.

The first feasibility study was reported by Vallencien in 1993 [43] who found evidence of ablation in 8 patients with renal tumours. The limitations of the technique include difficulty in lesion localisation and targeting, small ablation zones and side effects including bowel injury and skin burns. In addition, in renal tumours the overlying ribs and respiratory movements can present a problem [44].

One study applied HIFU to the healthy renal tissue of patients requiring nephrectomy for tumour and found the tissue effects were variable and did not correspond well with the

amount of HIFU energy applied [45]. Other studies have also not demonstrated complete tumour ablation [46, 47]. At present therefore, HIFU although very attractive is not recommended as a treatment option.

16. Microwave thermotherapy

Microwave thermotherapy involves the insertion of flexible antennae into tissue and the formation of a rapidly alternating electromagnetic field which in turn causes coagulative necrosis [48]. Experimental studies in rabbits have suggested oncological equivalence with nephrectomy [49].

The disadvantages of the limited zone of ablation and the large antenna size have limited the minimally invasive application of microwave thermotherapy. The technique remains largely experimental although some early clinical experience has been described [50, 51]. The technique has also been described as an adjunct to open partial nephrectomy in order to provide a bloodless plane before resection [52].

17. Interstitial LASER coagulation

Interstitial LASER therapy works by inserting a bare-tip laser fibre directly into tissue. Laser light is converted to heat $>55^{\circ}\text{C}$ and causes tissue necrosis. Nd-YAG and diode lasers have been used. Although the technique has been described experimentally with the laparoscopic approach [53] and in a small number of clinical studies [54] it should still be regarded as experimental.

18. Cyberknife (radiosurgery)

This technique was pioneered in neurosurgery for the treatment of intracranial tumours and uses stereotactic techniques to apply highly focused radiation. The cyberknife is a frameless image-guided radiosurgery device with a linear accelerator on a robotic arm which delivers an adequate conformal dose of radiation by focusing a large number of radiation beams at the target area such that each individual beam does not damage the surrounding normal tissue. Ponsky et al reported initial results in porcine kidneys and demonstrated complete fibrosis of the lesions with preservation of the surrounding normal tissue [55]. A phase 1 study of radiosurgery involving 3 patients showed necrotic tumour in 1 of the 3 patients at subsequent nephrectomy and further trials are awaited [56].

19. Conclusion

In this chapter we have discussed the management of small renal tumours by ablative therapies. These treatments have emerged as an alternative to nephron sparing or radical surgery. The most common ablative techniques are cryotherapy and radiofrequency ablation (RFA). The indications for ablative techniques are the same as for partial nephrectomy and the ideal lesion is a peripheral, enhancing, well circumscribed lesion less than 4cm in diameter (Fig2)

Cryotherapy is most commonly applied laparoscopically and depends upon repeated freeze thaw cycles to achieve tissue destruction. There is good oncological control in the medium term and long term data is continuing to emerge.

Radiofrequency ablation is more commonly applied percutaneously. Several meta-analyses have suggested that short term oncological outcomes are good but that local recurrence, retreatment rates and progression to metastatic disease may be higher than with cryotherapy.

Other ablative techniques include High Intensity Focused Ultrasound (HIFU), Microwave Thermotherapy, Interstitial Laser Coagulation and Radiosurgery (Cyberknife). Although attractive as treatment options these techniques are yet to be established as viable treatments for the small renal mass.

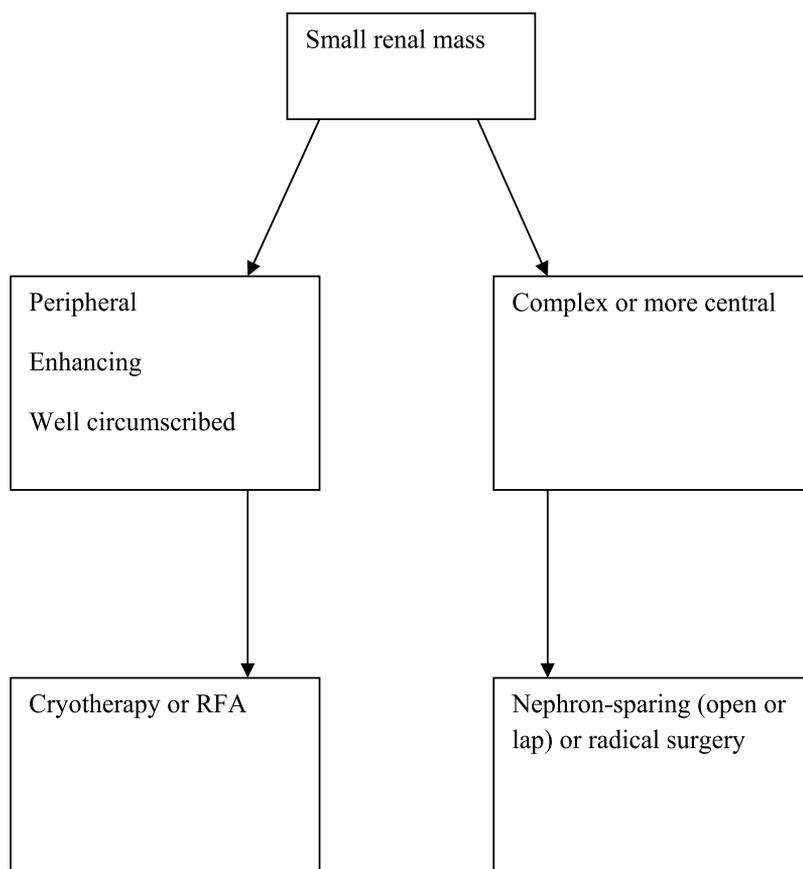


Fig. 2. The Management of Small Renal Tumours by Ablative Therapies

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Cancer Treatment with Hyperthermia

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1. Introduction

Broadly speaking, the term hyperthermia refers to either an abnormally high fever or the treatment of a disease by the induction of fever, as by the injection of a foreign protein or the application of heat. Hyperthermia may be defined more precisely as raising the temperature of a part of or the whole body above normal for a defined period of time. The extent of temperature elevation associated with hyperthermia is on the order of a few degrees above normal temperature (41–45°C) (Habash et al. 2006).

Hyperthermia is a type of cancer treatment in which body tissue is exposed to high temperatures, using external and internal heating devices. Hyperthermia is almost always used with other forms of cancer therapy such as radiation and/or chemotherapy. Research has shown that high temperatures can damage and kill cancer cells, usually with minimal injury to normal tissues. It is proposed that by killing cancer cells and damaging proteins and structures within the cells, hyperthermia may shrink tumors making the cells more sensitive to radiation therapy (RT) and/or chemotherapy (ACS 2009).

In some cases if the elevated temperature is utilized as the stand alone technique, tissue temperature above 48 degree would be the objective.

hyperthermia induces almost reversible damage to cells and tissues, but as an adjunct it enhances radiation injury of tumor cells and chemotherapeutic efficacy. Because of the results that high temperature may produce in tissues, one can refer to use of temperatures >50°C as coagulation, 60 to 90°C as thermal ablation, >200°C as charring (Chichel A., et al. 2007). Hyperthermia, as a novel concept for cancer treatment has already entered clinical practice.

2. History

The clinical use of hyperthermia in the system of traditional medicine (Ayurveda) began in India around 3000 years ago. It formed part of a clinical protocol developed called "Panchakarma" that was used in curative and preventive medicine. Hippocrates (540-480 B.C) said that incurable disease is cured by heat, and demonstrated it using the hot sand in the summer. Semantically, hyperthermia comes from the Greek word hyper ("raise") and therme (heat).

Furthermore, Parmenides, Greek philosopher and physician said: "Give me the power to produce fever and I will cure any disease".

Cancer treatment by hyperthermia was first mentioned in ancient Rome by Cornelius Celsus Aulus, Roman encyclopedic doctor (25 BC - 50 AD), who noted that the first stages of cancer are extremely thermosensible.

In the Middle Ages hyperthermia was described by Leonidas - Nicolaus Leonicensus (1428-1524), professor of medicine in Padua. Fever was considered to be an agent of purification and detoxification of the body. After the Renaissance, there were reports of spontaneous tumor regression in patients with smallpox, influenza, tuberculosis and malaria, accompanied by fever.

Enthusiasm for applying heat diminished after 1537, when Ambroise Paré, military surgeon, has demonstrated that medical treatment by cauterization unacceptable consequences. In 1779 Dr Kizowitz described the effect of hyperthermia on malignant tumors caused by malaria.

As in modern ages, the literature on the use of hyperthermia, either as an adjunct to other treatments or as the primary mode of tumor eradication, goes back to the last century. The first paper on hyperthermia was published in 1886 (Bush W., 1886). It was claimed that the sarcoma on the face of a 43-year-old woman was cured when fever was caused by erysipelas. A decade later, (Westermarck 1898) used circulating high temperature water for the treatment of an inoperable cancer of uterine cervix with positive results. Hyperthermia was already investigated for the treatment of malignancies more than a hundred years ago. Westermarck reported on the use of localized, nonfever-producing heat treatments that resulted in the long-term remission of inoperable cancer of the cervix. Hot baths, electrocautery, and surgical diathermy were employed to locally raise tumor temperature. In the early twentieth century, both applied and basic research on hyperthermia was carried out; however, the heating methods and temperature measuring technologies were not sufficiently advanced at that time and positive clinical application of hyperthermia treatment was not accomplished.

The concept of using heat to treat cancer has been around for a long time, but early attempts to treat cancer with heat had mixed results. Several clinical trials were developed in the 1980s. Given the difficulties with hyperthermia delivery with the available technology and lack of widely applicable quality assurance guidelines enthusiasm for hyperthermia was about to disappear in the late 1980s. Despite little clinical success, research continued in the early 1990s (Hurwitz, 2010).

Worldwide interest in hyperthermia was initiated by the first international congress on hyperthermic oncology in Washington in 1975. Research on hyperthermia rose sharply during 1970's (Kim & Hahn 1979 and references therein). In the 1970's hyperthermia was investigated for the treatment of muscle invasive bladder cancer. More than ten years ago Matzkin performed an in vitro study where solely hyperthermia (43.8C) was used in the treatment of superficial transitional cell carcinoma (TCC) of the bladder . Results of several clinical trials are encouraging [Colombo et al]

In the United States, a hyperthermia group was formed in 1981 and the European Hyperthermia Institute was formed in 1983. In Japan, hyperthermia research started in 1978 and the Japanese Society of Hyperthermia Oncology was established in 1984.

3. Reasons for hyperthermia

The simplest curative application of heat that possess physiological basis (physiological hyperthermia) is treatment of aches, pains, strains, and sprains via application of

temperature below 41°C for approximately an hour and use physiological mechanisms of increasing blood flow and metabolic rates (Roemer RB. 1999).

For cancer treatment purposes, there are reports that malignant cells are more sensitive to heat than are their normal counterparts (Cavalieri, R. et al. and Levine, E.M), although that finding is not universal.

By now, several clinical studies have demonstrated the beneficial effect of regional hyperthermia (ZIB report 2008). Tumor cell environment, such as hypoxia, poor nutrition, and low pH, while detrimental to cell kill by ionizing radiation, is beneficial to heat therapy. Acidic environment of tumor confers resistance to radiation but favors cell kill due to heat.

The effect of hyperthermia depends on the temperature and exposure time. For example, it has been demonstrated that with a non-specific HT-applicator already a significant increase in local control (from 24% for RT alone versus 69% for RT plus HT) is achieved for metastatic lymph nodes, without additional toxicity.

Report of long-term follow-up in a randomized trial comparing radiation therapy and radiation therapy plus hyperthermia to metastatic lymph nodes in stage IV head and neck patients (Valdagni R., 1994).

4. Types of cancers

Cancer tumors located in various organs might be treated with different hyperthermia techniques. The chance of successful treatment depends on the stage of cancer development, application of specific technique, and response of patient's physiology to treatment. The cancer types already cured by hyperthermia are:

sarcoma, carcinoma, melanoma, head and neck, Brain

thyroid, lung, esophagus, breast,

kidney, bladder, liver, appendix, stomach,

pancreas, endometrial,

ovarian, prostate, cervix,

peritoneal lining. (mesothelioma).

rectum, appendix

By definition, the term "head and neck cancers" usually excludes tumors that occur in the eyes, in brain and in skin. The most frequently occurring cancers of the head and neck area are located in the oral cavity and the larynx. Worldwide, around 3-5% of patients suffering from cancer have tumors in the head and neck (H&N) region. (Paulides, M., 2007)

5. Physiological and biological phenomena in hyperthermia

First of all, it is noteworthy that there is no individual cellular target of hyperthermia, in contrast to the well known DNA damage after irradiation with x-, gamma or hadron.

In cellular level, reproductive death starts at 40 °C which is progressive with increasing time at the elevated temperature. Cells when heated to a temperature of 45 °C tend to undergo apoptosis or mitotic death process. Heat in the range of 42 °C to 45 °C, can also sensitize cells exposed to ionizing radiation or chemotherapy. The cells' sensitivity to heat shock is a function of their position in the cell cycle but heat-injured cells are capable of repairing sublethal and potentially lethal damage. (Hahn, G., 1974)

Inactivation curves showing duration of hyperthermic shock versus logarithm of surviving fraction have a shape similar to that for X-ray killing, i.e., a shoulder, followed by an approximately linear portion of the survival curve.

The results indicate that the cells in solid tumors that are most difficult to kill with conventional radio-therapy or chemotherapy may be those most readily eliminated by hyperthermia. Data indicate that elevated temperatures (39-43° C) increase the sensitivity of the cells to at least low-dose-rate X-irradiation and probably to some chemotherapeutic agents. One of the important mechanisms of cell death is probably protein denaturation, observed at temperature above 40 °C, leading to changes in enzyme complexes for DNA synthesis and repair as well as alterations in structures like cytoskeleton and membranes. Heating cells at 42 °C for 10 minutes leads inhibition of DNA synthesis by 40 percent while, this figure amounts to 90 percent at 45 °C for 15 minutes. Heat can induce cell death in non-dividing cells by activation of various enzymes. Heat also affects cell membrane, a target not shared with ionizing radiation. Hypoxic cells, which are generally resistant to ionizing radiation, are sensitive to heat.

The cellular and molecular basis for this selective death of cancer cell has been studied. While inhibited RNA synthesis and mitosis arrest are reversible and nonselective results of hyperthermia, an increase in the number of lysosomes and lysosomal enzyme activity are selective effects in malignant cells. These heat-induced lysosomes are more labile in malignant cells and therefore result in increased destructive capacity. Furthermore, the microcirculation in most malignant tumors exhibits a decrease in blood flow or even complete vascular stasis in response to hyperthermia, which is in contrast to an increased flow capacity found in normal tissues. This, in combination with depression or complete inhibition of oxidative metabolism in tumour cells subjected to hyperthermia and unaltered anaerobic glycolysis, leads to accumulation of lactic acid and lower pH in the microenvironment of the malignant cell. (S. González-Moreno, et al, 2010)

Thus hyperthermia in the range of 42 °C-45 °C is good sensitizer of ionizing radiation. It is also shown to have synergistic effects with chemotherapy agents such as Bleomycin, Adriamycin and Platinol derivatives.

Increased perfusion due to warmer environment improves drug delivery to the tumor. It also ensures increased intracellular uptake of the drug as well as repair inhibition of DNA damage due to cytotoxic drugs. Combination of hyperthermia and chemotherapy also has shown decreased drug resistance. Interestingly an enhancement ratio of 23 was shown in cell lines when cell lines were treated with Melphalan and heat at 44 °C. (ACR 2008, ACR 2009).

Most of the biomolecules, especially regulatory proteins involved in cell growth and certain receptor molecules are largely influenced by hyperthermia. New insights from molecular biology have shown that a few minutes after hyperthermia, a special class of proteins are expressed into the cell, the so-called heat shock proteins (HSP). They protect the cell from further heating or subsequent thermal treatments and lead to an increase of cell survival after preheating, an effect called thermotolerance. Additionally, the activity of certain regulatory proteins is influenced by hyperthermia causes alterations in the cell cycle and can even induce apoptosis, the cell death driven by the cell regulatory system itself.

At tissue scale, primary malignant tumors have poor blood circulation, which makes them more vulnerable to changes in temperature (Habash 2006)

Cancer tissues accumulate lactic acid at an extremely increased glucose level, because cancer cells metabolize glucose to great extent into lactic acid, even in the presence of oxygen. This over-acidification makes the cancer cells more sensitive to hyperthermia. On the other hand, the normal cells are stabilized energetically by glucose in the presence of oxygen. Therefore,

in a temperature range between 41.9 and 42.5 °C, the cancer cells are destroyed or at least damaged. The normal tissues of the organism, however, are not affected.

HT kills cells itself, implicates radiotherapy by inducing reoxygenation, increases delivery of liposomally encapsulated drugs and macromolecules such as monoclonal antibodies or polymeric peptides, enhances cellular effects of chemotherapeutics and augments immune reactions against the tumor due to thermotolerance mediated by heat shock proteins (HSPs) (Mayerson, 2004).

The generation of free radicals by hyperthermia treatment (HT) in the membrane or cytoplasm of cancer cells, results in peroxidation of intracellular polyunsaturated fatty acids (PUFA), and is considered to be an important source of antitumor activity.

6. Thermal and electrical properties of tissue

	Relative Permittivity	Conductivity [S/m]
Muscle	62.8	0.72
Bone	14.6	0.068
Marrow	6.2	0.024
Skin	63.5	0.53
Blood	72.2	1.25
Tumor	74	0.89
Rest (high water content but includes some fat)	40	0.4

Table 1. Electrical properties of human tissues

	Mass Density [kg/m ³]	Thermal Conductivity [W/(m °C)]	Specific Heat [W/(kg °C)]
Muscle	1047	0.45	3550
Bone	1990	0.29	970
Marrow	1040	0.45	3550
Skin	1125	0.31	3000
Blood	1058	0.49	3550
Tumor	1047	0.55	3560
Rest (high water content but includes some fat)	1020	0.4	3200

Table 2. Thermal properties of human tissues.

7. Definition of radiofrequency, range of frequencies in hyperthermia

Designation	Frequency	Approximate wavelength
Radiofrequency (RF)	100 kHz	1000 m
	1 MHz	100 m
	10 MHz	10 m
	100 MHz	1 m
Microwave	1 GHz	10 cm
X-ray		

Table 3. Concise and approximate definition of frequency ranges in electromagnetic waves.

8. Treatment types: Local, regional, whole body

Depending on the organ bearing the cancerous tissue, stage of cancer development and method of energy delivery to patient's body, three kinds of hyperthermia techniques are recognized. This brings about various equipments and treatment works. These types are:

- Local hyperthermia
- Regional hyperthermia
- Whole-body hyperthermia

9. Local hyperthermia

Primary malignant tumors before the metastases stage are treated with local hyperthermia. Treatment is performed with superficial applicators of different shapes and kinds such as waveguide, spiral and current sheet placed on the surface of superficial tumors with an intervening layer called bolus. Energy sources could be RF, microwave, or ultrasound. When ultrasound is used, the technique is called high intensity focused ultrasound (HIFU). Heat is applied to a small area such as a tumor. The penetration depth depends on the frequency and size of the applicator; the clinical range is typically not more than 3–4 cm and the area is less than 50 cm². In local hyperthermia temperature rises to 42°C for one hour within a cancer tumor, hence the cancer cells will be destroyed.

There are several approaches to local hyperthermia including (ACS, 2009; NCI, 2004):

- External/Superficial: external applicator is used to deliver energy to the tumor below the skin
- Intraluminal or endocavitary: used to treat tumors within or near body cavities (e.g., rectum and esophagus) with placement of radiative probes inside the cavity.
- Interstitial: used to treat tumors deep within the body (e.g., brain tumors) with the use of anesthesia to place probes or needles into the tumor to deliver energy.

Candidates for local hyperthermia include chest wall recurrences, superficial malignant melanoma lesions, and lymph node metastases of head and neck tumors. Therapeutic depth is highly limited in regions with irregular surface, such as the head and neck (Habash et al, 2006)

10. Regional hyperthermia

For large, deeply seated, and inoperable tumors, regional hyperthermia is used. Cervical and bladder cancer are of this type. Another example is treatment of a part of the body, such as a limb, organ, or body cavity. In regional hyperthermia external applicators using microwave or radiofrequency energy are positioned around the body cavity or organ to be treated. (JJW Lagendijk et al, 1998).

A sub-group of regional hyperthermia is regional perfusion which is used to treat cancers in the arms and legs (e.g., melanoma) or cancers in some organs (e.g., liver and lung). Some of the patient's blood is removed, heated, and then pumped or perfused back into the limb or organ. Anticancer drugs are usually administered during this treatment.

Another kind of regional hyperthermia, hyperthermic intraperitoneal chemotherapy (HIPEC), also referred to as intraperitoneal hyperthermic chemotherapy (IPHC), has been proposed as an alternative for the treatment of cancers within the peritoneal cavity, including primary peritoneal mesothelioma and gastric cancer. The HIPEC is applied during surgery, via an open or closed abdominal approach. The heated chemolytic agent is infused into the peritoneal cavity, raising the temperature of the tissues within the cavity to 41-42°C. (ACS 2009)

Whole Body Hyperthermia (WBH): WBH, achieved with either radiant heat or extracorporeal technologies, elevates the temperature of the entire body to at least 41 °C. There are various techniques of heating systemically. Immersion in temperature controlled hot water bath and radiant heat with U.V. are the usual techniques for whole body hyperthermia.

In radiant WBH, heat is externally applied to the whole body using hot water blankets, hot wax, inductive coils, or thermal chambers. The patient is sedated throughout the WBH procedure, which lasts less than four hours. The patient reaches target temperature within approximately 1 hour, is maintained at 41.8 °C for one hour, and experiences a one-hour cooling phase. During treatment, the esophageal, rectal, skin and ambient air temperatures are monitored at 10-minute intervals. Small probes may be inserted into the tumor under a local anesthetic to monitor the temperature of the affected tissue and surrounding tissue. Heart rate, respiratory rate, and cardiac rhythm are continuously monitored. Patients are returned to regular situation in patient rooms after hyperthermia and discharged after 20-24 hours of observation (Robins, et al., 1997; Green, 1991).

Extracorporeal WBH is achieved by reinfusion of extracorporeally heated blood. A circuit of blood is created outside the body by accessing an artery, usually the femoral artery, and creating an extracorporeal loop. The circulating blood is passed through a heating device, usually a water bath or hot air, and the heated blood is then reinjected into a major vein. The desired body temperature is adjusted and controlled by changing the volume flow of the warmed reinfused blood (Wiedemann, et al., 1994).

Extracorporeal hyperthermia treatments are conducted under general anesthesia. To counteract the activation of coagulation by the hemodialyzer, high-dose heparin is administered. An extracorporeal WBH treatment session typically lasts four hours. Target temperature is reached in two hours and is maintained for one hour, followed by a cooling period of one hour. Subsequently, the patient is infused with normal saline to maintain systolic blood pressure above 100 mm Hg. The patient is then monitored weekly for complications (Kerner, et al., 2002; Wiedemann, et al., 1994).

11. Treatment planning and simulation

Due to varying tumor location and geometry and different size and shape of patients, individual therapy planning is necessary. The first step of hyperthermia treatment planning is the generation of a patient model by segmentation of images from computerized tomography (CT) or magnetic resonance imaging (MRI) scans. In some cases, online parameter identification based on MRI is performed. A model of the applicator and this segmentation are used to calculate the power absorption (PA, [W/m³]) or specific absorption rate (SAR, [W/kg]) distribution in the patient by electromagnetic models. These EM models for treatment planning are commonly based on the finite-element (FE) method or finite-difference time-domain (FDTD) method. A temperature distribution in the patient can be calculated from the power absorption distribution by applying Pennes' bio-heat equation (PBHE), or more elaborate algorithms including the blood vessel network, i.e. discrete vasculature (DIVA) models, down to vessel sizes in the millimeter range. The main problems with these thermal methods are long time-requirements for the generation of a vessel network and the large, poorly-predictable, variations in thermal properties of tissues. The target in treatment planning is to heat a particular tumor and delivering at least 43°C to 90% of its volume for cumulative in multiple treatments for longer than 10 minutes corresponds to doubling of the probability for complete response and duration of response to hyperthermia and radiotherapy versus radiotherapy alone (Oleson et al. 1993).

The thermal iso-effect dose is an established quantity for assessing the therapeutic benefit of a treatment. As for now CEM 43°C T90 (cumulative equivalent minutes at a standard targeted treatment temperature of 43°C obtained within 90% of the tumour volume) appears to be the most useful dosimetric parameter in clinical research. Treatment planning based on the tumor cell survival has been proposed for thermoseed placement, but up to now rather ad hoc cost functional based on the temperature distribution or on the absorption rate density have been used for regional hyperthermia. (J. van der Zee et al 2007)

In local-regional hyperthermia therapy planning using RF as the heat source the therapeutically optimal antenna parameters for the applicator are determined for each patient. The specific absorption rate values are obtained by solving the Maxwell equations, and the temperature distribution is predicted by variants of the bio-heat transfer equation. Although this can be a demanding task, a planning tool greatly improves the medical treatment quality with a virtual experiment to model, simulate and optimize the therapy with high precision. [J Crezee et al. 2005]

12. Motivations for simulation

- Provide better heating through treatment preplanning.
- Optimize setups for treatment cases.
- Assist new applicator design in the future.

On the other hand, the perfusion depends on the temperature due to autoregulation capabilities of the tissue. Moreover, at least in abdominal hyperthermia, the systemic thermoresponse seems to play a significant role. Different perfusion models have been proposed, covering a broad spectrum of homogenized and discrete vascular models.

A mathematical model of the clinical system (radio frequency applicator with 8 antennas, water bolus, individual patient body) involves Maxwell's equations in inhomogeneous media and a so-called bio-heat transfer PDE describing the temperature distribution in the

human body. The electromagnetic field and the thermal phenomena need to be computed at a speed suitable for the clinical environment.

Finally, in all treatment planning works an upper bound is imposed on the temperature: $T < T_{lim}$. Typical values for T_{lim} are 44 C for muscle, fat, and bone tissues, and 42 C for more sensitive organs such as bladder or intestine. (M. Weiser, 2008).

13. Instrumentation: Applicator, bolus, temperature measurement and monitoring

Hyperthermia can be applied by whole-body, external or interstitial/intracavitary techniques (applicators). External HT applicators use ultrasound (US) or electromagnetic (EM) waves to direct energy to the target region. US provides similar heating options as EM but results in more bone-pain complaints during treatment (Ben-Yosef et al. 1995).

In general, two types of probes are required in hyperthermia. One to deliver energy to the tissue, another to monitor the tissue temperature. The temperature in the tumor is measured by temperature sensors during the treatment. The temperature is then optimized continuously using automatic computer-controlled regulation of the applicator power output. Commercially Available Thermometer probes are :

Thermocouples

Thermistors

Non-perturbing probes

Thermistor sensor with high-resistance plastic leads containing graphite

Optical fibres

Liquid Crystal sensor

Birefringent sensor of LiNbO_3

Fluorescent-type sensor made of two phosphorus

Semiconductor crystal, gallium-arsenide (GaAs) as a temperature sensor

Multichannel systems with non-perturbing multisensor probes

Multi-GaAs-sensors as a linear array with up to 8 sensor points in one probe

Multiple fluorescent phosphor sensors

Multiple thermistor sensors with high-resistance leads

Non-invasive thermometry

- Microwave multi-frequency Radiometry
- Computerized tomography (CT) for thermometry in vivo
- Nuclear Magnetic Resonance (NMR)
- Electrical impedance tomography (EIT)

The thermometers based on optical fibers offer the advantage of not possessing metallic components, and therefore they do not disturb the electromagnetic fields

The probe that delivers energy to the patient's body, usually referred to as applicator, ordinarily is in touch with skin. Every applicator includes a bolus which is placed on the patient's skin. For treatment, this bolus is filled with circulating water that can be heated as necessary. The bolus serves to physically couple the electromagnetic waves to the patient's body, and hence reduce the reflection and waste of energy.

Heating could be capacitative or inductive. Heating could be with external antennae or with interstitial and intracavitary probes. Intracellular heating with ferromagnetic material subjected to alternating magnetic field can also generate localized heating. RF at

8-12 MHz is useful for heating of the deep-seated tumor while, microwave heating at 434 MHz to 915 MHz is useful in surface tumors. Heating with ultrasound is also feasible. Mechanical ultrasonic waves delivered at 0.2-5MHz and can effectively heat a small volume at various depth.

In case of shallow tumors, the energy source is microwave 915 MHz generator. Commonly, it has eight channels, with phase and amplitude of each adjustable individually. Using a three-way splitter up to 24 antennas can be powered. The eight signals from the individual channels (each capable of delivering up to 50 watts) can be combined to provide a total output of up to 400 watts.

In order to deliver an optimal therapy, the phased array applicator needs to be controlled in such a way that the tumor is maximally heated without damaging healthy tissue by excessive temperatures. Pain and unpredictable heat deposition at tissue bone or tissue air cavity can be a limiting technological factor. Scanning transducers can overcome this difficulty. Ultrasound heating, unlike imaging has not gained popular use in the clinic.

14. Clinical techniques

Hyperthermia is mostly applied within a department of radiation oncology under the authority of a radiation oncologist and a medical physicist. It is always implemented as part of a multimodal, oncological treatment strategy, i.e., in combination with radiotherapy or chemotherapy. (Habash 2006).

In a hyperthermia clinic, the treatment starts with a comprehensive medical consultation with previous medical-imaging reports such as sonographics, X-rays, CTs, MRIs, nuclear medical images. If further examinations would be prescribed if necessary.

Depending on the indications, the hyperthermia treatment is given once or twice a week. Due to thermotolerance a general phenomenon pertaining to transient resistance to additional heat stress, it is impractical to apply two different HT sessions with an interval shorter than 48-72 hours, until the resistance decays to a negligible level. Total number of sessions depends on the tumor characteristics and varies between 5 and 10 per patient. chemotherapy is administered concurrently; radiation therapy must closely precede or follow the hyperthermia treatment by up to 120 minutes.

First, the patient is placed in a horizontal position. The temperature sensors are affixed to the skin above the tumor or inserted into the tissue through an implantable catheter. The number of temperature sensors used depends on the size of the tumor. The applicator, which is selected on the basis of the size and location of the tumor lesion, is held in the treatment position with the aid of either a support arm or holding straps.

On the day of main treatment, patients come at 8:00 to the hyperthermia-clinic with an empty stomach (on the day prior to the treatment, eating is permitted until 8:00 p.m. and drinking until midnight), a premedication (a sedative injection) is given in the morning before plus the attachment of an indwelling bladder catheter.

During an approximately 60-minute controlled infusion period, still at normal body temperature, the blood glucose level is increased by the three to four-fold of the initial value (by continuing the infusion during the TCHT main treatment, the blood glucose level attains a five to six-fold level of the initial value). Then, the body-warming-up process (hyperthermia) begins at approximately the same time as a moderate anaesthesia (neuroleptic analgesia at maintained spontaneous respiration; intratracheal intubation only if

necessary) which acts over a time frame of approximately 6 hours. By means of infrared-A (short-wave part of the infrared spectrum) the body-core temperature is raised to 42.0°C (107.6 °F) within about 90 to 120 minutes. The chemotherapy is administered during the warming-up phase just before the body reaches 42.0°C

In the following so-called temperature-plateau-phase, a main body temperature of 42.0°C to 42.5°C is constantly maintained over 60 to 90 minutes. The cooling-off phase lasts for approximately another 90 to 120 minutes and uses the same monitoring measures as the warming-up and the plateau phase. An anti-emetic (a means to reduce vomiting) is added to the infusion during the last phase.

During the TCHT main treatment, lasting altogether approx. 8 hours, two doctors and two nurses are constantly at the patient's side (one doctor and a nurse continuously during the night and the next morning) then the patient will be transferred to the adjoining intensive care unit, an intensive care phase follows. The next morning at about midday the patient will be transferred by an accompanying doctor to the convenient private hospital to recover. For about 5 days the patients need infusions and medicines for recovery and initial daily blood sampling.

In a detailed report which you will take along, we recommend the follow-up checks as an outpatient later at the home town.

To make a general sense of external hyperthermia using RF, one could say the heat session is started by applying 80 W of total power with the power and phase control system (Bakker *et al* 2010), using the optimized phase and power settings from HTP.

Power is increased subsequently in steps of 30W, usually around one step per minute, till one of the tolerance limits is reached (40 °C in myelum indicative, 60Wkg⁻¹ in myelum predicted by HTP, 43 °C in other tissues) or the occurrence of a hot spot indicated by the patient at a site without thermometry. Two phases of a treatment are defined for data analysis: (1) 'warm-up phase' and (2) 'plateau-phase', and the transition is assumed to be always after 15 min of heating.

The increased oxygen saturation in the blood results in a stabilization of the cardiac functions, the circulatory system, the respiratory system and the central nervous system. Some cytostatics act better in an acid environment, so that the efficacy of chemotherapy can be increased through overacidification of the tumor. Hyperthermia itself also increases the efficacy of some cytostatics. Some side effects of chemotherapy can be alleviated by relative hyperoxemia. On the basis of this complex interaction, an individually adapted chemotherapy in combination with the hyperthermia is highly effective and, in general, well tolerated.

External local hyperthermia is utilized or heating of small areas (usually up to 50 cm²) to treat tumors in or just below the skin up to 4 cm. This can be used alone or in combination with radiation therapy for the treatment of patients with primary or metastatic cutaneous or subcutaneous superficial tumors (such as superficial recurrent melanoma, chest wall recurrence of breast cancer, and cervical lymph node metastases from head and neck cancer). Heat is usually applied using high-frequency energy waves generated from a source outside the body (such as a microwave or ultrasound source).

Intraluminal or endocavitary methods may be used to treat tumors within or near body cavities. Endocavitary antennas are inserted in natural openings of hollow organs. These include (1) gastrointestinal (esophagus, rectum), (2) gynecological (vagina, cervix, and uterus), (3) genitourinary (prostate, bladder), and (4) pulmonary (trachea, bronchus). Very

localized heating is possible with this technique by inserting an endotract electrode into lumens of the human body to deliver energy and heat the area directly.

The transient phase of heat distribution in patient's body takes about 15 minutes, while the duration of a single treatment session is about two hours. For this reason, usually only the steady state of the temperature distribution is optimized, which results in a significantly simpler optimization task.

Hyperthermia is most effective when the area being treated is kept within an exact temperature range for a defined period of time without affecting nearby tissues. This is challenging since not all body tissues respond in the same way to heat. Small thermometers on the ends of probes are placed in the treatment areas to monitor the desired temperature. Magnetic resonance imaging (MRI) is proposed as a replacement of the probes to monitor the temperature (ACS, 2009).

The following measures are taken in order to intensively monitor all the body functions. Two peripheral venous accesses in the form of flexible soft-tip catheters are attached for infusions, intravenous injections and blood sampling. The painless localization of the thermometric probes (rectal, axillary, as well as on the skin of the stomach and the back), of the pulse oxymeter (on the right middle finger) and of the ECG miniature adhesive electrodes complete the intensive medical monitoring. During the whole treatment time, the ECG and oxygen saturation are very closely observed and all the relevant parameters are monitored by means of blood samples every 15 minutes. Continuous blood pressure measurements as well as regular blood-gas analysis are monitored. In this way possible deviations are recognised and corrected early. Serious disturbances can thus be averted to the greatest possible extent.

15. Case studies

In a study involving 109 patients with superficial tumors, patients mostly suffering from breast wall recurrence due to mammary carcinoma, the enhancement effect of hyperthermia in combination with radiation therapy was demonstrated. Previously irradiated patients who underwent a second round of radiation therapy in conjunction with hyperthermia responded significantly better to this therapy. Complete remission was achieved in 68% of those treated with hyperthermia plus radiation while in the control group who did not receive hyperthermia, complete remission was observed in only 24% of the patients. (Jones, E.2005)

The effectiveness of hyperthermia treatment in cases of advanced head and neck tumors has been confirmed (R. Valdagni and M. Amichetti 1994). With radiation therapy alone, complete remission was achieved in 41% of patients, while the combination of radiation therapy and hyperthermia increased the remission rate to 83%. In addition, the 5-year survival rate for these patients was increased from zero to 53% from the addition of hyperthermia to radiation therapy.

There are nearly 24 randomized studies reported to which 18 have reported a positive benefit in combining hyperthermia with radiation. Patients with cervical nodes were randomized to radiation alone to a dose of 64-70 Gy and radiation with hyperthermia. Hyperthermia was delivered twice a week. The initial response was reported to have improved from 41% to 83% with a 5 year overall local control increasing from 24% to 69% and survival from none to 53%. Addition of hyperthermia to radiation in cancer of cervix was reported to have improved outcome as compared to radiation (N. G. Huilgol)

Bladder cancer at various stages was studied for 358 patients from 1990 to 1996. Patients were divided to two groups. One group underwent radiotherapy (median total dose 65 Gy) alone (n=176) another group radiotherapy plus hyperthermia (n=182). Complete-response rates were 39% after radiotherapy and 55% after radiotherapy plus hyperthermia. The duration of local control was significantly longer with radiotherapy plus hyperthermia than with radiotherapy alone. The 3-year overall survival was 27% in the radiotherapy group and 51% in the radiotherapy plus hyperthermia group (Van der Zee 2000).

For ovarian cancer, *in vitro* studies have shown that hyperthermia produces a dose-enhancement effect (i.e. a thermal enhancement ratio of approximately 3 for a 60 min heat exposure) besides, it is shown that hyperthermia can overcome acquired drug resistance . (A.M.Westerman 2001)

Around 70% of all initial responders to chemotherapy make no improvement and subsequently require additional therapy. The classic treatment includes aggressive tumor reductive surgery (TRS) followed by platinum based combination chemotherapy, using cisplatin or carboplatin combined with a taxane. As an adjunct to traditional therapy, hyperthermia has been shown to enhance cisplatin cytotoxicity. Mild hyperthermia (39–43°C) has successfully been utilized in combination with chemotherapy to increase cellular sensitivity to anticancer drugs mainly using an intraperitoneal approach. The interaction between heat and chemotherapeutic agents results in increased drug uptake by accelerating the primary step in a drug's efficacy and increasing the intracellular drug concentration. Therefore, the combination of hyperthermia and anti-cancer drugs may reduce the required effective dose of the anti-cancer drug, and it could enhance the response rates in ovarian cancer cells. (Amber P. et al, 2007)

Local recurrence rates of breast cancer after mastectomy alone have been reported as high as 45%. This high rate of failure can be reduced to 2–15% with the addition of postmastectomy radiation therapy (PMRT) and usually chemotherapy as well, with a corresponding improvement in overall survival. With its radiosensitizing properties, hyperthermia presumably lowers the radiation dose needed to achieve durable local control, which in turn has potential implications for decreased long-term toxicities in patients with a prior history of radiotherapy. The addition of concurrent chemotherapy to hyperthermia and radiation therapy, constituting thermochemoradiotherapy (ThChRT)), has been evaluated in phase I/II trials by several researchers and found to be well-tolerated, with moderate success. (Timothy M. Zagar et al 2010)

16. Side effects

The possible side effects of hyperthermia depend of the technique being used and the part of the body being treated. Pain, thermal burns or blisters are the limiting adverse events of the techniques. Fewer side effects are observed with improvement in technology along with better skills and improved technology (ACS, 2009; ECRI, 2007). Post surgical site may be more susceptible to heat due to poorly vascularised state. Skin and subcutaneous tissues are generally susceptible to increased power deposition when heated with microwave and radiofrequency waves. Thermal burns which are superficial and generally heal quickly are seen in 2-15% of the patients (N. G. Huilgol)

In case of thermochemotherapy (TCHT) during the first days after the main treatment, the occurrence of fever up to 39 °C can be observed as an expression of a strong

immunostimulation and is desirable in most cases. At this time, though, exhaustion, weakness,

Nausea, vomiting, headaches, diarrhoea and herpes labialis (blisters on the lips) can also occur.

Cases requiring treatment are, however, observed in less than 3 % of the therapies.

In rare single cases after TCHT treatment, an increased amount of oncolytical products can lead to an overstrain of the excretory mechanism (liver, kidney). As a consequence, temporary jaundice (icterus) as well as an increase of the liver and kidney values may occur. Although the side effects of most of the cytostatics are milder than those of conventional chemotherapy, toxic effects of isolated cytostatics caused by the TCHT are observed in very few cases. Temporary functional disturbances of the peripheral nerves can, though, occur with temporary strength reductions.

During the TCHT main treatment, a moderate anaesthesia is given. The patient is unable to drive for at least three days after the main treatment. In the following days, due to various reasons (e.g. after-effects of the chemotherapy or additional medications), reaction times can be deteriorated and, therefore, driving ability is considerably limited. Most of these side effects are temporary.

17. Engineering aspects of hyperthermia: Modeling, computation of temperature distribution, computer applications

Energy absorption in cancerous tissue provides heat required for temperature increase. Predominantly, electromagnetic waves in various frequency ranges are utilized. Thus, Maxwell's equations must be solved for the specific geometry with estimated electrical properties at the given anatomy. This computation process leads to SAR (Specific Absorption Rate) in Watts per unit mass of tissue. Then heat transfer equation must be solved to reproduce the temperature distribution in cancerous tissue and the adjacent healthy tissue. In contrast to RF ablation and focused ultrasound therapies, electrical and thermal properties of tissue do not change significantly over 37 - 45 temperature range. For this reason, these values are simply taken as constants depending only on tissue type.

Due to irregular geometry, modeling errors for computing the electrical field exists. Thus, even accurate solution of Maxwell's equations will not provide the actual electrical field. As a reliable tool, MRI can be used for monitoring the temperature distribution and identification of the applicator parameters. The 3D voxel data can be obtained approximately every other minute from proton resonance frequency shifts.

When using RF as the energy source, a time-harmonic electrical interference field is generated by a phased array of antennas which can be controlled individually by variations in amplitude and phase of each source.(M. Werser 2008).

A system for local hyperthermia consists of a generator, control computer applicator, and a scheme to measure temperature in the tumor. The therapy system is controlled automatically by the computer, which can be operated either via a touchscreen or by means of a mouse and keyboard.

To produce a visualization of the model from the patient's anatomy, the computer in clinic is equipped with GPU (Graphical Processing Unit). The GPU is the heart of a graphics card. Due to the application in multibillion game market, GPUs have quickly evolved into powerful devices available at a low price. This makes them attractive not only for graphics of video games, but also for scientific computing. GPUs are so fast because they are inherently parallel: while CPUs have 2 to 4 cores, GPUs have up to 128 arithmetic units.

18. Application of nanoparticles in hyperthermia

The difficulty in limiting heating close to the tumor region without damaging the healthy tissue is a technical challenge in hyperthermia. The use of magnetic nanoparticles can overcome the difficulty in spatial adjusting of power absorption by cancerous tissue. Application of magnetic materials in hyperthermia was first proposed in 1957 (Gilchrist R. K., et al. 1957)

Magnetic induction hyperthermia is a technique for destroying cancer cells with the use of a magnetic field. The temperature of the cancer tissue can be raised in the range of 42–46 C, by indirect heating produced by various magnetic materials introduced into the tumor. Depending on increase in temperature, cell damage (necrosis) or even its direct destruction (thermoablation) may be promoted.

A large number of magnetic materials for magnetic induction hyperthermia have been developed. The major part of ferro-, ferri-, as well as superparamagnetic materials are suitable for this specific application. An important requirement of all these materials is biocompatibility. MgO-Fe is common material of choice in recent research.

The heating capacity depends on the material properties, such as magnetocrystalline anisotropy, particles size and microstructure. To enable them to penetrate into smallest part of every tissue or even into cancer cell, magnetic material is made with nanometer size, called magnetic nanoparticle (MNP).

Cancerous cells typically have diameters of 10 to 100 micrometers. This has produced the motivation to use MNP to penetrate into a cell.

The particles used in hyperthermia exhibit ferro- or ferrimagnetic properties. These particles have permanent magnetic orientations or moments and some kinds display magnetism even in the absence of an applied magnetic field (Pankhurst *et al.*).

Magnetic nanoparticles are designed to selectively be absorbed in tumor. Once in the tumor, they agitate under an alternating magnetic field and generate heat within tumor. Heat generation is due to different magnetic loss processes such as moment relaxation (Ne' el), mechanical rotation, (Brown) or domain wall displacements), leading to the destruction of the tumor, whereas most of the normal tissue remains relatively unaffected.

Particles with diameters of 10 nanometers or less typically demonstrate superparamagnetic properties. The magnetic moments of superparamagnetic nanoparticles are randomly reoriented by the thermal energy of their environment and do not display magnetism in the absence of a magnetic field. Unlike ferro- and ferrimagnetic materials, they do not aggregate after exposure to an external magnetic field (Berry and Curtis). Aggregation can hinder the body's efforts to remove the nanoparticles. Therefore, superparamagnetic nanoparticles are ideal candidates for hyperthermia cancer treatment.

Nanoparticles can also effectively cross the blood-brain barrier, an essential step in treating brain tumors (Koziara *et al.*). Finally, nanoparticles can be coupled with viruses (20-450 nm), proteins (5-50 nm), and genes (10-100 nm long) (Pankhurst *et al.*).

In practice, MNP is introduced into patient's body by injecting a fluid containing magnetic nanoparticles. This technique is called Magnetic fluid hyperthermia (MFH). When placed in an alternating magnetic field with frequencies in tens to hundreds MHz, MNP begin to agitate and produce enough heat inside the tumor. In this technique, only the magnetic nanoparticles absorb the magnetic field. No heat generation in healthy tissue is the advantage of this technique over other hyperthermia techniques such as laser, microwave, and ultrasound.

Magnetic nanoparticles are evenly dispersed in water or a hydrocarbon fluid. Small size of dissolved particles leads to little or no precipitation due to gravitational forces. For medical applications, the biocompatibility of both the fluid and nanoparticles must be considered. The fluid must have a neutral pH and physiological salinity. In addition, the magnetic material should not be toxic. The established biocompatibility of magnetite (Fe_3O_4) makes it a common choice.

The heating ability of MNPs is expressed by the specific absorption rate (SAR), which is equal to the power loss per material mass. Generally, it is advantageous to achieve the temperature enhancement needed for any application with as low as possible MNPs concentration. For a specific nanoparticle system, SAR is directly related to the applied field amplitude and frequency as well as to geometrical (size, shape) and structural features of the particle. Although various magnetic nanomaterials present high SAR values, the demand for low MNPs concentration and biocompatibility issues restricts significantly materials choice. An alternate route towards larger SAR values is expected to be the enhancement of magnetic moment per particle, e.g. the use of Fe particles coated by a biocompatible shell such as MgO, instead of iron oxides, besides the higher magnetization, it also provides a satisfactory solution to the problem of chemical stability and biocompatibility. Water soluble Fe/MgO nanoparticle is the basis of magnetic hyperthermia. The use of zero-valence iron particles, instead of iron oxides, provides improved magnetization values while the MgO coating serves as a satisfactory solution for the achievement of chemical stability and biocompatibility. The non-toxicity of magnesium-based materials, their corrosion resistance and antimicrobial action are fields of intense research. (A. Chalkidou, et al. 2011) and (O. Bretcanu, et al, 2006)

19. Hyperthermia in combination with other modalities

As an adjunct to traditional cancer therapy, hyperthermia has been shown to enhance cytotoxicity of chemotherapy agents. Since adequate heating of the whole tumor volume is difficult except for superficially located small tumors, and in general the reported response duration is short, the use of hyperthermia alone is not recommended (van der Zee, et al., 2008). Mild hyperthermia (39–43°C) has successfully been utilized in combination with chemotherapy to increase cellular sensitivity to anticancer drugs mainly using an intraperitoneal approach. The interaction between heat and chemotherapeutic agents results in increased drug uptake by accelerating the primary step in a drug's efficacy and increasing the intracellular drug concentration. Therefore, the combination of hyperthermia and anti-cancer drugs may reduce the required effective dose of the anti-cancer drug, and it could enhance the response rates in cancer cells. The results of combined application of chemotherapy and hyperthermia has been satisfactory .

The thermo-chemotherapy (TCHT) is a combined modality treatment with high tolerance for malignant tumors of the mammary gland, of the whole gastric intestinal tract (specially pancreatic cancer), of the lungs, of the urogenital tract (specially ovarian-cancer), of the skin, bones and soft-tissues as well as oral and neck advanced malignant tumors (specially node metastasis). In principle, adenocarcinoma and squamous epithelium carcinoma with metastasis (as well bone metastasis) or without metastasis, osteo sarcoma and soft-tissue sarcoma of nearly all localisations, the malignant melanoma and non-Hodgkin lymphoma and also pleural malignant mesothelioma can be treated. The TCHT main treatment, which

lasts several hours, is followed by approximately 24 hours of intensive care treatment in the specially equipped hyperthermia-clinic.

The treatment itself is based on a controlled interaction between whole-body hyperthermia (body warming-up), induced hyperglycaemia (increasing of the blood glucose level), relative hyperoxemia (oxygen enrichment of the blood) and pre-arranged with the patient modified chemotherapy. Thanks to this multistep therapy, one has the chance to positively influence the course of the illness - even when tumors have not previously responded to radiotherapy, to cytostatics or to hormones.

20. A brief overview of important softwares

In order to permit a patient-specific treatment planning, a special software system (HyperPlan) has been developed.

COMSOL is a general purpose software to compute electromagnetic fields interaction with matter.

SEMCAD X takes the segmentation and a CAD implementation of applicators as input and tissue and material properties are assigned to the solid models. With a proper set-up, the electric fields for each antenna are calculated using the electromagnetic solver of SEMCAD X.

The position of tumor in the patient's body along with neighboring organs and tissues can be reconstructed by Ansoft Human-Body Model. The accuracy of this software is at millimeter level. There are more than 300 objects defined in this model including bones, muscles and organs. Frequency-dependent material parameters are included as well.

21. Prospects

- Heating deep seated tumors effectively still remains an unsolved technical problem.
- Hyperthermia may find additional indications in gene therapy, stem cell purging, drug targeting with heat sensitive liposomes and potentiation of immunity in HIV.
- The reliability of the mathematical optimization depends on the accuracy of the models describing the physical situation. In particular the physiological parameters are individually varying to a significant amount, such that a priori models are subject to significant modelling errors.
- The physical processes of field interference and heat distribution inside the very heterogeneous human body is too complex to be optimized manually. Thus, optimization algorithms are required for therapy planning,

22. Conclusion

Although basically an old and historic approach for treatment of cancer, hyperthermia is not a well-known modality among patients and medical experts. On the other hand, it has proved a very successful therapy method in combination with radiation therapy and / or chemotherapy. The precise mechanism of cancer development and destruction is not known; especially the response of different patients at similar situation is quite unpredictable. At clinical stage, the mathematics behind the treatment planning is difficult to implement for complicated geometry of tumor. The heat transfer and SAR parameters are not identical among patients. Up to the best knowledge of authors of this chapter, these parameters might not be measured with a proper precision, especially at clinical practice.

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Antiangiogenic Treatment Concepts in Gynecologic Oncology

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1. Introduction

Gynecologic malignancies count for about 80,000 of all new cancer diagnoses in women in the United States (Jemal et al., 2010). In the US almost 20,000 patients a year are diagnosed with epithelial ovarian cancer, 11,000 with cervical cancer and 42,000 with endometrial cancer (Jemal et al., 2010). A relevant part of these tumor situations are found in an early-stage disease setting, for instance most of all newly diagnosed endometrial cancers. But frequently there is no possibility for an early-stage diagnosis and the tumor is already advanced when primarily detected, like in the majority of all cases with epithelial ovarian cancer (EOC).

Though tumor biology and standard treatment concepts are different for all three entities, there is a common need for new therapeutic approaches to improve the patients' outcome. Many preclinical studies have suggested that antiangiogenic strategies are beneficial against these cancers (Delli Carpini, 2010). One reason may be the fact that these tumors are able to form large single tumor nodulations during intraabdominal spread or local progression with hypoxic cores triggering tumor-associated neo-angiogenesis (Bryant et al., 2010). Furthermore, the frequently seen phenomenon of peritoneal carcinosis, closely related to gynecologic malignancies, induces angiogenesis to preserve nutritive supply to all metastatic tumor nodes (Fagotti et al., 2010, Figure 1).

Since Folkman first proposed the strategy of targeting the tumor vasculature as a novel therapeutic strategy, considerable progress has been made to understand the underlying mechanisms of angiogenesis (Folkman, 1971). The control of angiogenesis is under the influence of both pro- and anti-angiogenesis factors. Of these, vascular endothelial growth factor (VEGF) and its family of receptors play a key role in the regulation of angiogenesis. The VEGF gene family consists of several members including placenta growth factor (PlGF), VEGF-A, VEGF-B, VEGF-C and VEGF-D, but VEGF-A (often referred to as VEGF) is the dominant protein (Ferrara, 2003). The VEGF receptor family consists of three members including VEGFR-1(Flt-1), VEGFR-2 (KDR or Flk-1), and VEGFR-3 (Flt-4). Given the key role of VEGF and its family of receptors in regulating angiogenesis, inhibitors of both VEGF and its receptors are actively being developed as anti-cancer therapies. To inhibit the VEGF pathway two different options are used:

1. VEGF ligand inhibition by antibodies or soluble receptors
2. VEGF receptor inhibition by tyrosine kinase inhibitor (TKIs) or receptor antibodies

Several further antiangiogenic targets beside the VEGF pathway are identified and explored (Figure 2).

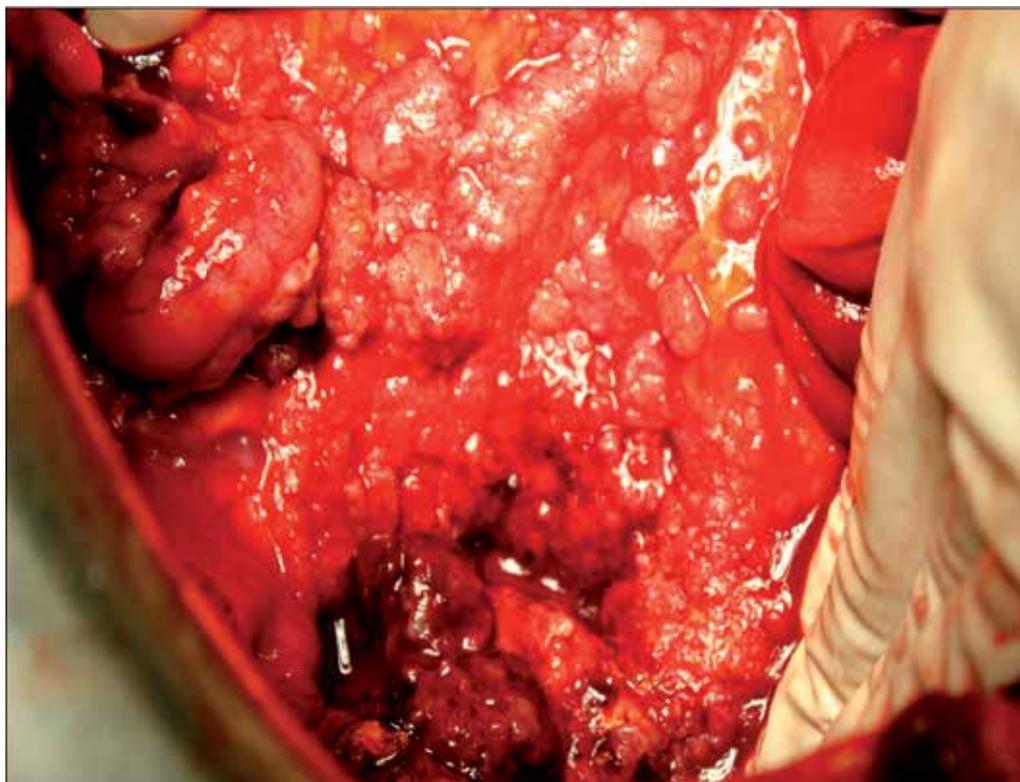


Fig. 1. Peritoneal carcinosis and Angiogenesis.

The clinical evaluation of antiangiogenic drugs revealed new, unique toxicity profiles and potential side-effects, such as hypertension, proteinuria and GI-toxicities that have so far not been in the focus of the oncologist and need to be understood and closely considered during therapy. However, under careful and responsible advise most of the established and documented treatment concepts can be performed without difficulties.

In summary, antiangiogenic drugs are reasonable and promising new therapeutic strategies under clinical investigation. Subsequently, current antiangiogenic treatment strategies against the three main gynecologic malignancies ovarian cancer, cervical cancer and endometrial cancer will be presented and discussed.

2. Ovarian cancer

2.1 Background

In the US about 21,880 women a year develop a malignant tumour of the ovary. The incidence of ovarian carcinoma has remained unchanged in the last few decades. With more than 13,850 deaths, it is the fifth highest cause of cancer-related mortality in women. Symptoms of the disease usually develop at a very late stage. For this reason about 70% of patients are already in an advanced stage of the tumor at the time of diagnosis (FIGO III or

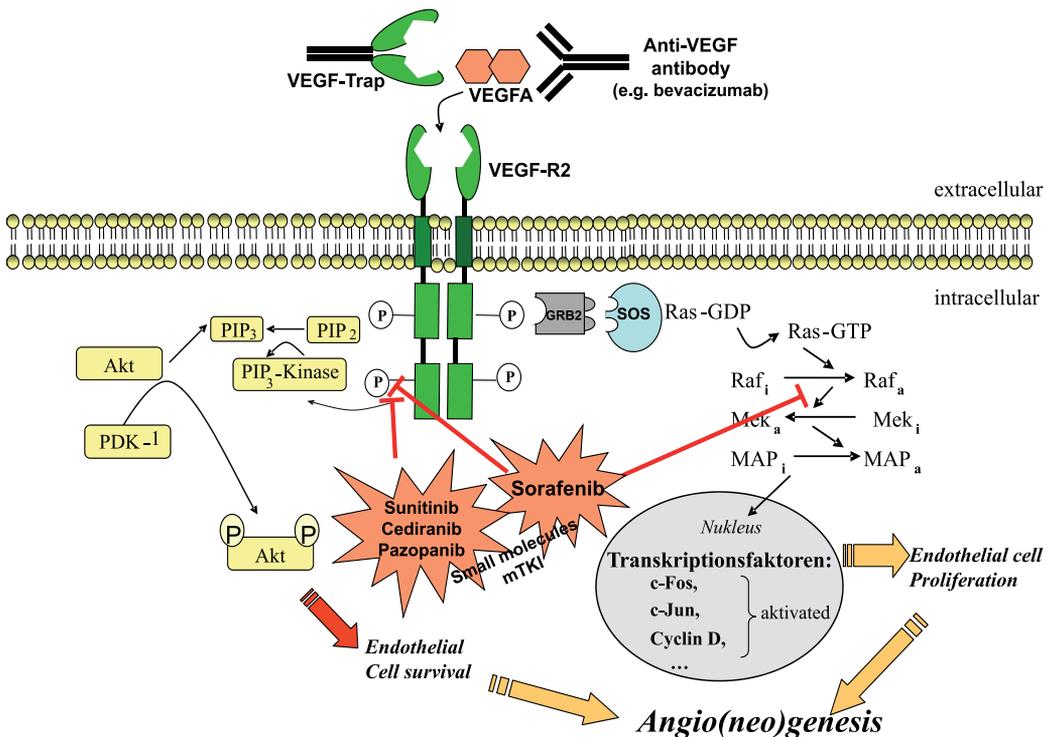


Fig. 2. Molecular mechanisms in tumor-associated angiogenesis and main targets of current investigations.

IV). Surgical tumor removal is the primary treatment. Whether and to what extent residual tumour formation is present postoperatively is the deciding factor in the subsequent prognosis for the patient. After surgery, chemotherapy involving paclitaxel plus carboplatin is generally indicated in the event of an initially advanced tumour stage (McGuire et al., 1999; McGuire et al., 2001; Parmar et al., 2003). Despite improved surgical procedures and a high primary response to chemotherapy, about 70% of patients with advanced ovarian carcinoma develop a tumor relapse and die from the disease.

Angiogenesis is a critical pathway in the development and progression of ovarian cancer. Therefore, identification and development of novel agents with limited toxicity that target mechanisms of tumor progression such as angiogenesis are of high priority. Data from numerous preclinical and clinical trials support the assumption of VEGF/VEGFR, PDGFR as well as FDGF as target molecules for the treatment of ovarian cancer (Burger, 2010). Beyond these currently intensively investigated targets, further antiangiogenic pathways are explored, such as angiopoetin or vascular disrupting agents (VDA) (Theo et al., 2010; Zweifel et al., 2011).

Anti-angiogenic agents in investigational clinical trials

Several anti-angiogenic agents are evaluated in investigational clinical trials to improve the therapy of recurrent and also primary ovarian cancer. Results from a number of phase I and phase II trials are available and also phase III trials have been performed and have led to first reported data, in particular the GOG 218 as well as the ICON-7-trial.

Author	patients	Treatment	ORR [%]	Median OS [months]	Median PFS [months]	
Micha 2007	n=20 (first line, advanced EOC, PPC, FTC)	Paclitaxel i.v. (175 mg/m ²) and carboplatin i.v. (AUC %) q3w for 6 cycles and bevacizumab (15 mg/kg) at cycles 2-6.	80	NR	NR	48,3 % neutropenia of 116 cycles 10% hypertension
Burger 2007	n=62 persistent/recurrent EOC/PPC	bevacizumab 15 mg/kg i.v. q3w	21	17	4,7	9,4% hypertension 6,4% GI events (nausea, emesis) 4,8% pain 3,2% allergic
Cannistra 2007	n=44 platinum-resistant EOC/PPC	bevacizumab 15 mg/kg i.v. q3w	16	10,7	4,4	9,1% hypertension 15,9 % proteinuria 2,3% bleeding 2,3 % wound-healing complications 11,4% GI perforation
Garcia 2008	n=70 recurrent EOC/PPC	bevacizumab 10 mg/kg i.v. q2 w and cyclophosphamide 50 mg/d p.o.	24	16,9	Median TTP: 7,2	19,4 % lymphopenia 18,6% pain 8,6% fatigue Bevacizumab-related: 15,7% hypertension 5,7% GI-Perforation or fistula 4,3 proteinuria
Tillmanns ^a 2010	n=48	bevacizumab 10 mg/kg i.v. q2 w and nab-paclitaxel 100 mg/m ²	46	16,5	8,3	3,8% nausea 3,8% nosebleed 3,8% bowel obstruction 2,8% neuropathy 2,6% neutropenia 2,4% anemia 1,6% infection
Penson 2010	n=62 newly diagnosed	Carboplatin i.v. (AUC5), paclitaxel i.v.(175 mg/m ²) and bevacizumab 15 mg/kg i.v. for 6-8 cycles on day 1 every 21 days. Bevacizumab was omitted in the first cycle and continued as a single agent for 1	76	Not reached	29,8	Chemotherapy phase: 22,6 % neutropenia 12,9% metabolic 9,7% hypertension 6,5% thrombocytopenia,

Table 1. Antiangiogenic drugs currently under investigation in EOC.

Bevacizumab

The most clinical experiences have been collected targeting VEGF with the recombinant humanized monoclonal anti-VEGF antibody (bevacizumab, avastin®).

Several phase II studies showed efficacy and tolerability as well in the palliative as in the adjuvant setting. Recently, bevacizumab in combination with the standard chemotherapy of carboplatin/paclitaxel was evaluated in two phase III trials demonstrating a significant improvement in PFS compared to the standard chemotherapy treatment (GOG 218 and ICON 7). Table 1 provides an overview of all published phase II and III studies (Table 1).

Treating ovarian cancer patients with bevacizumab single agent regimens demonstrated very promising response rates (Table 1), furthermore some combined therapies including cytotoxic drugs seemed to enhance not only direct antitumoral efficacy but also antiangiogenic potentials. This is in particular the case for so called metronomic treatment strategies, where chemotherapeutic agents are administered in low dosages in regular, short time intervals, for example, cyclophosphamide in daily oral application (Sanchez-Munoz et al., 2010).

The main toxic effects of bevacizumab as documented in the available trials are hypertension, headache, proteinuria, thrombosis and hemoptysis (Table 1). A particular attention has been drawn on GI perforations that have been observed and seem to be a specific phenomenon on bevacizumab-therapies in patients with EOC. The exact pathomechanism of this potentially fatal complication is not fully understood though there are several explaining theories (Richardson et al., 2010). Currently, the average risk of developing a GI perforation during bevacizumab-therapy can be estimated at 7-8 % for patients with EOC (Richardson et al., 2010, Tanyi et al., 2011).

VEGF trap

VEGF trap (aflibercept®), a molecular fusion protein, inhibits VEGF-mediated events as a high affinity VEGF decoy. It is also able to bind to other VEGF family members e.g. PlGF. Tew et al. published a multicenter phase II study in women with recurrent ovarian cancer who were treated with 2mg/kg or 4mg/kg aflibercept, but the study only showed a moderate response rate (ORR 7 %, Moroney et al., 2009)

Toxic effects of aflibercept are hypertension, headache, fatigue and GI -perforations (1,8%). Colombo et al. could show that aflibercept reduces ascites in patients with advanced ovarian cancer. They achieved a response in terms of less often repeated paracenteses.

Ramucirumab

Ramucirumab (IMC-1121B) is a novel fully human antibody targeting VEGFR2. It showed significant antitumor activity in several mouse tumors and human tumor xenografts by inhibiting angiogenesis mediated by reduction of microvessel density, tumor cell apoptosis and necrosis, as well as a decreased tumor cell proliferation (Prewett et al., 1999; Spratlin, 2011).

Ramucirumab is currently being explored in phase III trials in hepatocellular carcinomas (ImCLON HCC-Ramucirumab), NSCLC (Lilly study I4T-MC-JVB(a)), in previously untreated patients with HER2-negative, unresectable, locally recurrent or metastatic breast cancer (Trio-012) and in patients with metastatic colorectal carcinoma. A phase III study is designed in patients with metastatic adenocarcinoma of the stomach. The safety and efficacy of Ramucirumab is also evaluated in a randomized phase II trial in patients with metastatic melanoma with or without dacarbazine (Carvajal et al., 2010), as second-line therapy in

patients with locally advanced or metastatic transitional cell carcinoma of the bladder, urethra, ureter, or renal pelvis and metastatic androgen-independent prostate cancer with or without mitoxantrone and prednisone.

At the time of publication, ramucirumab was undergoing assessment in a non-randomized, open-label, multicenter phase II study as a monotherapy in the treatment of persistent or recurrent EOC, FTC, or PPC. Ramucirumab is given at 8mg/kg q2w. The results of these trials will tell whether ramucirumab is a useful addition to current antiangiogenic therapies and an option in the treatment of patients with ovarian cancer.

Tyrosine kinase inhibitors

Tyrosine kinase inhibitors repress the VEGF pathway by binding directly to the VEGFRs.

Sorafenib

Sorafenib is an oral tyrosine kinase inhibitor which not only targets VEGFR2 and 3 and PDGFR-beta but also the Ras/Raf/Mek/ERK pathways. In the GOG 170 trial Matei et al. evaluated Sorafenib as a single agent treatment in patients with recurrent ovarian cancer. In this phase II trial preliminary results showed a progression free survival for at least six months in 12 of 59 patients, partial response in 2 patients and stable disease in 20 patients. Progressive disease was determined in 30 patients (Matei et al., 2010).

Siu et al. tested Sorafenib in combination with gemcitabine in a phase I trial in solid tumors and could show that the combination was well tolerated (Siu et al., 2006). In contrast to this and in line with Matei et al. Pölcher and colleagues pointed out that sorafenib showed markable toxicity in ovarian cancer study protocols, thus this drug needs to be further evaluated in clinical trials (Pölcher et al., 2010).

Sunitinib

Sunitinib (Sutent®) is also an oral tyrosine kinase inhibitor that binds to VEGFR 1-3 as well as PDGFR-alpha and beta. Sunitinib is approved for the treatment of advanced or metastatic renal cell carcinoma as first line therapy, of nonresectable gastrointestinal stromal tumours and for the treatment of patients with unresectable, locally advanced, or metastatic pancreatic neuroendocrine tumors (pNET). There are lots of clinical trials to determine the efficacy and safety of sunitinib in several tumor entities e.g glioblastoma, colon cancer and breast cancer. [<http://clinicaltrialsfeeds.org/clinical-trials/results/?term=sunitinib>]

In a preclinical study sunitinib inhibited tumor growth and reduced peritoneal metastasis of human ovarian cancer in xenografted mice. (Bauerschlag et al., 2010). The following phase II study of sunitinib (initial dose 50mg/d p.o) in 30 patients with recurrent EOC or PPC showed only a partial response (3,3%). Hand-foot-syndrome, hypertension, fatigue and gastrointestinal symptoms but no gastrointestinal perforation were the main toxicities. (Biagi et al., 2011).

Pazopanib

Pazopanib is an investigational, oral, angiogenesis inhibitor targeting VEGFR, PDGFR and c-kit. Pazopanib is currently being studied in a number of different tumour types; clinical trials are currently underway in renal cell carcinoma (Phase III), breast cancer (Phase III in inflammatory breast cancer), ovarian cancer, STS, NSCLC, cervical cancer and other solid tumours. It is being evaluated as a monotherapy, in combination with targeted therapies and in combination with cytotoxic chemotherapy.

Author	patients	Treatment	ORR [%]	Median OS [months]	Median PFS [months]	
Welch 2010	n=33 recurrent EOC	Gemcitabine 1000mg/m ² i.v. weekly for 7 of 8 weeks in the first cycle, then weekly for 3 weeks of each subsequent 4-week cycle. Sorafenib 400 mg p.o.	4,7	13	Median TTP: 5,4	hand-foot syndrome, fatigue, hypokalemia, diarrhea
2010	n=102 (was planned),4 were enrolled neoadjuvant advanced EOC	Carboplatin AUC5 and Paclitaxel 175 mg/m ² preoperatively and concomitant sorafenib 400 mg twice daily. (After four cycles of postoperative chemotherapy, a maintenance phase of single agent oral Sorafenib through 1 year was planned)	-	-	-	3 patients had life threatening events (cardiac output failure, myocardial infarction, anastomotic leak): The study was interrupted!
Matei 2011	n=71 recurrent/persistent EOC/PPC	Sorafenib 400 mg twice daily.	2,8	NR	At 6 months (n=12)	14% metabolic 12,6% hand-foot syndrome 9,9 % rash
Biagi 2011	n=30	Sunitinib 50 mg daily, 4 of 6 weeks	3,3	NR	4,1	fatigue, gastrointestinal symptoms, hand-foot syndrome and hypertension.
Matulonis 2009	n=46 recurrent EOC/FTC/PPC	Cediranib 45 mg daily (Because of toxicities the dose was lowered to 30 mg)	17,4	Not yet reached	5,2	46% hypertension 24% fatigue 13% diarrhea
Friedlander 2010	n=31 recurrent EOC/FTC/PPC	pazopanib 800 mg once daily	18			

Table 2. Overview of published phase II and phase III trials on bevacizumab in patients with ovarian cancer.

VEG104450 is a Phase II study to assess the biochemical response rate (determined by CA-125 response) to pazopanib monotherapy in subjects with epithelial ovarian, fallopian tube, or primary peritoneal carcinoma that have responded to standard treatment and who have a high risk of recurrence due to a rising CA-125. Data reported recently (Friedlander, 2008) showed that, in 36 ovarian subjects with biochemical (i.e. CA-125) recurrence after < 2 treatment regimens, the most frequent AEs (reported by more than 20% of subjects) are diarrhoea, fatigue, nausea (all 47%), hypertension and abdominal pain (31%), AST and ALT increase (25%), anorexia and vomiting (22%). These were primarily Grade 1 and 2, with only one Grade 4 event reported in the study (of peripheral oedema, considered unrelated to the study drug); there were no Grade 5 events and no bowel perforations reported in this study. Currently, the German PACOVAR-trial analysis in a phase I/II setting evaluates activity and tolerability of pazopanib combined with orally administered metronomic cyclophosphamide in patients with recurrent, intensively pretreated ovarian cancer. First results of the phase I are expected in 2012.

Endometrial cancer

Endometrial cancer is the most common gynecologic malignancy with a peak incidence between the ages of 55 and 65. Worldwide about 142.000 women are affected by endometrial cancer every year (Jemal et al., 2010). It is frequently diagnosed at an early stage as women affected often present by abnormal vaginal bleeding. At an early stage endometrial cancer can be treated surgically with curable intention (van Wijk et al., 2009). In patients with high-risk endometrial cancer postoperative pelvic radiotherapy, adjuvant radiation therapy or adjuvant chemotherapy have shown to improve outcome (Ray et al., 2009).

However, about 13% of all patients with endometrial cancer develop recurrent disease. (Van Wijk et al., 2009). In the treatment of recurrent endometrial cancer different therapeutic modalities consisting of radiotherapy, surgery and systemic therapies as chemotherapy and hormone therapy are in use (Ray et al., 2009). Clinical trials evaluating chemotherapeutic regimen for patients with endometrial cancer include combinations of doxorubicin and cisplatin, cyclophosphamide or paclitaxel and carboplatin, most of them administered in palliative situation (Ray et al., 2009).

Due to promising results of antiangiogenic treatment concepts in many solid tumors, some efforts have already been made to elucidate the role of VEGF in endometrial cancer. Kamat et al. examined serum samples of endometrial cancer patients and established an endometrioid orthotopic mouse model to approach the role of VEGF in endometrial cancer. The authors found significantly increased levels of VEGF in approximately half of the tumors. These high levels were independently associated with a poor outcome of the affected patients, as an overexpression of VEGF enhances tumor growth. Moreover, in a published mouse model study the combination of docetaxel and bevacizumab has proven a greater therapeutic efficacy than docetaxel or bevacizumab alone (Kamat et al., 2005).

Recently, Aghajanian et al published data of a phase II GOG trial of bevacizumab in patients with recurrent or advanced endometrial cancer. In a series of n=56 patients the authors could demonstrate that a treatment regimen consisting of 15mg/kg bevacizumab i.v. every three weeks led to an ORR of 13.5% and a PFS at 6 months of 40.4% (Aghajanian et al., 2010). Furthermore, it could be shown that high VEGF-A immunohistochemical staining in archival tumors was associated with a reduced risk of death but that high circulating VEGF-A levels were associated with poor outcome. For possible explanation authors suggested that VEGF staining in tumor did not reflect the state of the tumor before treatment when

VEGF-levels in plasma where measured. Finally, in 2011, Reinhardt and colleagues published a case report presenting the successful remission in a patient with recurrent, heavily pretreated endometrial cancer using a combination regimen with bevacizumab and metronomic cyclophosphamide (Reinhardt et al., 2011). Adverse events of bevacizumab therapies in endometrial cancer patients include GI-hemorrhage, proteinuria, hypertension, thrombosis and pulmonary embolism. So far no GI-perforations or fistulae have been reported (Aganajian et al., 2010)

Beside bevacizumab as antiangiogenic treatment concept the oral tyrosine kinase inhibitors of multiple VEGF receptors sunitinib and sorafenib have been evaluated in clinical studies for the treatment of endometrial cancer.

Sorafenib showed minimal activity with PFS at 6 months of 29% and median overall survival of 11.4 months. As adverse events hypertension, hand-foot-syndrome, anemia, thrombosis, fatigue and bleeding were found (Nimeiri et al., 2008).

Accordingly, in a phase II study in recurrent or metastatic endometrial cancer sunitinib demonstrated activity with an ORR of 15% and median overall survival of 19 months. Adverse events were fatigue and hypertension. (Correa et al., 2010)

Cervical cancer

Cervical cancer is the second most cause of female cancer mortality worldwide with 288 000 deaths every year. About 510.000 cases of cervical cancer are reported each year with nearly 80% in developing countries. Cervical cancer is preventable and generally curable if diagnosed at early stage (Wright et al., 2006). Surgery is the goldstandard in early lesions, whereas locally advanced lesions are managed with concurrent cisplatin chemotherapy and pelvic radiation (Monk et al., 2009). Metastatic disease or recurrent lesions not amenable to radical local excision or regional radiation are treated with palliative chemotherapy (Tewari et al., 2009). Patients diagnosed with locally advanced or metastatic cancer of the cervix have a very poor prognosis with a 5-year survival between 5 and 15 % (patients with stage IV disease) (Takano et al., 2009).

Recurrent tumors within the irradiated and therefore devascularized fields likely have microenvironment changes that make chemotherapy delivery far from optimal. For these reasons, this patient population is not one that is ideally suited to receive multiple lines of chemotherapy. Biologic therapies offer another therapeutic strategy that has demonstrated effectiveness in tumors resistant to chemotherapy (Monk et al., 2010).

Angiogenesis seems to play an important role in the development and progression of cervical cancer. Evidence that angiogenesis plays an important role in locally advanced cervical cancer has been shown in recent years (Mackay et al., 2010; Carpini et al., 2010). In one study of 111 patients with cervical cancer, Cooper et al identified tumor angiogenesis (as reflected by the tumor microvessel density) as a significant prognostic factor within a Cox multivariate analysis, where it was associated with poor locoregional control and overall survival. Data from prospective studies in women with advanced cervical cancer, treated with anti-angiogenic therapy, are limited to a few small studies.

Bevacizumab

Corresponding to other solid tumors, most experiences are also reported in targeting VEGF with bevacizumab.

Wright et al first evaluated the effect of the VEGF-Inhibitor bevacizumab in a retrospective trial in women with recurrent cervical cancer (Wright et al., 2006). Five patients were treated

with bevacizumab and 5-fluoruracil, one patient with bevacizumab and capecitabine, respectively. The median age of these patients was 43 years, the stage distribution was IB2, IIB and IIIB at 2 patients a throw. All of the patients had received prior platinum-based chemotherapy. Bevacizumab was given intravenously every other week to five of the six patients with a starting dose of either 5 mg/kg or 10 mg/kg. One patient was given bevacizumab at a dose of 15 mg/kg every three weeks. A total of 30 doses of bevacizumab was given. The regimen was well tolerated. There was a grade 4 neutropenic sepsis encountered in one patient after 2 cycles of bevacizumab and one extremity thrombosis in another subject. The overall response rate (ORR) was 33 % (2 of the 6 patients). One of these patients showed a complete response, the other one a partial response. There was a stable disease in two patients (33 %). The median time to progression for the four patients with clinical benefit (CR, PR, SD) was 4.3 months. None of the patients demonstrated a progression free interval > 6 months. Though only a small number of patients was observed, this study indicated that the combination of bevacizumab and 5-fluoruracil-based chemotherapy seems to be feasible and associated with significant activity in patients with recurrent cervical cancer.

In 2009 Monk published a phase II-trial of bevacizumab in the treatment of persistent in recurrent squamous cell carcinoma of the cervix (GOG protocol 227C) (Monk et al., 2009). 46 patients were randomized to the trial and received bevacizumab in a dose of 15 mg/kg intravenously every 3 weeks. Primary endpoints of the study were PFS for at least 6 months and evaluation of adverse events. A total of 254 cycles of bevacizumab as a single agent therapy were administered with a median of 4 cycles per patient. Eleven patients (23.9 %) experienced a progression free survival of > 6 months, whereas five patients (10.9 %) showed partial responses. Median overall survival for all patients was 7.29 months. There were several grade 3/ 4 adverse events demonstrated in this trial, including hypertension (n = 7), thromboembolism (n = 5), gastrointestinal complaints (n = 4), anemia (n = 2), vaginal bleeding (n = 1), neutropenia (n = 1) and fistula (n = 1) No unusual toxicities were noted. This trial provided that bevacizumab as a single agent therapy was relatively well tolerated, safe and demonstrated remarkable activity. Importantly, the results of this protocol constitute the first prospective clinical trial of a biologic agent that shows clinical activity in cervical cancer (Tewari et al., 2009). Exploratory analyses suggested an increased risk of progression (or death) for those who are African- American, are young, and have a poor performance status. Additional analyses also suggested an increased risk of death for those who have more prior chemotherapy regimens.

Takano et al (Takano et al., 2009) reported of two cases of patients with cervical cancer treated with bevacizumab (2 mg/kg), paclitaxel (80 mg/m²) and carboplatin (AUC = 2.0). Therapy consisted of carboplatin/paclitaxel weekly on days 1, 8, 15 and bevacizumab weekly on days 1, 8, 15, 21, q28d. In both patients full remission was notified and there was no evidence of disease for > 10 months as well as no adverse events higher than grade 3.

Based on the findings in the studies described, a phase III trial with bevacizumab for treatment of cervical cancer is planned by the GOG. In GOG 240, patients will be randomized to one of four regimens: paclitaxel/cisplatin, paclitaxel/cisplatin plus bevacizumab, paclitaxel/topotecan and paclitaxel/topotecan plus bevacizumab.

Also, other anti-angiogenesis compounds will soon be investigated by the GOG. For example, brivanib, a highly potent dual inhibitor of VEGFR and fibroblast growth factor receptor (FGFR) will be under study (Monk et al., 2010).

Sunitinib

There is one phase II study published in 2010 by Mackay et al. that evaluates the activity of sunitinib, a tyrosine kinase inhibitor in the treatment of locally advanced or metastatic cervical cancer (Mackay et al., 2010) (NCIC CTG Trial IND.184). Sunitinib is an oral, multi-targeted tyrosine kinase inhibitor, that inhibits receptors for VEGF, c-Kit and platelet-derived growth factor. 19 patients received sunitinib at a dose of 50 mg/day in 6-week cycles (4 weeks, followed by 2 weeks off treatment). 16 patients (84 %) showed stable disease, but there was no objective response noted in any of the patients. However, 4 patients developed fistulae during treatment and one patient had an enterocutaneous fistula 3.5 months post-study. All of the fistulae occurred within the previous radiation fields. Although SD-rate was 84 %, the overall progression free survival was only 3.5 months in this study, which compares with the progression free survival in the phase II trial published by Monk and described before. But the observation of five cases of fistulae (26 %) was of particular concern, so that sunitinib as a single agent therapy does not have enough sufficient activity in women with advanced cervical cancer to recommend further study.

3. Conclusion and future perspectives

There is consensus that angiogenesis is a crucial phenomenon in the progression of gynecologic tumors. Many promising efforts have been made so far demonstrating that ovarian cancer as well as uterine cancers can be targeted by antiangiogenic treatment strategies.

As gynecologic tumors still often respond very differently to antiangiogenic therapies future studies will have to work on identifying patients that will likely respond to specific antiangiogenic regimens. Existing and already proven active therapies will have to be confirmed in further long-term experiences, in particular regarding their potential long-term toxicity or side-effects and finally new, additional targets beyond the VEGF/ VEGFR-mechanism have to be evaluated. Finally, a new attention should be paid to the concept of maintenance treatments, in particular in EOC, as antiangiogenic treatment concepts, might be especially useful in keeping a major cytotoxic therapeutic success. A particular role may be attributed in the future to combined antiangiogenic and metronomic cytotoxic regimens.

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Photodynamic Therapy in Combination with Antiangiogenic Approaches Improve Tumor Inhibition

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1. Introduction

Photodynamic therapy is a non-surgical and minimally invasive procedure that is rapidly developing as a cancer treatment modality. It involves the administration of a photosensitizer that selectively accumulates in the tumor tissue, which is subsequently activated with light of specific wavelength that interacts with molecular oxygen to form toxic, short-lived species known as singlet oxygen, which causes tumor cell death (Macdonald & Dougherty, 2001). The evident advantage of PDT over other conventional cancer treatments such as chemotherapy and radiotherapy is its selective targeting and reduced toxicity (Dolmans et al., 2003). The treatment is relatively non-invasive as it usually only requires targeted illumination of the tumor site. PDT can also be repeated without detrimental consequences to the patients. Currently, PDT is being successfully used for the treatment of early lung cancers (Moghissi et al., 2007; Usuda et al., 2006) and in dermatology for the treatment of non-melanoma skin cancers and precancerous diseases (Klein et al., 2008). PDT has also been successfully employed to treat early carcinomas of the oral cavity and larynx to preserve normal tissue and improve cure rates (Biel, 2007). In the past 20 years, PDT has been successfully used for the treatment of dermatological diseases, ophthalmic diseases, head and neck cancers, brain tumors, pulmonary and pleural mesothelial cancer, cardiovascular disease, gastroenterological cancer, urological disease and gynaecological cancer (Z. Huang, 2005).

However, PDT is an oxygen consuming modality, and an inherent consequence of PDT is local hypoxia. This condition arises either due to direct oxygen consumption during treatment or indirectly due to the destruction of tumor vasculature. As a result, cells under hypoxic stress may switch to an adaptive response by inducing hypoxia inducible factor like

HIF-1 α thus triggering angiogenesis. Angiogenesis is the formation of new blood vessels from pre-existing vessels. It is a vital process in the progression of cancer from small, localized neoplasms to larger, growing, and potentially metastatic tumors (Folkman, 2002). Therefore, the process of tumor angiogenesis is triggered by the tumor's release of pro-angiogenic signals such as vascular endothelial growth factor (VEGF), which bind to receptors on nearby vessel endothelial cells. VEGF is a potent regulator of tumor angiogenesis that plays a critical role by increasing blood vessel permeability, endothelial cell growth, proliferation, migration and differentiation (Ferrara, 2004). It is upregulated in response to hypoxic conditions in tumor via the transcription of hypoxia-inducible factor (HIF-1) (Pugh & Ratcliffe, 2003). Cellular and circulating levels of VEGF have been elevated in haematological malignancies and are adversely associated with prognosis (Giles, 2001). Reports on tumors treated with PDT showed an upregulation of various angiogenic factors like VEGF, HIF-1 α , cyclooxygenase-2 (COX-2), basic fibroblast growth factor (bFGF) and matrix metalloproteinases (MMPs) (Solban et al., 2006; Yee et al., 2005). Studies have shown the upregulation of HIF-1 α , VEGF, COX-2 and bFGF after hypericin-mediated PDT treated tumors, suggesting that PDT-induced damage to tumor microvasculature and the resultant hypoxia upregulated the expression of certain proangiogenic factors (Zhou et al., 2005). They also reported that the inclusion of various angiogenic inhibitors along with PDT treatment enhanced the PDT effectiveness. Currently, anti-angiogenesis agents are being developed to target different growth factors and molecular pathways that play a major role in tumor angiogenesis.

This chapter evaluates expression of VEGF after PDT and also the efficacy of PDT by combining monoclonal antibodies (angiogenesis inhibitors) against VEGF and epidermal growth factor receptor (EGFR) to improve the overall bladder tumor responsiveness. The following approaches were adapted in this study: (i) evaluating the expression of VEGF after PDT, (ii) targeting the VEGF pathway using monoclonal antibody, Avastin, to inhibit tumor angiogenesis and also to study the effect of Avastin on other angiogenic growth factors; (ii) targeting the EGFR pathway, using the monoclonal antibody Erbitux to inhibit tumor angiogenesis and to assess its effect on the EGFR pathway and finally (iii) combining both Avastin and Erbitux with PDT to assess the importance of blocking the two major angiogenic pathways, VEGF and EGFR, to improve treatment outcome.

PDT followed by Avastin inhibited VEGF expression and other important growth factors to improve tumor response in bladder carcinoma xenografts. In a similar way, PDT and Erbitux suppressed growth factors related to the EGFR pathway to produce better treatment outcome. It was noticed that PDT induced tumor destruction can be maintained and significantly enhanced by the administration of Erbitux. VEGF and EGFR pathways play a major role in angiogenesis of bladder tumors. Combining angiogenic inhibitors with PDT protocol to block VEGF and EGFR pathways has proven to be effective in controlling tumor regrowth. Therefore, antiangiogenesis agents may augment the activity of PDT by inhibiting its counterproductive upregulation of VEGF and EGFR. The success achieved by combining angiogenic inhibitors with PDT can provide information for potential target mechanisms, which can be translated into clinical studies with better response rate, less local and systemic toxicity and improved overall survival in patients.

2. Photodynamic therapy induced VEGF

Vascular endothelial growth factor is one of the most important regulators of angiogenesis that acts as a switch to trigger tumor recurrence by promoting proliferation, migration and

tube formation of endothelial cells. Moreover, VEGF binds to the tyrosine kinase receptors, VEGFR-1 and VEGFR-2 thus initiating a downstream signaling cascade that promotes angiogenesis (Kowanetz & Ferrara, 2006). In vitro studies have clearly demonstrated that VEGF is a potent mediator of angiogenesis as it helps in the proliferation and migration of the endothelial cells to form tube like capillaries (Bernatchez et al., 1999). Studies have reported that hypoxia plays a major role in the expression of VEGF in tumor tissue (Robbins et al., 1997). It has also been reported that PDT produced significant increases in VEGF within treated lesion (Ferrario & Gomer, 2006). The expression of VEGF in areas surrounding tumor necrosis has also suggested that hypoxia within tumors played a major role in angiogenesis (Senger et al., 1986; Shweiki et al., 1992).

Photodynamic therapy can produce a significant effect on the expression profile of VEGF in serum and tumors. Experiments were conducted in a xenograft model to evaluate VEGF expression at 24 h, 48 h and 72 h after treatment to understand the initiation of regrowth post hypericin PDT (Bhuvaneswari, Gan et al., 2007). Controls in the experiments were animals with untreated tumors. As human nasopharyngeal carcinoma cells was used as xenografts in a mouse model, both human and mouse VEGF were estimated in serum and tumor tissue. The decrease in mouse VEGF in serum immediately post treatment was not significant, but it reached control levels within 72 h. Greater amount of mouse VEGF compared to human VEGF in serum could indicate the involvement of host environment in modulating the PDT response of the tumor (Figure 1). At 24 h post PDT, both the mouse and human VEGF levels in tumor tissue decreased compared to the control group but elevated by 72 h (Figure 2). The decrease of VEGF observed at 24 h post PDT could be explained through the postulation that the residual tumor cells from the initial PDT treatment could be reoxygenated after 24 h following PDT (Uehara et al., 2001) or may be due to reversal of temporary vascular occlusion (Tsutsui et al., 2002). Downregulation of VEGF immediately after PDT and its subsequent upregulation at 72 h could indicate that regrowth in tumors after PDT begins as early as 72 h. It can be argued that both tumor angiogenesis and recurrence may therefore be mediated by PDT via the enhancement of VEGF expression within the treated tumor mass (Tsutsui et al., 2002).

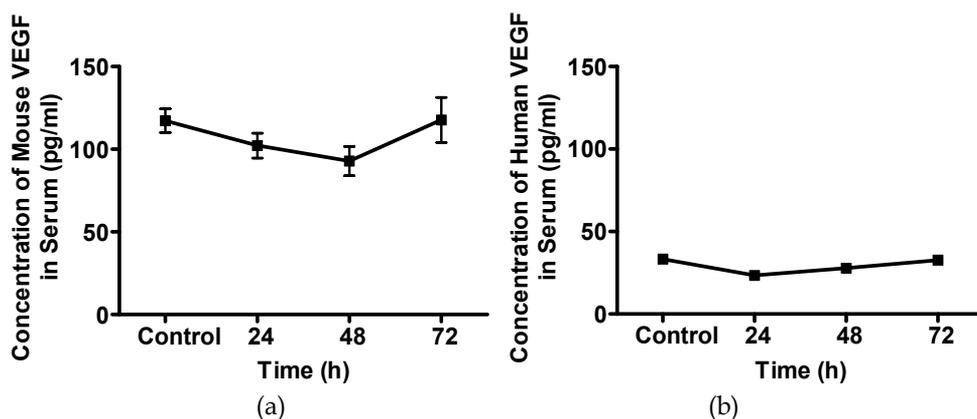


Fig. 1. Concentration of (a) mouse VEGF and (b) human VEGF in serum in the control and at 24 h, 48 h and 72 h post PDT. Error bars represent the standard error of the mean concentration of mouse VEGF in serum at 24 h, 48 h and 72 h, $n = 8$. The mouse VEGF in serum decreased at 24 h and 48 h post treatment compared to the control group. However at 72 h post PDT the mouse VEGF levels increased and were comparable to the control group.

Mouse VEGF levels were found to be significantly lower than human VEGF in the tumor tissue and this could be attributed to the number of host cells versus the number of tumor cells present within the treated region. Similar observations were reported by Gomer et al. (Gomer et al., 2006). Detection of VEGF has long been known as a potential serum diagnostic marker for malignant diseases. Increased serum VEGF concentrations have been measured in various types of cancer, including brain, lung, renal and ovarian cancer (Kondo et al., 1994). High serum VEGF has been strongly associated with poor clinical outcome in lymphoma patients (Salven et al., 2000). Overexpression of VEGF is known to be common in NPC, which is related to hypoxia up-regulated expression involving a HIF-dependent pathway, and is associated with poor prognosis. Targeting the hypoxia pathway may be useful in the treatment of NPC (Hui et al., 2002). Patients with nasopharyngeal carcinoma having high VEGF levels in serum have been associated with a worse progression-free survival. A recent study has also shown increased microvessel density in oral cancer tissues in VEGF-positive tumors and indicated that upregulation of VEGF was correlated with tumor angiogenesis and disease progression (Shang et al., 2007).

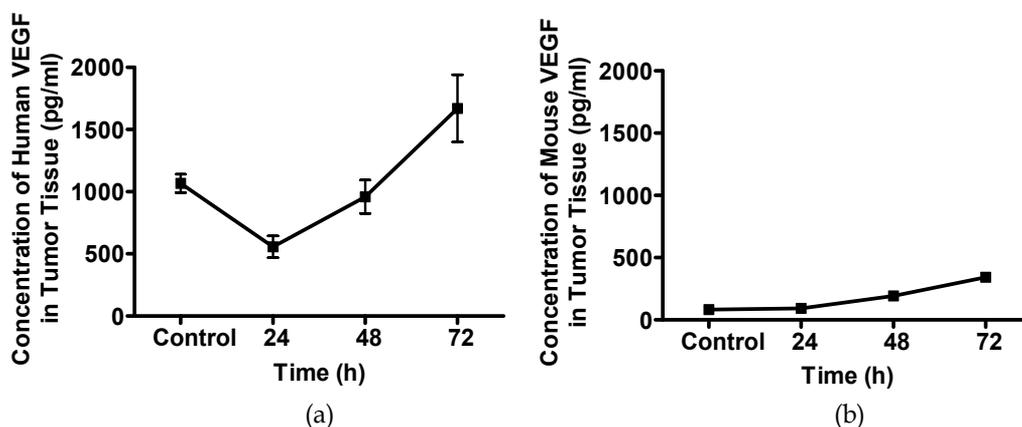


Fig. 2. Concentration of (a) mouse VEGF and (b) human VEGF in tumor tissue in control and at 24 h, 48 h and 72 h points post PDT. Error bars represent the standard error of the mean concentration of human VEGF in tumor tissue at 24 h, 48 h and 72 h, $n = 8$. Mouse and human VEGF were significantly higher in the tumor tissue (320-1700 pg/ml) compared to serum (33-110 pg/ml). Mouse VEGF in tumor tissue increased at 48 h and 72 h post PDT. The increase in VEGF levels from 24 h to 72 h was found to be statistically significant ($p < 0.05$) (Figure 2b). Controls were animals with untreated tumors.

Immunofluorescence results also confirmed the increased expression of VEGF post PDT in the tumor tissue (Figure 3). Several groups have reported the upregulation of VEGF following PDT (Bhuvaneswari, Gan et al., 2007; Ferrario et al., 2006; Uehara et al., 2001; Yee et al., 2005). Ferrario et al. revealed that PDT-mediated hypoxia and oxidative stress could be involved in photofrin-mediated PDT induced expression of HIF-1 α and also increased protein levels of the HIF-1 target gene VEGF, in treated mouse mammary carcinoma xenografts (Ferrario et al., 2000). In a similar study, the same group also reported significant overexpression of HIF-1 α and VEGF after photofrin-mediated PDT in a xenograft model of Kaposi's sarcoma (Ferrario et al., 2006). Increased expression of VEGF was noticed from 0 h to 6 h in tumors treated with haematoporphyrin mediated PDT compared to control tumor

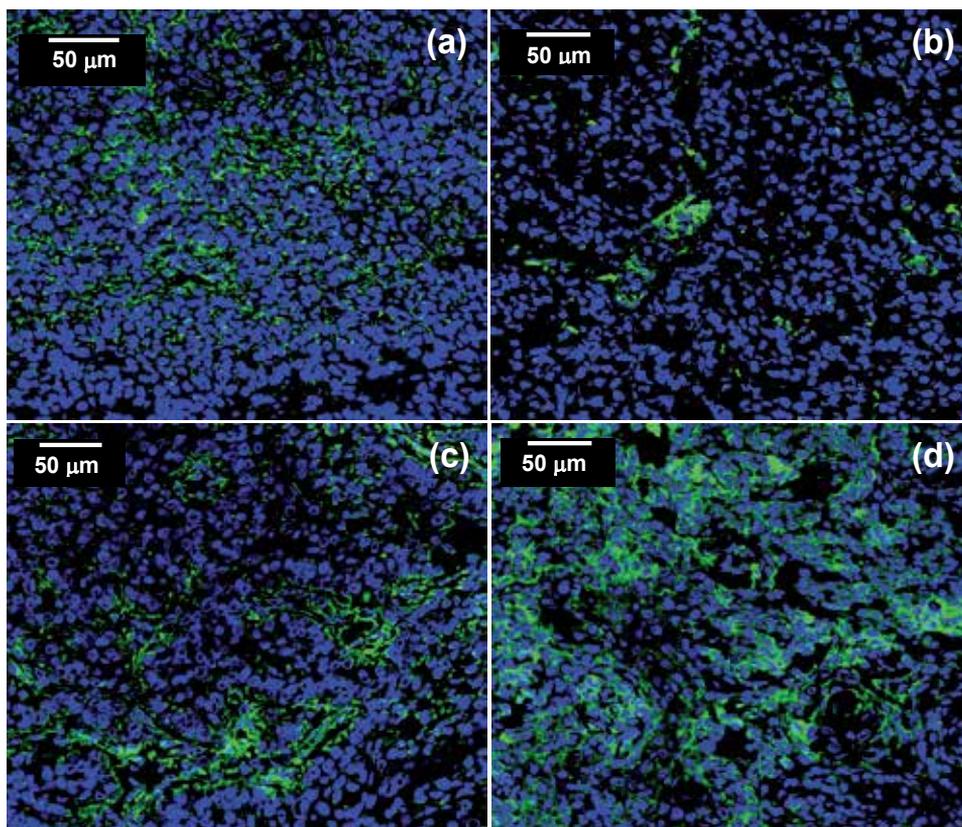


Fig. 3. Immunofluorescence was performed to confirm the expression of VEGF in tumor tissue at different time points post PDT. In the confocal images, the green FITC fluorescence staining indicated the expression of VEGF. (a) control, (b) 24 h post PDT, (c) 48 h post PDT and (d) 72 h post PDT. Magnification: 200X, scale bar = 50 mm. Around 13% and 15% (IF score 2) of scattered staining in certain regions of the cytoplasm was observed in the control (Figure 3a) and at 48 h post PDT (Figure 3c). Minimum VEGF expression of 5% (IF score 1) was observed at 24 h post PDT (Figure 3b). Maximum VEGF staining of 26% (IF score 3) was noticed at 72 h time point (Figure 3d).

in a mouse squamous cell carcinoma model (Uehara et al., 2001). Similar observations were noted by Jiang et al. whereby VEGF levels significantly increased after photofrin-PDT in intracranial glioblastoma xenografts (Jiang et al., 2008). In earlier studies, the same research group had reported increased VEGF levels in normal rat brain that induced the formation of aberrant new vessels following treatment with high dose PDT. In another study it was demonstrated that low dose PDT increases endothelial cell proliferation and VEGF expression in nude mice brain (Zhang et al., 2005). In addition, the upregulation of VEGF in photofrin mediated PDT was also observed in the brain tissue adjacent to tumor in a dose dependent manner (Jiang et al., 2004). Solban et al. investigated the effect of subcurative PDT using photosensitizer benzoporphyrin derivative (BPD) in an *in vivo* orthotopic model of human prostate cancer that demonstrated increased VEGF secretion 24 h following PDT and suggested vascular damage and/or a direct effect of BPD to be responsible for this

increase (Solban et al., 2006). Kosharsky et al. observed increases in not only VEGF secretion but also incidences of lymph node metastases after subcurative PDT in an orthotopic model of prostate cancer (LNCaP), that created conditions favorable for enhanced tumor growth and metastasis (Kosharsky et al., 2006). The same group also investigated the use of an optical molecular imaging strategy to monitor VEGF expression *in vivo* and effectively labeled and imaged bound VEGF released from the extracellular matrix in response to photodynamic therapy (Chang et al., 2008). Increased secretion of HIF-1 α and its target gene VEGF has been observed in hypericin-mediated PDT in both nasopharyngeal and bladder carcinoma (Bhuvaneswari, Gan et al., 2007; Bhuvaneswari, Yuen et al., 2007). Moreover, cellular mediated long drug light interval (DLI) hypericin-PDT induced greater expression of pro-angiogenic growth factors compared to vascular mediated short drug light interval PDT in bladder carcinoma (Bhuvaneswari et al., 2008). Zhou et al. (Zhou et al., 2005) demonstrated that the expression of HIF-1 α and VEGF increased in PDT-treated tumor samples collected 24 h post-PDT in a mouse model of human nasopharyngeal carcinoma. Mono-L-aspartyl chlorin e6 (NPe6) PDT of cytokine-overexpressing Lewis lung carcinoma (LLC/IL-2) tumors revealed that the expression of GADD-5 α and VEGF are induced after PDT and in particular the expression levels were much higher as compared with those in LLC tumors, 12 h after PDT (Ohtani et al., 2008). However, the application of ALA-PDT resulted in a lowered rate of metastatic spreading and decreased VEGF level in blood serum of 3LL-bearing mice that has been attributed to vascularization disturbances in tumor tissue (Lisnjak et al., 2005). Hypocrellin mediated PDT in human brain tumor cells induced expression of proangiogenic VEGF and of antiangiogenic SFH-1, angiostatin, p43, allograft inflammatory factor-1 and connective tissue growth factor suggesting favorable and deleterious effects of hypocrellin-PDT on tumor outgrowth (Deiningner et al., 2002). Based on the above studies, it can be inferred that PDT using photosensitizers i.e., photofrins, hypericin, hypocrellins and chlorin e6 increases VEGF concentrations within the tumor tissue and acts as a key regulator of angiogenesis and tumor recurrence post treatment.

3. PDT in combination with Avastin

Combination of anti-angiogenic agents with the PDT regime has been shown to be effective in inhibiting tumor regrowth and improving tumor response (Bhuvaneswari et al., 2009). Studies have reported that transplantable BA mouse mammary carcinoma treated with PDT and non-specific antiangiogenic peptides, IM862, a dipeptide and EMAP-II, a single chain polypeptide, increased tumor regression by inducing apoptosis and inhibiting VEGF production. However, the anti-angiogenic agents by themselves did not produce the desired outcome (Ferrario et al., 2000). Use of novel antiangiogenic monoclonal antibodies, MF1 and DC101 along with PDT against vascular endothelial growth factor receptors VEGFR-1 and VEGFR-2, respectively, reduced the tumor volume significantly and prolonged the survival time of glioma-implanted animals (Jiang et al., 2008). PDT followed by administration of an antiangiogenic agent, TNP-470, abolished the increase in VEGF levels caused by subcurative PDT and reduced local tumor growth in an orthotopic model of prostate cancer (LNCaP) (Kosharsky et al., 2006). Synthetic RTK inhibitors SU5416 and SU6668 when combined with hypericin PDT significantly extended survival of tumor-bearing host mice (Zhou et al., 2005). Combining PDT with humanized monoclonal antibody Avastin (bevacizumab)

resulted in significant increase in long-term responsiveness of treated Kaposi's sarcoma tumors when compared to monotherapies (Ferrario et al., 2006). Chang et al. (Chang et al., 2008) used an *in vivo* optical imaging technique that produces wavelength-resolved fluorescence hyperspectral images to study changes in tumoral VEGF concentration following PDT and Avastin treatment. The *in vivo* antigen blocking experiment showed that Avastin pretreatment before imaging blocked the tumoral VEGF, and also that VEGF-specific contrast agent labeling decreased in tandem with the pretreated Avastin dose, demonstrating that VEGF-specific contrast agent specifically binds to the VEGF protein.

Since VEGF and its receptors represent central molecular targets for antiangiogenic intervention, addition of Avastin (bevacizumab) along with PDT can increase the treatment efficacy. Avastin is a recombinant, partially humanized, monoclonal IgG1 antibody that binds to and inhibits the biological activity of human VEGF thus preventing interaction with its receptors. Avastin along with chemotherapy has been approved in the United States of America (USA) for the treatment of colorectal cancer and NSCLC and in other countries for the treatment of breast cancer, prostate cancer and renal cell carcinoma (Shih & Lindley, 2006).

In this study, the potential of combining anti-angiogenic agent Avastin that is specific to VEGF, with photodynamic therapy to enhance treatment efficacy by improving the tumor responsiveness was investigated. As Balb/c nude mice are immunocompromised, human bladder carcinoma cells were injected to establish subcutaneous tumor grafts. Subcutaneous models were used for our experiments because of the simple inoculation procedure, reproducibility of tumor growth and easy accessibility of the tumor for measurement and treatment. MGH bladder tumors form vascularized solid tumors.

The tumor regression experiments conducted in the xenograft model clearly indicated that combining Avastin with PDT can impede the angiogenesis process and improve the response of treated tumors. This has been demonstrated by the significant decrease in the tumor volume of the combination therapy group of PDT + Avastin compared to the control and high dose PDT groups (Figure 4). This demonstrates that by targeting the VEGF pathway, post-PDT angiogenesis can be reduced. It should be noted that Avastin is a monoclonal antibody that targets human VEGF and not mouse VEGF. The tumor volume of high dose PDT treated group was significantly greater than the low dose PDT group and this could be due to the difference in fluence rates administered. High fluence rate can deplete tumor oxygen to a greater extent, thereby reducing the primary cytotoxic processes of PDT and affecting tumor control. On the other hand, low fluence rate treatments can be more effective in decreasing vascular lesions even if the same overall fluence is maintained. Other studies have concluded that lower fluence rate treatments can preserve the status of oxygen for a more effective PDT (Chen et al., 2002; Sitnik et al., 1998; Tromberg et al., 1990) and it is well recognized that light fluence rates play a major role in the tumor oxygenation status during PDT exposure. But we should also understand that oxygen-conserving low fluence rate PDT cannot always be effective if it is unable to produce the desired tumor damage and it is essential to estimate the lower limits of fluence rate that would be required for effective treatment thus potentially allowing the tailoring of treatment to specific situations (Henderson et al., 2006; H. W. Wang et al., 2004). The group of animals that were administered only Avastin showed greater tumor response compared to the high dose PDT and control groups, this could be due to the fact that Avastin by itself can target and bind to human VEGF. Though not statistically significant, better tumor response was observed in the combination therapy group compared to Avastin only and low dose PDT groups, however a greater sample size would be required to establish these findings. Complete cure was not achieved in these groups by end of 30-day post treatment, as Avastin does not

target mouse VEGF produced by the host environment. Tumors were excised to analyze the expression of VEGF and other angiogenic proteins at the end of the 30-day tumor growth experiments. This time point was chosen to study the long-term effect of Avastin on the expression profile of angiogenic proteins.

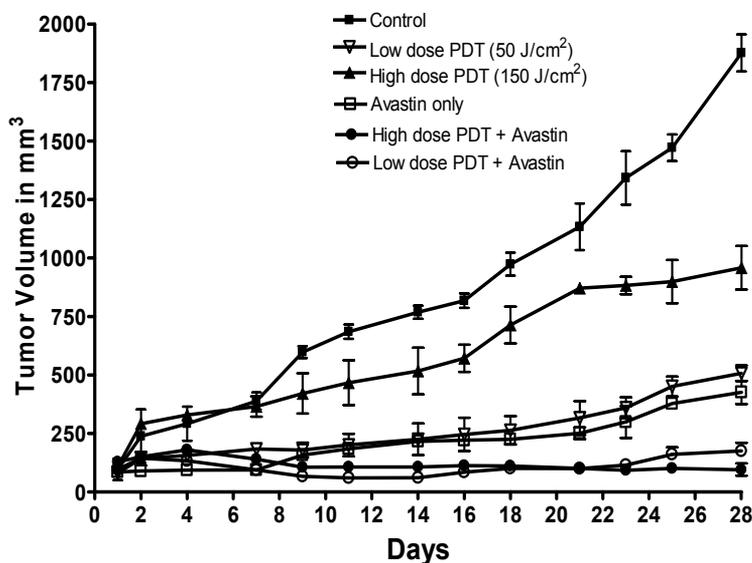


Fig. 4. Tumor volume charted against days, to assess the tumor response in various treatment groups. The combination therapy group of PDT and Avastin exhibited greater tumor response in comparison with other groups. Each group represents the mean (bars, SE) of 10 animals.

Next, circulating human VEGF concentrations in mice was investigated to analyze tumor-derived VEGF. A low but detectable amount of human VEGF was observed in most tumors (Figure 5). It has been shown that high fluence rates during PDT can influence the inflammatory responses associated with PDT (Henderson et al., 2004). In the same way, the results also suggest that high dose PDT could have triggered inflammatory responses within the treated tumors that may enhance VEGF secretion. Though it was expected that Avastin would specifically bind to the circulating human VEGF, measurable amount of VEGF was documented in the animals that received Avastin alone. One of the reasons for detecting circulating VEGF in all the treatment groups could be the extended period of VEGF transcriptional activation, (Liang et al., 2006) and it is likely that low level of VEGF can be generated, continuously or in pulses, during the angiogenesis process that could vary significantly from tumor to tumor. Also, the data did not seem to exhibit any correlation between VEGF secretion and tumor volume. The smaller tumors in the Avastin only group expressed relatively greater amount of VEGF compared to the tumors in the control and high dose PDT treated groups, this observation can be attributed to multiple factors such as tumor vascularization, tumor invasiveness, tumor infiltrating macrophages and the production of cytokine IL-1 α that has been shown to influence the secretion of VEGF (Borg et al., 2005).

Immunohistochemistry was performed to detect VEGF and this method has been used in earlier studies for quantifying VEGF (Harper et al., 1996; Saito et al., 1999). As VEGF is a

secreted protein, it was observed mainly in the cytoplasm and the extracellular matrix (Figure 6). These data demonstrate that tumors under oxidative stress express greater amounts of VEGF. Also, significantly lower occurrence of VEGF was observed in the combination therapy of PDT and Avastin. These results are consistent with an earlier report by Solban et al. (Solban et al., 2006) on subcurative PDT performed on an orthotopic model of prostate cancer that showed increased VEGF secretion and also demonstrated that VEGF induction can be abolished by administering p38 MAPK inhibitor along with PDT.

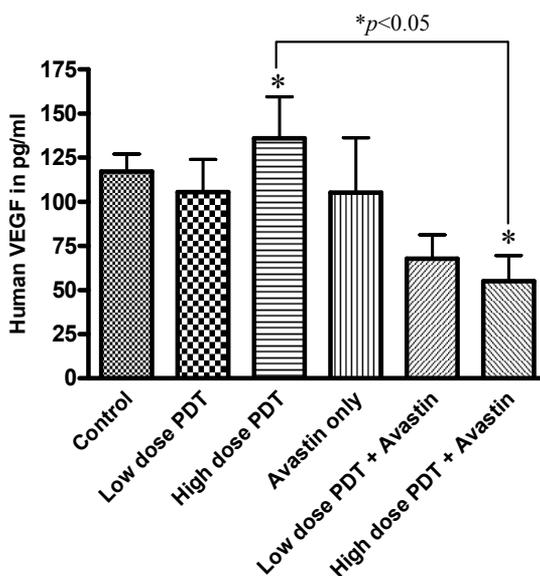


Fig. 5. Relative concentration of human VEGF measured in pg/ml in serum for various treatment groups. Greater expression of VEGF was observed in the high dose PDT group compared to the combination therapy group of high dose PDT + Avastin. Each group represents the mean (bars, SE) of 10 animals.

The effect of different treatment regimes on the expression profiles of angiogenic proteins was also investigated. The study established differential expression of proteins in the angiogenesis pathway as different PDT combinations were administered (Figure 7). The protein angiogenin initiates cell migration, proliferation and induces neovascularization *in vivo* (Hartmann et al., 1999). In our experiments it was upregulated in high-dose PDT treated tumors compared to all other groups and this may be due to hypoxia induced production of angiogenin. Similarly studies have established positive correlation between hypoxia and angiogenin expression in human malignant melanoma, (Hartmann et al., 1999) and human primary breast carcinoma (Campo et al., 2005). A study on gastric carcinoma cancer has shown angiogenin expression in cancer tissues to be positively correlated with VEGF (Chen et al., 2002) and our results show that blocking the VEGF pathway using PDT with Avastin does downregulate the expression of angiogenin. Both bFGF and VEGF seem to differentially activate the Raf pathway in the angiogenesis process (Alavi et al., 2003) and as bFGF has shown to promote angiogenesis indirectly by the upregulation of VEGF in endothelial cells, (Pepper et al., 1992) the reduced expression of bFGF in the combination

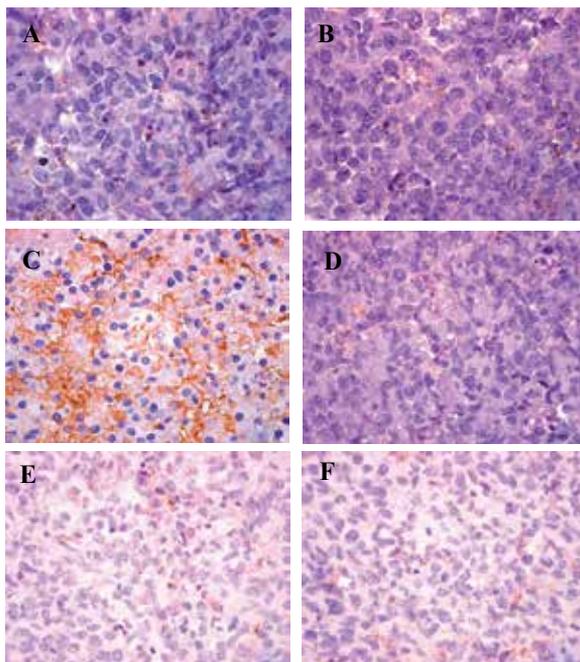


Fig. 6. VEGF expression was assessed in tumors treated with various treatment regimens using immunohistochemistry A - Control (untreated tumor), B - Low dose PDT, C - High dose PDT, D - Avastin only, E -Low dose PDT + Avastin and F - High dose PDT + Avastin. VEGF, a secreted protein was observed in the cytoplasm and extracellular matrix. 30% Immunostaining for VEGF was observed in high dose PDT treated tumors. Control, low dose PDT and Avastin only groups exhibited <5% of staining. Minimal staining of less than 2% was observed in the combination therapy groups of PDT and Avastin. All sections are shown at a magnification of $\times 630$.

therapy groups could mean that inhibiting VEGF may possibly attenuate bFGF expression as well. Amplification of bFGF by a HIF-1 α -dependent pathway, (Calvani et al., 2006) may be one of the reasons for the upregulation of bFGF in PDT treated tumors. Similarly EGF, a key EGFR ligand that promotes angiogenesis (Ciardiello, 2005) and was upregulated in high dose PDT treated tumors, is known to be HIF-1 α regulated (Vaupel, 2004). We observed downregulation of EGF in the combination therapy groups suggesting that VEGF and EGFR pathways are closely related, sharing common downstream signaling pathways. (Taberero, 2007) PIGF-1 is expressed in placental tissues, colon and mammary carcinomas and it belongs to the VEGF family (Cao et al., 1996). No PIGF expression was noted in the low dose PDT treated group as the oxidative stress in these tumors was expected to be minimal due to low fluence rate administered during PDT. However, the Avastin only and combination therapy treated tumors expressed minimal PIGF, which may suggest that Avastin, which binds to VEGF, has negligible effect on PIGF. Furthermore as PIGF binds only to VEGF receptors, it has been documented that PIGF can be downregulated by blocking the VEGFR-1/FLT1 receptor pathway (Ahmed et al., 2000). As both EGF and PIGF was not observed in the control tumors and induced only post PDT treatment, we conjecture that these proteins may not play a major role in angiogenesis of MGH bladder tumors, based on our experimental data that show increased tumor volume in control groups. After PDT

treatment, the tumors are in hypoxic condition which is one of the factors causing cytokine expression (Gomer et al., 2006). The role of IL-6 in angiogenesis is mediated through the induction of VEGF (T. Cohen et al., 1996). The increased expression of interleukins in the control and PDT treated groups may have resulted due to greater tumor volume and PDT induced inflammation, respectively. Compared to high dose PDT group, the tumors in combination therapy group produced lower levels of cytokines and this we theorize to be the role of Avastin in reducing angiogenesis by binding to VEGF, thus reducing the expression of post PDT inflammatory proteins. Expression of IL-6 was elevated in most of the treatment groups compared to IL-8 suggesting its importance in PDT-induced inflammation.

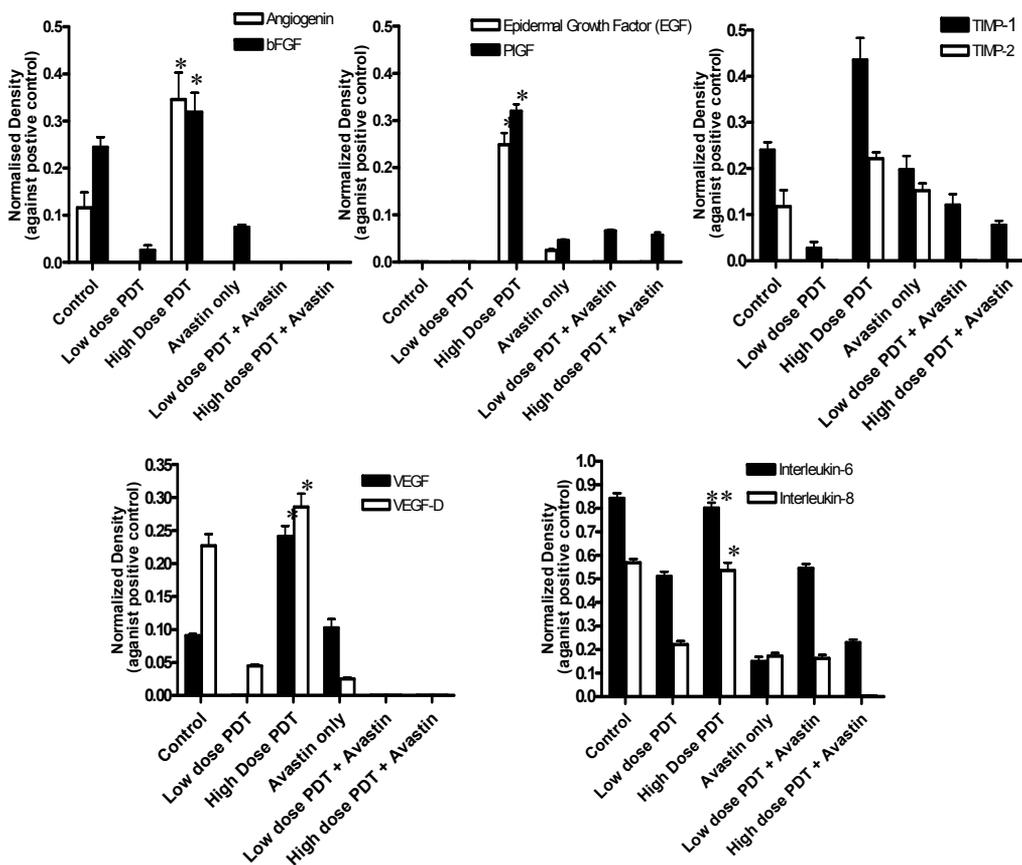


Fig. 7. Antibody arrays were used to analyze the expression of angiogenic proteins in the treated tumors. Density of proteins was plotted and normalized against the positive control Actin, (a) Angiogenin and bFGF, (b) EGF and PIGF, (c) TIMP-1 and TIMP-2, (d) VEGF and VEGF-D and (e) IL-6 and IL-8. Each group represents the mean (bars, SE) of 5 tumors (i.e. one membrane was used per tumor). Statistical analysis was performed using one-way ANOVA with Bonferroni's multiple comparison tests. * = p < 0.001, ** = p < 0.01 when high dose PDT group was compared with the combination therapy groups of low dose PDT + Avastin and high dose PDT + Avastin.

On the other hand, TIMPs are natural inhibitors of MMPs. A stimulatory role of TIMP-1 in angiogenesis has been proposed in an earlier study (Wurtz et al., 2005) which reports that by inhibiting MMPs, TIMP-1 may prevent angiostatin and endostatin production, thus playing a positive role in tumor angiogenesis. In contradiction to an earlier report (Ferrario et al., 2004) that PDT suppresses TIMP-1 expression in mouse mammary carcinoma, we noticed upregulation of TIMP-1 in the high dose PDT treated tumors. Nevertheless, this dissimilarity can be attributed to the different tumor systems and the PDT protocol administered. VEGF which is also known as VEGF-A is involved in angiogenesis and lymphangiogenesis and VEGF-D, a secreted protein stimulates lymphangiogenesis and metastasis in tumors (Hoeben et al., 2004). Reports have shown that subcurative PDT in an orthotopic model of prostate cancer increases VEGF secretion and also cause lymph node metastasis. It was also demonstrated that the administration of anti-angiogenic agent TNP-40 abolished this increase and reduced tumor growth (Kosharskyy et al., 2006). In our study VEGF was not detected in low dose PDT group and that could be due to lower oxidative insult to the tumor tissue compared to the high dose PDT group. Furthermore, downregulation of VEGF levels was observed in the PDT + Avastin treated tumor as the combination treatment effectively suppressed the VEGF signalling cascade. However, we noticed minimal expression of VEGF using IHC and ELISA and this we attribute to the different tumor microenvironment as tumors were collected from different animals though they were treated with the same treatment protocol. In conclusion, the results demonstrate that by targeting the VEGF pathway, post-PDT angiogenesis can be inhibited. Furthermore, suppressing the VEGF pathway can also downregulate other angiogenic mediators.

4. PDT in combination with Erbitux

Erbitux was approved by the US Food and Drug Administration (FDA) for use in combination with irinotecan for the treatment of metastatic colorectal cancer and it is also being used for the treatment of metastatic squamous cell carcinoma of the head and neck (SCCHN) (Wong, 2005). Results of a large phase II study on irinotecan-refractory, colorectal cancer patients have shown a significant response of 22.9% when Erbitux was combined with chemotherapy agent, irinotecan (Cunningham et al., 2004). In another study, the response rate was significantly improved when Erbitux was combined with cisplatin in the first-line treatment of recurrent or metastatic SCCHN (Burtness, 2005). A randomized trial that compared radiotherapy plus Erbitux with radiotherapy alone in patients with stage III or IV non-metastatic SCCHN, demonstrated significantly longer locoregional control with radiotherapy plus Erbitux than with radiotherapy alone; moreover, progression-free survival were significantly longer and the overall response rate was significantly better with the combination therapy (Griffin et al., 2009). Erbitux given concurrently with radiotherapy yields a significant clinical benefit over radiotherapy alone without any increase in radiotherapy-associated toxicity, this was demonstrated in the results of a recent phase III randomized study (Bernier & Schneider, 2007).

In the *in vivo* tumor regression study, we demonstrate that the combination therapy of Erbitux with PDT can improve the tumor response by attenuating the angiogenic process (Figure 8). A similar study conducted on a mouse model of human ovarian cancer in which C225 (Erbitux) was combined with PDT regimen produced synergistic reductions in mean tumor burden and significantly greater median survival (del Carmen et al., 2005). In this study, PDT treated tumors did not exhibit significant tumor regression compared to combination therapy groups

and this could be attributed to the high fluence rate that was administered during PDT. High fluence rate can deplete tumor oxygen to a large extent, thereby stimulating the production of stress induced survival molecules that reduce the effectiveness of PDT and affect tumor control (Henderson et al., 2004). More importantly, the use of high light dose for this experiment was to test our hypothesis that combining PDT with Erbitux can improve tumor control and also to evaluate the effectiveness of Erbitux in reducing EGFR concentrations. The investigations have indicated that Erbitux alone as monotherapy was not effective in controlling tumor growth. One of the possible reasons for this observation could be the fact that tumors overexpressing EGFR might not be sensitive to Erbitux. Although we would assume that tumors overexpressing EGFR would respond well to anti-EGFR therapy, studies have demonstrated that the level of EGFR expression does not have any impact on tumor response rates as a significant number of EGFR-positive tumors could be resistant to Erbitux (Ellis & Hoff, 2004; Vallbohmer et al., 2005). The group that received the combination therapy of PDT and Erbitux exhibited accelerated growth a week after PDT which could be due an increase in the expression of angiogenic growth factors either due to hypoxia induced by oxygen depletion during PDT light irradiation or incomplete treatment. Our earlier results have shown increased expression of angiogenic growth factor VEGF at 72 h post PDT (Vallbohmer et al., 2005). In this study, the regular administration of Erbitux after PDT treatment could have blocked the EGFR pathway and reduced angiogenesis. Therefore, our data supports the hypothesis that combination therapy of PDT and Erbitux would be more effective in preventing angiogenesis compared to monotherapy alone.

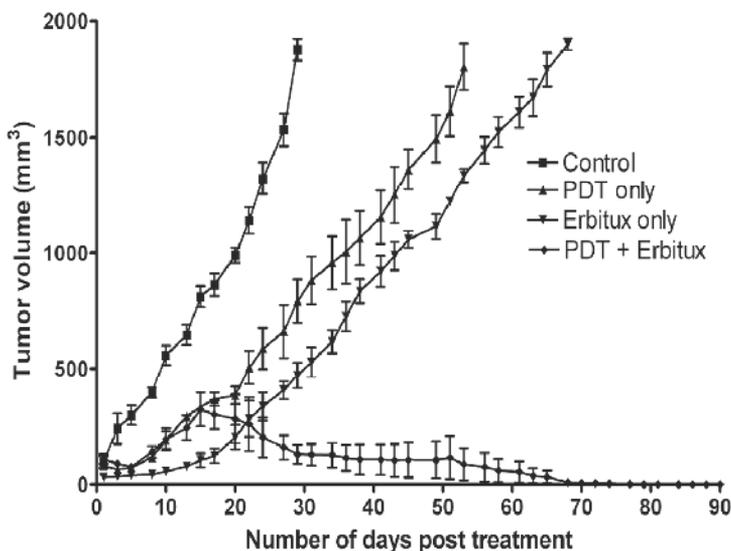


Fig. 8. Mean tumor volume charted against number of days post treatment, to assess the tumor response in various treatment groups. The combination therapy group of PDT and Erbitux exhibited greatest tumor response in comparison with all other groups. Each group represents the mean response (bars, SE) of 10 animals.

To further substantiate our results we performed western blotting and immunohistochemistry to determine the EGFR levels in all the treatment groups. EGFR

immunoreactivity was localized mainly in the cell membranes and to a lower extent in the cytoplasm as well (Figure 9). It has been well established that the core of solid tumors is hypoxic, and that hypoxic tumor environment is sufficient to trigger EGFR expression in tumors (Franovic et al., 2007). Previous studies have reported the downregulation of EGFR after PDT (Ahmad et al., 2001; Tsai et al., 2009); in marked contrast our results demonstrated an increase in EGFR expression post hypericin-mediated PDT. This observation could be attributed to numerous reasons such as the light/drug dosage, the complexity of tumor microenvironment and the properties of the photosensitizer (Henderson et al., 2004). Combined antitumor activity of Erbitux with standard chemotherapy and radiotherapy is well documented in the treatment of different types of tumors and is reported to be more efficacious than individual monotherapies (E. E. Vokes & Chu, 2006). In this study, combination modality of PDT and Erbitux was effective in reducing the expression of EGFR and that could have lead to the regression of tumors in this group.

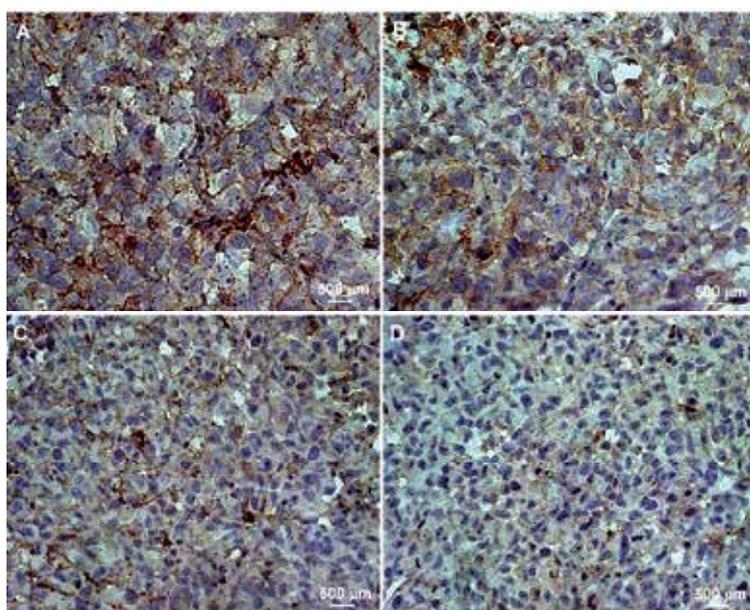


Fig. 9. EGFR expression was assessed in tumor sections using immunohistochemistry. The brown colored membrane staining indicates EGFR positive immunoreactivity. (A: Control, B: PDT, C: Erbitux and D: PDT +Erbitux). PDT and Erbitux (D) resulted in significant reduction of EGFR expression of 4-6% (EGFR score 1) compared to monotherapy (B: PDT and C: Erbitux) and control groups (A). Maximum EGFR tumor cell membrane staining of 21-24% (EGFR score 3) noticed in the untreated tumors. The monotherapy groups of PDT only and Erbitux only, exhibited 15-17% (EGFR score 2) and 11-13% (EGFR score 2) staining respectively. Magnification: 630X.

In the current study, we have also shown that PDT plus Erbitux increased apoptosis in the treated tumors compared to PDT only and inhibitor only monotherapies (Figure 10). Erbitux has been known to increase apoptosis in various tumor models by different mechanisms, including upregulation of pro-apoptotic Bax protein (Mahtani & Macdonald, 2008), decrease in the expression of anti-apoptotic molecule Bcl-2 (S. M. Huang et al., 1999) and the activation of

pro-apoptotic caspases (Iwase et al., 2008). Hypericin-PDT is also known to induce apoptosis in a dose-dependent manner with higher doses leading to necrosis. Based on the lack of tumor inhibition in the monotherapy groups, it can be noted that tumors treated with PDT alone and Erbitux alone induced limited apoptosis in bladder carcinoma tumors. Therefore in this investigation, we observe that the combination therapy has significantly increased tumor cell apoptosis and inhibited tumor progression. Preclinically, many studies have shown that treatment with Erbitux in combination with radiotherapy or chemotherapy enhances apoptotic cell death than individual therapies. In a similar manner, PDT induced apoptosis, could have been enhanced by the combination of Erbitux to the treatment regime.

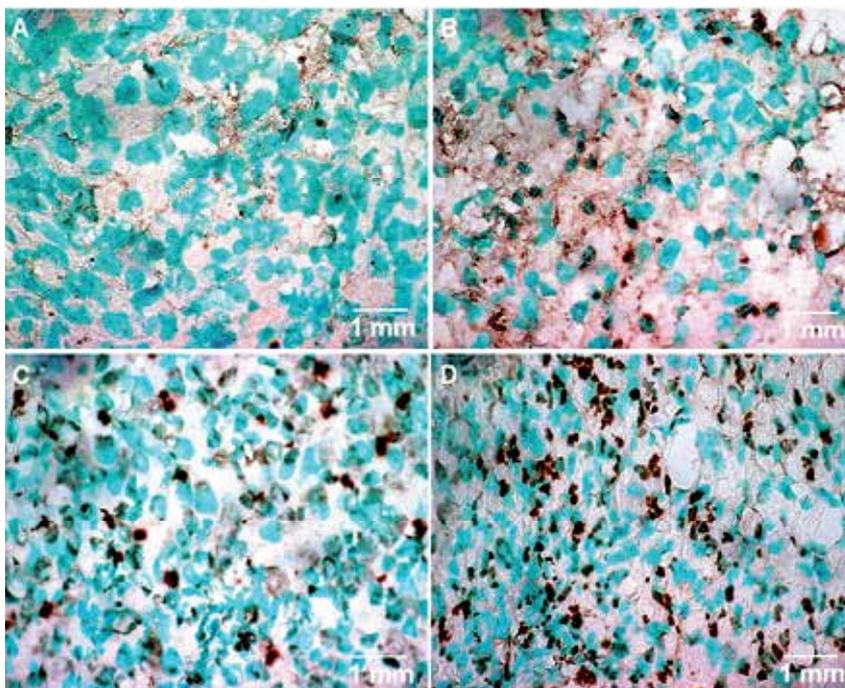


Fig. 10. The tunnel assay was performed on the tumors that were harvested from the animals at the end of the treatment. Few isolated positive nuclei were noticed in (A) untreated tumors, (Apoptotic index (AI) - 6%). Both (B) PDT only (AI - 14%) and (C) Erbitux only (AI - 16%) treated tumors showed increased apoptosis compared to control. High levels of apoptotic nuclei were clearly exhibited by tumors treated with the (D) PDT plus Erbitux combination therapy (AI - 32%, $p < 0.001$). Magnification: 630X.

By using EGF phosphorylation antibody array membranes, we examined the relative level of phosphorylation of specific sites for human EGFR receptors. Interestingly, we noted the phosphorylation of Threonine 686 site of ErbB2 in all the groups. Studies have suggested that the dysregulation of cellular protein kinase C (Ouyang et al., 1996) and protein kinase A (Monje et al., 2008) activity could phosphorylate ErbB2 on Thr-686 for the activation and proliferation of tumor cells (Figure 11). However, our findings suggest that ErbB2 on Thr-686 may not be essential for regulation of tumor proliferation, as tumor control was observed in the PDT + Erbitux treated group. Phosphorylation of EGFR tyrosine 845, only noticed in control tumors, is implicated in the stabilization of the activation loop, providing a binding

surface for substrate proteins and is capable of regulating receptor function and tumor progression (Cooper & Howell, 1993). c-Src is known to be involved in the phosphorylation of EGFR at Tyr845 (Biscardi et al., 1999). The major autophosphorylation sites of ErbB2 are Tyr1248 and Tyr1221/1222 that lead to Ras-Raf-MAP kinase signal transduction pathway (Kwon et al., 1997). In control tumors, ErbB2 was phosphorylated at tyrosine 1221/1222 and is associated with high tumor grade and with shorter disease-free survival and overall survival (Frogne et al., 2009). Similarly, ErbB4 is able to induce phosphorylation of phosphatidylinositol 3-kinase regulatory subunit which is a pro-survival protein that prevents apoptosis (B. D. Cohen et al., 1996; Gallo et al., 2006). Our data suggests that dephosphorylation of ErbB4 tyrosine 1284 is critical for tumor regression in the dual treatment group.

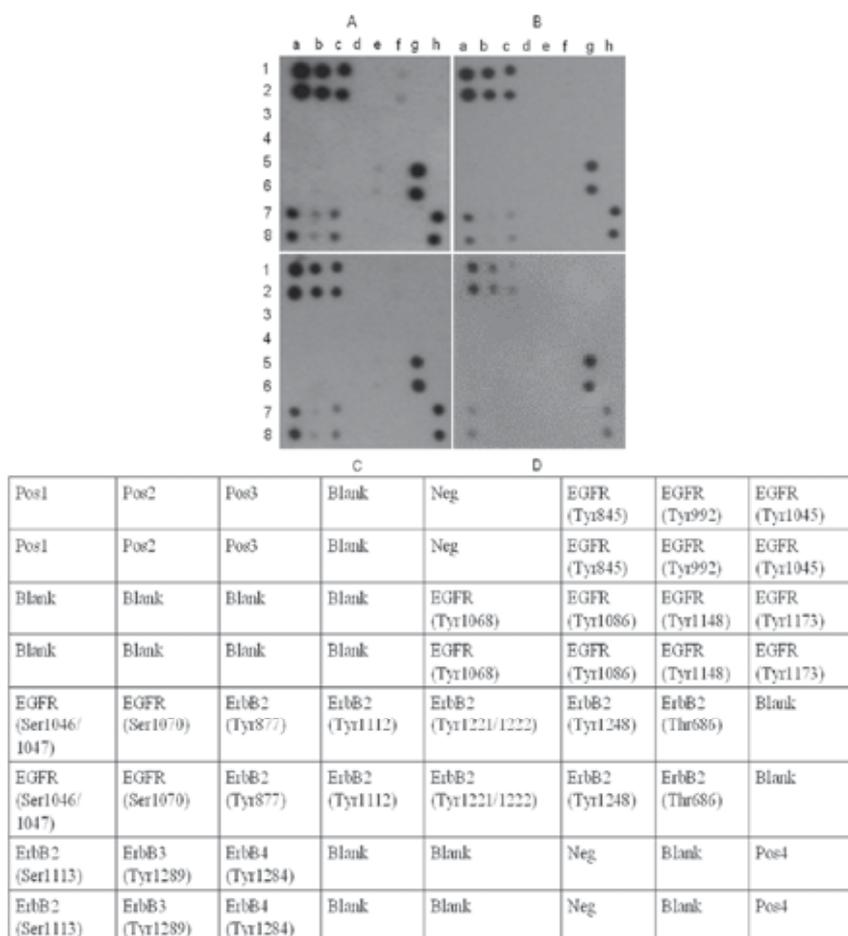


Fig. 11. Phosphorylation statuses of EGFR sites were determined using antibody arrays. Increased phosphorylation of ErbB2(Thr686), ErbB2(Ser1113) and limited phosphorylation of EGFR(Thy845), ErbB2(Tyr1221/1222), ErbB3(Tyr1289) and ErbB4(Tyr1284) sites was seen in the control group. In the monotherapy groups, ErbB2(Thr686), (Ser1113) and ErbB4(Tyr1284) sites were phosphorylated. Inhibition of most of the EGFR phosphorylation sites was observed in combination therapy groups except for ErbB2(Thr686) and (ser1113).

EGFR-mediated Ras-Raf-MEK-ERK and PI3K-PTEN-AKT pathways play an important role in transmission of signals from membrane receptors to downstream targets that regulate apoptosis, cell growth and angiogenesis. Components of these pathways include genes such as Ras, B-Raf, PI3K, PTEN and Akt that can be mutated or aberrantly expressed in human cancer. Though we did not investigate these genes, it should be noted that they could cause resistance to anti-EGFR therapy. Numerous studies have reported Kras mutations as a predictor of resistance to Erbitux therapy and are associated with poor prognosis in colorectal cancer (Lievre et al., 2006) and non-small cell lung carcinoma (Riely et al., 2009). In a similar way, Braf mutation is also known to cause resistance to anti-EGFR therapy in colorectal cancers (Li et al., 2006) and primary lung adenocarcinomas (Schmid et al., 2009). Mutation of PTEN tumor suppressor gene in human cancer cells leads to activated EGFR downstream signaling including PI3-kinase/AKT and have been linked to resistance to anti-EGFR targeted therapies (M. Y. Wang et al., 2006). However, in this study we investigated the role of EGFR target genes cyclin D1 and c-myc that are involved in cell proliferation. Our RT-PCR results showed downregulation of cyclin D1 and c-myc in the tumors treated with the combination therapy (Figure 12). Amplification of cyclin D1, a key cell cycle regulatory protein, appears to be an important event in bladder cancer and is often associated with cell proliferation and poor prognosis in human tumors (Le Marchand et al., 2003). In our study, downregulation of EGFR also resulted in reduction of cyclin D1. This observation could be due to the administration of Erbitux, that is known to cause cell cycle arrest in the G(1)/G(0)-phase, also increases the expression of cyclin-dependent kinase inhibitors (Huether et al., 2005). c-myc, another EGFR target gene that can obstruct the induction of apoptosis in tumor cells and lead to uncontrolled cell growth was reduced in the PDT + Erbitux treated tumors. Over-expression and amplification of c-myc can play an important role in metastatic progression that indicates poor prognosis in different cancers (Peng et al., 1997). These results suggest that EGFR target genes could play a role in tumor inhibition in bladder cancer by arresting cell cycle growth and inducing apoptosis.

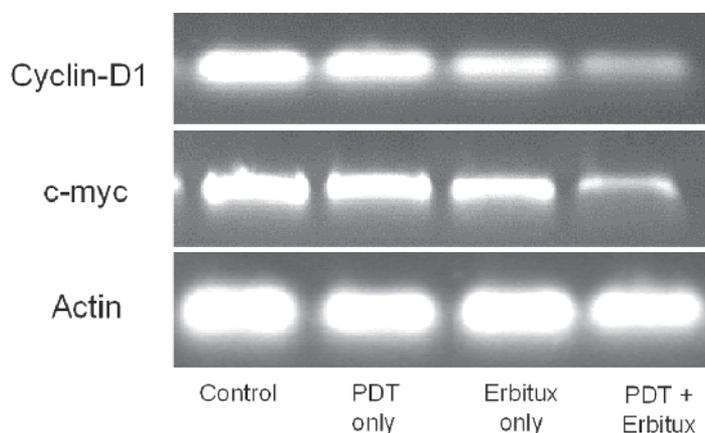


Fig. 12. The effect of EGFR inhibition on target genes cyclin-D1 and c-myc was evaluated at the RNA level. Cyclin D1 is an important regulator of G1 to S-phase transition and overexpression of cyclin D1 has been linked to the development and progression of cancer. c-Myc is activated in a variety of tumor cells and plays an important role in cellular proliferation, differentiation, apoptosis and cell cycle progression. Downregulation of cyclin-D1 and c-myc was observed in the tumors treated with PDT and Erbitux ($p < 0.05$) when compared with the other groups.

5. PDT in combination with Avastin and Erbitux

In this study, the potential of combining anti-angiogenic agent Avastin that is specific to VEGF and Erbitux that targets EGFR along with PDT to improve bladder tumor response was investigated. To achieve this, the inhibitory effect of the anti-angiogenic compounds was tested using angiogenic assays. Cell migration is a highly integrated multistep process that is essential for invasion and metastasis of tumors. Directed cell migration is normally initiated in response to extracellular cues such as chemoattractants, growth factors and the extracellular matrix (Ridley et al., 2003). In this study, stimulation with VEGF, a proangiogenic protein, increased the migration of tumor cells. On the other hand, antiangiogenic agents, Avastin and Erbitux reduced the migratory potential of the tumor cells. Avastin is known to significantly reduce proliferation and migration capacity, and increase apoptotic rates in endothelial cells (Carneiro et al., 2009). A similar study has reported 40-60% inhibition of cell migration of SCC cells when incubated with Erbitux in a dose dependent manner (S. M. Huang et al., 2002). One of the most important and crucial events in cancer metastasis is the invasion of basement membrane. The invasion assay results in this study have clearly demonstrated the stimulatory effect of VEGF on endothelial cell migration (Figure 13). VEGF plays an important role in tumor invasion and metastasis through its specific action on endothelial cells in tumor tissue (Khosravi Shahi & Fernandez Pineda, 2008). *In vitro* studies have clearly demonstrated that VEGF is a potent mediator of angiogenesis as it helps in the proliferation and migration of the endothelial cells to form tube-like capillaries (Bernatchez et al., 1999). Tumor cells exposed to hypoxia induced by PDT triggers the expression of VEGF mRNA that in turn releases the VEGF protein (Dvorak et al., 1995; Shweiki et al., 1992).

Endothelial barrier disruption by VEGF-mediated Src activity has been shown to potentiate tumor cell extravasation and metastasis (Weis et al., 2004). Another study has reported that interference with VEGF function could be sufficient to abrogate tumor invasion (Skobe et al., 1997). Reduced invasion of endothelial cells through the basement membrane was observed in the Avastin and Erbitux treated cells, suggesting their role in preventing the disruption of the basement membrane. Another important step in angiogenesis is the formation of a functional vascular system for tumor growth and metastasis. Endothelial cell tube formation is a consequence of various biological activities, including cell migration, vacuolization, cell-cell junction formation and cell elongation. It is well established that VEGF plays an important role in the process of angiogenesis by facilitating endothelial cell migration and tube formation similar to the observations in this study (Ferrara, 2004). Another report has also identified Hedgehog signaling as an important component of the molecular pathway leading to vascular tube formation (S. A. Vokes et al., 2004). Avastin and Erbitux were used successfully to reduce and inhibit tube formation, thus signifying their role in blocking major angiogenic processes. Similarly, another study demonstrated that bevacizumab could significantly impair tube formation capabilities in tumor derived endothelial cells and also noted a continuing effect after 14 days of treatment even after omitting the antibody (Grau et al., 2011). Treatment with Cetuximab has also shown to reduce cell-to-cell interaction of human umbilical vascular endothelial cells (HUVEC), resulting in disruption of tube formation (S. M. Huang et al., 2002). Further substantiating these *in vitro* findings, Avastin and Erbitux also inhibited angiogenesis in a mouse plug matrigel assay, as evaluated by haemoglobin content levels.

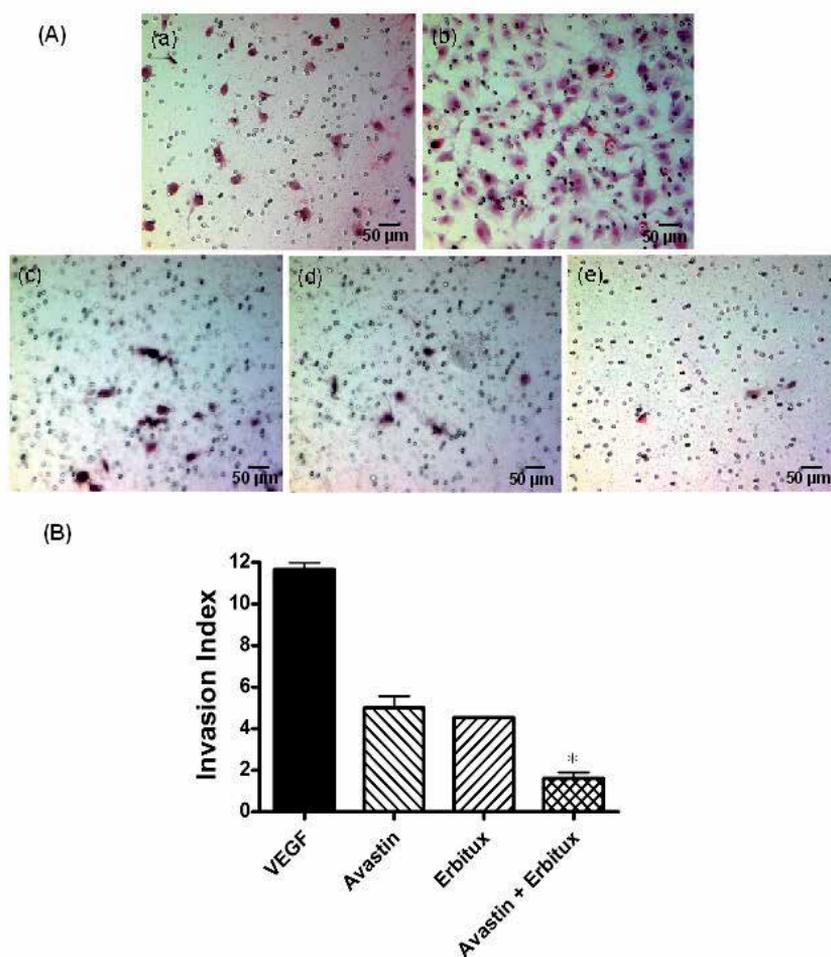


Fig. 13. Effect of angiogenesis stimulator and inhibitors on HUVECs *in vitro*. (a) control, (b) VEGF, (c) Avastin (d) Erbitux and is (e) Avastin + Erbitux. (A) High invasion of endothelial cells through basement membrane were noticed in VEGF treated wells Avastin and Erbitux inhibited the migration of endothelial cells. The figures are representative of the results from three separate experiments. (B) The invasion index was calculated based on the number of cells in the test samples compared to the control samples. Calculations for each group were performed in triplicate. Invasion index for VEGF was high and was lowest for the combination of angiogenesis inhibitors ($*=p<0.001$). Error bars represent the standard error of the mean invasion index in comparison to control in all the groups, $n = 6$.

Avastin and Erbitux monotherapies and also Avastin + Erbitux remarkably suppressed the sprouting of endothelial cells and induction of new blood formation in the matrigel plugs (Figure 14). It has been demonstrated that the effects of blocking angiogenesis can be observed on tumor transplanted onto animals (O'Reilly et al., 1994). The antiangiogenic activities of Avastin and Erbitux may be explained by their inhibitory action on the proliferation, migration and differentiation of the tumor cells, by inhibiting VEGF and EGFR respectively.

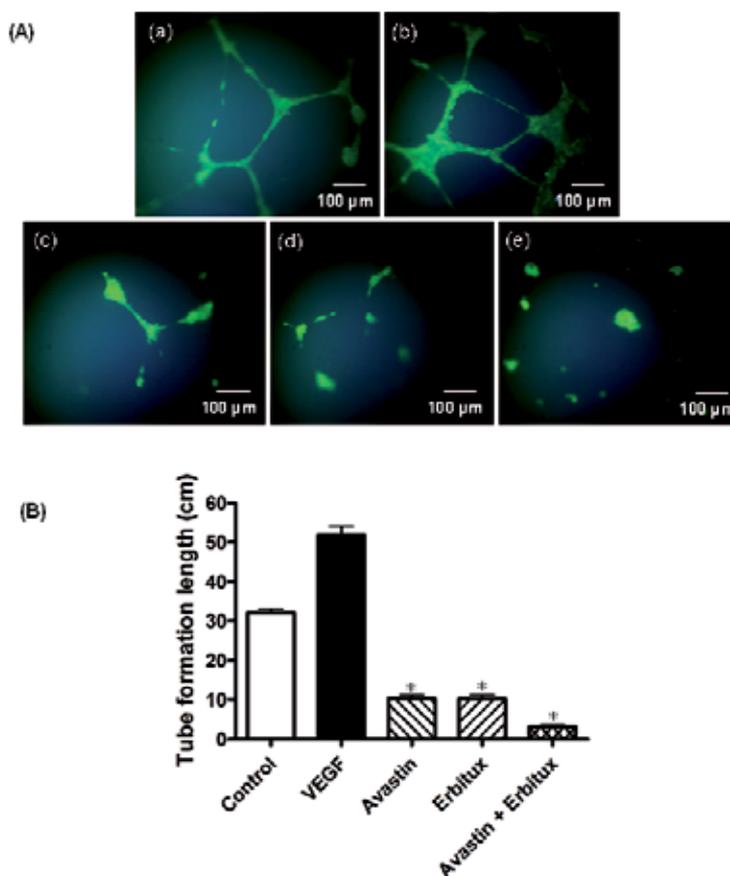


Fig. 14. (A) Endothelial cell tube formation was assessed using VEGF, Avastin and Erbitux. (a) control, (b) VEGF, (c) Avastin (d) Erbitux and (e) Avastin + Erbitux. VEGF induced tube formation and Avastin and Erbitux decreased growth inhibited tube formation of endothelial cells. Combining Avastin and Erbitux, almost completely abrogated tube formation (B) The total tube length of each treatment group was quantified by the software, Datinf Measure (Tubingen, GmbH, Germany). The figures are representative of the results from three separate experiments. Error bars represent the standard error of the mean invasion index in comparison to control in all the groups, $n = 8$.

The tumor regression data demonstrated that combining Avastin + Erbitux with PDT can impede the angiogenic process and improve the response of treated tumors (Figure 15). The tumor volume of the PDT treated group was significantly greater than the Avastin treated and Erbitux treated groups as high fluence administered during treatment can deplete tumor oxygen to a large extent, releasing stress induced survival molecules that reduce the effectiveness of PDT and affect tumor control (Gomer et al., 2006). Although tumor regression was also observed in Avastin + Erbitux only treated group, complete cure was not observed. Thus targeting EGFR and VEGF without PDT treatment might not be sufficient to cause regression of most bulky tumors. One of the possible explanations for this observation may be related to pericytes that respond to angiogenic stimuli and promote

endothelial stability through matrix deposition, and have macrophage-like function (Lu et al., 2007). Also, the tumors overexpressing EGFR might not be sensitive to Erbitux. Although it is normally assumed that tumors overexpressing EGFR would respond well to anti-EGFR therapy, studies have demonstrated that the level of EGFR expression does not have enough impact on tumor response rates as a significant number of EGFR-positive tumors could be resistant to Erbitux (Ellis et al., 2004; Vallbohmer et al., 2005). Complete cure was noted in tumors treated with PDT and continued Avastin + Erbitux therapy. Thus the data from the present study supported the hypothesis that Avastin and Erbitux are capable of binding and neutralizing secreted VEGF and EGFR respectively, thus causing regression of tumor vessels, and preventing tumor recurrence.

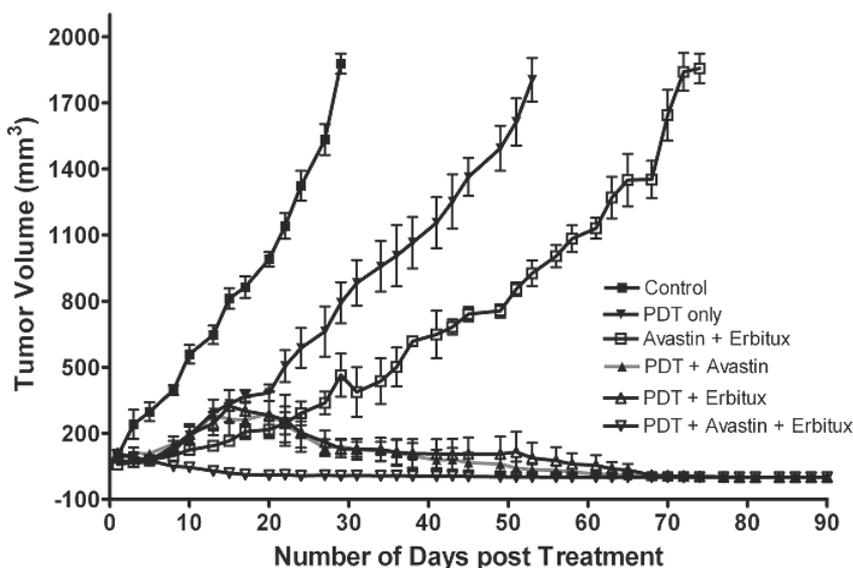


Fig. 15. Tumor volumes were charted against days to assess the tumor response in various treatment groups. The combination therapy groups of PDT + Avastin, PDT + Erbitux and PDT + Avastin + Erbitux exhibited greater tumor response in comparison with other groups. Each group represents the mean (error bars, SE) of 10 animals.

VEGF and EGFR expression was suppressed in the tumors treated with PDT and inhibitors (Figure 16). The data in this study demonstrate that tumors treated with PDT expressed greater amounts of VEGF, which is consistent with an earlier report by Solban et al. (Tortora et al., 2008) on subcurative PDT performed on an orthotopic model of prostate cancer that showed increased VEGF secretion. Also, significantly lower occurrence of VEGF was observed in the combination therapy of PDT and Avastin. On the other hand, previous studies have reported the downregulation of EGFR after PDT (Ciardiello et al., 2006), in marked contrast the results of this study demonstrated an increase in EGFR expression post hypericin-mediated PDT. This observation could be attributed to numerous reasons such as the light/drug dosage, the complexity of tumor microenvironment and the properties of the photosensitizer (Henderson et al., 2004). Combined antitumor activity of Avastin and Erbitux with standard chemotherapy and radiotherapy is well documented in the treatment

of different types of tumors and is reported to be more efficacious than individual monotherapies (Press & Lenz, 2007).

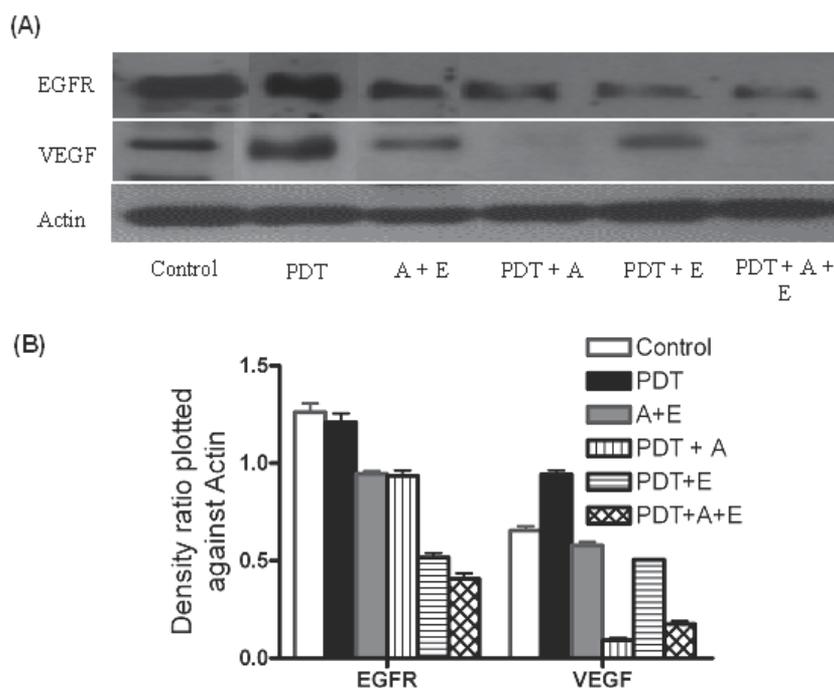


Fig. 16. (A) Expression of EGFR and VEGF was detected in the treatment groups using western immunoblot analysis. Expression of actin was used to monitor protein loading. (B) Ratio of EGFR and VEGF density was plotted against actin.

In this study, combination modality of PDT + Avastin + Erbitux was effective in reducing the expression of VEGF and EGFR which could have led to the greater tumor regression in this group. Combination of Avastin and Erbitux with PDT also improved treatment efficacy by suppressing angiogenic proteins. Though immediate tumor inhibition was noticed in the groups treated with both the inhibitors and PDT, the overall outcome post 90-day treatment for both single agent and double-agent inhibition remained the same. Although tumor regression was also observed in Avastin + Erbitux only treated groups, complete cure was not observed. Thus targeting EGFR and VEGF without PDT treatment might not be sufficient to cause regression of most bulky tumors. One of the possible explanations for this observation may be related to pericytes that respond to angiogenic stimuli and promote endothelial stability through matrix deposition, and have macrophage-like function. Also, the tumors overexpressing EGFR might not be sensitive to Erbitux. Although it is normally assumed that tumors overexpressing EGFR would respond well to anti-EGFR therapy, studies have demonstrated that the level of EGFR expression does not have enough impact on tumor response rates as a significant number of EGFR-positive tumors could be resistant to Erbitux. Complete cure was noted in tumors treated with PDT and continued Avastin + Erbitux therapy.

6. Conclusion

In conclusion, it has been demonstrated that VEGF is upregulated due to hypoxic conditions induced by hypericin-mediated PDT. Also, VEGF acts as a potent angiogenesis-stimulating factor that has potential as a tumor biomarker to determine the outcome of photodynamic therapy. Combination treatment of PDT with Avastin that binds to VEGF and blocks receptor binding improved the tumor response of bladder carcinoma xenografts and suppressed the VEGF pathway by causing the downregulation of important angiogenic mediators. In the similar way, the regular administration of Erbitux, an EGFR inhibitor after PDT treatment can block the EGFR pathway and reduce angiogenesis. Therefore, the combination therapy of PDT and Erbitux was more effective in preventing angiogenesis compared to monotherapy alone. In another study the combination of both Avastin and Erbitux with PDT was capable of binding and neutralizing secreted VEGF and EGFR respectively, thus causing regression of tumor vessels, normalizing surviving mature vasculature and preventing tumor recurrence.

To summarize, combining angiogenesis inhibitors with PDT increased therapeutic efficacy and this method is a promising approach to cancer therapy. The challenge is to choose the appropriate anti-angiogenic agent in combination with optimal light dosimetry PDT for potential clinical application. The success seen with the combination of inhibitors with conventional treatments can provide information for potential target mechanisms, which may translate into better response rate with less local and systemic toxicity and improved overall survival rates.

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Cancer Gene Therapy: The New Targeting Challenge

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1. Introduction

Cancer is characterized by genetic alterations due, for instance, to mutations in genomic DNA caused by chemicals (mutagens such as pollutants or nitrosamines, and polycyclic aromatic hydrocarbons), radiations (e.g., prolonged exposure to ultraviolet radiation from the sun, which can lead to melanoma or other skin malignancies), and viral infections (e.g., papilloma virus; human T-cell leukemia viruses 1, 2, 3, and 4; and herpes simplex virus). Mutations in genes involved in cell proliferation, tumor suppressor genes, or proto-oncogenes may lead to uncontrolled cell proliferation into a tumor. Currently, the most widely used treatments for cancer are combinations of surgery, radiotherapy and chemotherapy. However, the effectiveness of these treatments is variable. Consequently, means of potentiating conventional treatments, as well as new strategies, need to be developed.

Gene therapy is generally perceived as a treatment for rare genetic diseases, in which replacing the deficient gene by its normal counterpart has proved successful, most notably in severe combined immunodeficiency (SCID) (Fischer et al., 2010), adrenoleukodystrophy (Cartier et al., 2009), and β -thalassemia (Cavazzana-Calvo et al., 2010). However, cancer is the main focus of basic and clinical research on gene therapy (<http://www.wiley.com//legacy/wileychi/genmed/clinical/>). Variable levels of success have been achieved using a broad range of genes encoding tumor suppressor proteins such as p53, antiangiogenic proteins such as anti-vascular endothelial growth factor (VEGF), inflammatory cytokines, and other proteins (Lane et al., 2010), (Candolfi et al., 2010), (Adachi et al., 2010).

One of the main hurdles in gene therapy is selective delivery of recombinant vectors to the target tissue. In cancer gene therapy, administration of the vector within the tumor may be of interest, but some tumors are not readily accessible and vector dissemination to healthy cells cannot be ruled out. Today, accurate tumor targeting is a major goal of cancer gene therapy.

In this chapter, we will focus on the methods developed to improve targeting in cancer gene therapy, most notably gene-directed enzyme prodrug therapy (GDEPT), which is a major focus of research at our laboratory.

2. Gene-directed enzyme prodrug therapy (GDEPT)

Cytotoxic chemotherapy is often associated with severe systemic toxicities. Gene-directed enzyme prodrug therapy (GDEPT) or suicide gene therapy consists in selective delivery to

the tumor of a gene encoding a drug-metabolizing enzyme that catalyzes the *in situ* conversion of a non-toxic prodrug to a toxic active drug (Figure 1). GDEPT can be used to increase the levels of an enzyme produced by the tumor or to introduce an enzyme that is not expressed endogenously. The local production of the cytotoxic drug within the tumor is expected to result in greater effectiveness and less toxicity, compared to systemic drug delivery.

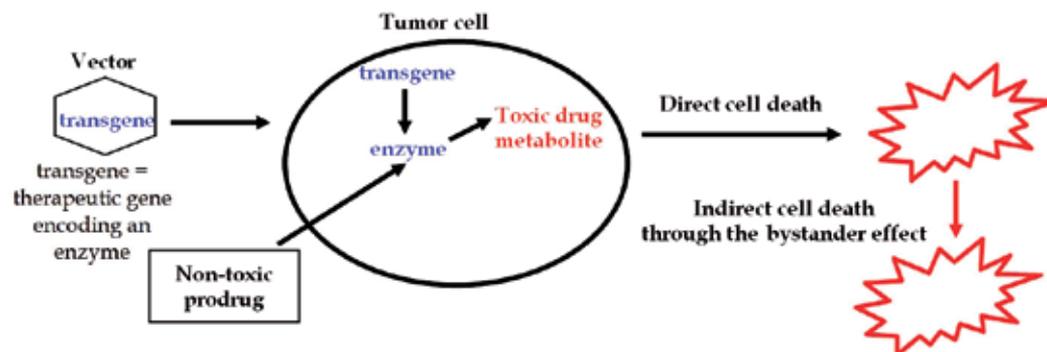


Fig. 1. Principle of gene-directed enzyme prodrug therapy (GDEPT)

Several studies have been performed with different enzyme and prodrug combinations. The most widely studied combinations are herpes simplex thymidine kinase/ganciclovir, cytosine deaminase/5-fluorouracil, and cytochrome P450 (CYP)/oxazaphosphorines (cyclophosphamide [CPA] and ifosfamide) (Altaner, 2008) (Table 1)

Enzymes	Source	Prodrug	Drug	Indication
Herpes simplex thymidine kinase	Herpes simplex virus	Ganciclovir	Ganciclovir triphosphate (GCV-TP)	Glioma, pancreatic cancer
Cytosine deaminase	<i>Escherichia coli</i>	5-Fluorocytosine (5-FC)	5-Fluorouracil (5-FU)	Glioblastoma, Colorectal cancer
Cytochrome P450	Rat/human/dog	Cyclophosphamide (CPA)	4-OH Cyclophosphamide (4-OH CPA)	Head and neck cancer, lung cancer, Burkitt's lymphoma
Nitroreductase	<i>Escherichia Coli</i>	CB1954	N-acetoxy derivatives	Cancer cells in general

Table 1. Enzyme/prodrug combinations used in GDEPT

2.1 Cytochrome P450 (CYP)/cyclophosphamide (CPA) combination

The chemotherapeutic prodrug CPA is widely used for the treatment of both solid tumors and hematological malignancies. Enzymatic bioactivation, chiefly via human CYP2B6 (Gervot et al., 1999), produces the metabolite 4'-OH-CPA, which undergoes spontaneous decomposition to acrolein and phosphoramidate mustard. Phosphoramidate mustard is an

electrophilic alkylating agent that causes the formation of intra- and interstrand DNA cross-links, which eventually lead to apoptotic cell death (Schwartz & Waxman, 2001). In patients treated with CPA, this prodrug is activated by CYP2B6 in the liver, and the active metabolites enter the bloodstream, which transports them not only to the tumor but also to healthy tissues where they may cause severe side effects including cardiotoxicity, renal toxicity, bone marrow suppression, and neurotoxicity (Fraiser et al., 1991) (Langford, 1997). To prevent these side effects, CYP2B-based gene-directed enzyme prodrug therapy was developed by D.J. Waxman and colleagues and, more recently, by our group (Waxman et al., 1999), (Jounaidi, 2002), (Jounaidi et al., 2006), (Tychopoulos et al., 2005). CYP2B expressed in tumor cells results in the *in situ* conversion of CPA to cytotoxic metabolites. Moreover, the diffusible 4'-OH-CPA metabolite can enter neighboring cells, where it is converted to phosphoramidate mustard, leading to the death of nontransfected tumor cells (Wei et al., 1995), (Tychopoulos et al., 2005). This bystander effect plays a major role in the CYP2B-based GDEPT strategy, and several studies of various suicide gene and prodrug combinations have shown that complete eradication of the tumor is possible even when the suicide gene product is expressed by less than 10% of the cells (Portsmouth et al., 2007)

In our laboratory, we are developing a GDEPT strategy based on human CYP2B6, the human CYP isoform that preferentially metabolizes CPA (Gervot et al., 1999). One of the main difficulties is the relatively low affinity of CYP2B6 for CPA. Modifications aimed at increasing the efficiency of CYP2B6 (V_{\max}/K_m) in catalyzing the 4-hydroxylation of CPA have therefore been evaluated. We used site-directed mutagenesis of the active site of CYP2B6 to produce a double mutant (I114V/V477W) characterized by a 4-fold increase in CPA-4-hydroxylation efficiency compared to the wild-type CYP2B6 (CYP2B6wt), ascribable chiefly to an increase in enzyme affinity (Nguyen et al., 2008). Recently, we obtained a triple CYP2B6 mutant (CYP2B6TM) that is 8 times more efficient than CYP2B6wt (unpublished results from our laboratory)

Another means of improving the efficiency of CYP2B6-mediated GDEPT is co-expression in the tumor cells of NADPH-cytochrome P450 reductase (RED). RED is a FAD- and FMN-containing enzyme that catalyzes the transfer from NADPH of electrons required for CYP-dependent enzyme reactions. Within tumors, where RED expression is heterogeneous (Fitzsimmons et al., 1996; L. J. Yu et al., 2001), CYP-GDEPT results in high levels of CYP expression, and RED availability can limit the rate of CYP-catalyzed enzyme reactions and, therefore, of prodrug bioactivation. To ensure the production of both CYP2B6 and RED by the same cancer cell, a CYP2B6wt-RED fusion protein having both 4-hydroxylase activity and reductase activity was built. This fusion protein proved more efficient than CYP2B6wt alone for metabolizing CPA in several pulmonary cell lines (Tychopoulos et al., 2005). Recently, we produced a CYP2B6TM-RED fusion protein that is 10 times more efficient than CYP2B6wt-RED in activating CPA (unpublished results from our laboratory).

These studies show that improving the efficiency of CYP2B6 is feasible. This method may allow the use of lower CPA dosages with no loss of cytotoxic effectiveness within the tumor but with less activation by hepatic CYP2B6 and, therefore, a possible decrease in cytotoxic effects on non-tumor tissue. Preliminary results in various human pulmonary and head-and-neck cancer cell lines show that expression of the CYP2B6TM-RED protein sensitized the cancer cells to lower doses of CPA compared to expression of CYP2B6wt-RED (unpublished results from our laboratory).

3. Gene therapy vectors

The most important step in any gene therapy protocol is the development of efficient vectors for delivering the transgene to its target. The ideal vector should be administered by a non-invasive route, penetrate only into the targeted cells in order to limit adverse side effects, and express the transgene in amounts sufficient to produce strong therapeutic effects. A wide range of vectors have been developed including viral vectors, polymers, liposomes, nanoparticles, and bare DNA.

Today, about 70% of clinical gene therapy trials worldwide use viral vectors such as retroviruses, adenoviruses, and adeno-associated viruses (AAV) or lentiviruses (Table 2) to transfer transgenes and 64.5% of these trials are conducted in patients with cancer (<http://www.wiley.com//legacy/wileychi/genmed/clinical/>).

However, retroviral vectors used to treat SCID have been responsible for leukemia caused by transgene insertion into proto-oncogene regions (Hacein-Bey-Abina et al., 2003). This side effect has severely slowed the development of gene therapy. However, we now have safer vectors such as the lentivirus used for gene therapy of adrenoleukodystrophy (Cartier et al., 2009) and β -thalassemia (Cavazzana-Calvo et al., 2010). Transgenes from recombinant lentivirus may be integrated mainly within intragenic or intronic regions (S. H. Yang et al., 2008).

Here, we will focus on three viruses that are presently widely used in gene therapy, namely, adenoviruses, AAVs, and lentiviruses.

3.1 Adenoviruses

Adenoviruses cause mild upper airway diseases. They are non-enveloped icosahedral viruses composed of a nucleocapsid and double-stranded linear DNA genome of about 35 kb with inverted terminal repeat (ITR) sequences at each end. There are 51 classified human adenovirus serotypes; serotypes 2 and 5 are those used most widely in *ex vivo* and *in vivo* gene therapy. They are very convenient vectors, because they can accommodate relatively large segments of DNA, up to 8 kb. Moreover, their transduction efficiency is high. To avoid a strong immune response after vector delivery, non-replicative recombinant adenoviruses lacking some of the early genes involved in the immune response are used. Deletion of the E1 sequence renders the virus unable to produce infectious viral particles in infected cells, and the E3 region is not necessary for viral production since it encodes proteins involved in evading host immunity. Thus, deletion of E1 and E3 is used to decrease the host immune response to the viral proteins (Alba et al., 2005).

Adenoviral vectors allow episomal and, therefore, transient transgene expression by infected cells (no integration of the foreign DNA into the genome of the host cell) (Russell, 2009) (Alemany & Curiel, 2001).

To infect cells, adenoviruses use the coxsackie-adenovirus receptor (CAR) and integrins as primary cell surface attachment components (Figure 2). The adenovirus (Ad) fiber knob binds with high-affinity to the CAR receptor and the viral penton base interacts with integrins (Bergelson, 1999). CAR plays a significant role in liver transduction and, consequently, most of the adenoviral particles administered intravenously are sequestered in the liver (Vrancken Peeters et al., 1996). However, the mechanism of adenoviral infection *in vivo* is controversial, especially as the introduction of mutations that abrogate CAR binding does not significantly impact the infectivity of adenoviral vectors.

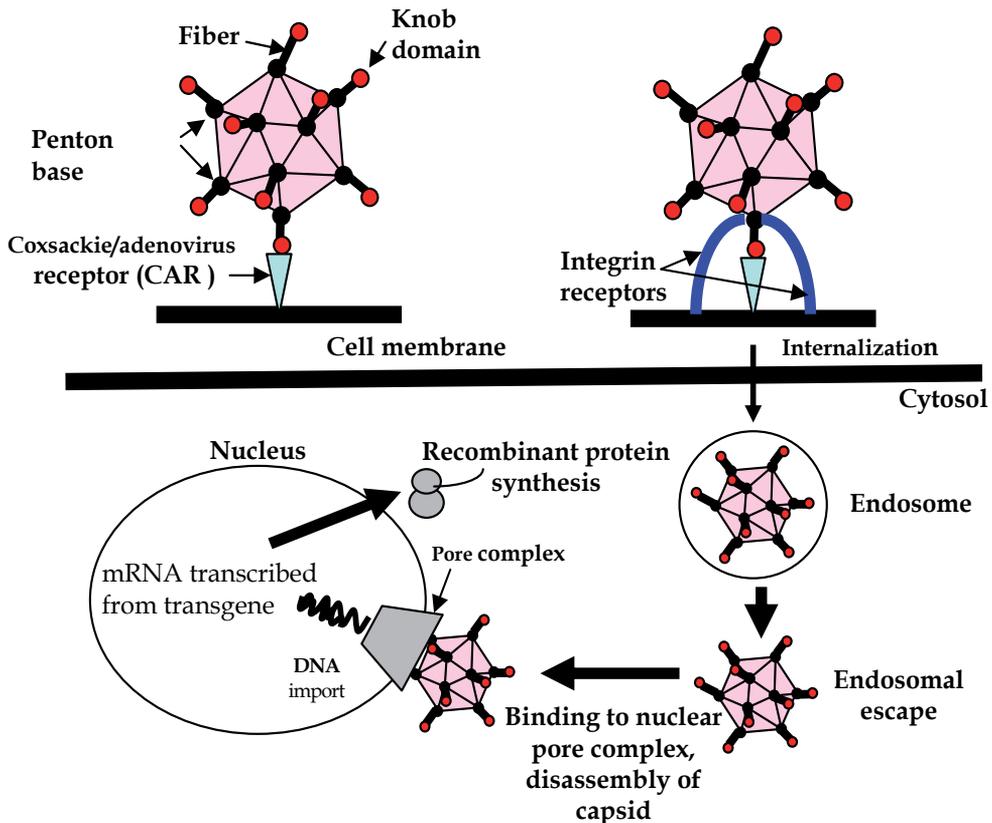


Fig. 2. Schematic representation of adenoviral attachment and internalization

Although immune responses have been limited, they have sometimes restricted the efficiency of adenoviral vectors in clinical trials. Increased immunogenicity has been reported, and many patients have pre-existing immunity to the adenoviral serotypes used in gene therapy. Cell-mediated recognition of the viral capsid components or nucleic acids has received considerable attention and is thought to be chiefly regulated by toll-like receptors (TLRs). Innate immune responses to viruses are initiated by the infected cells, which activate the interferon response to block viral replication, while simultaneously releasing chemokines that attract neutrophils, mononuclear cells, and natural killer cells. In 2010, adenoviruses were still the most widely used vectors for gene therapy. Nevertheless, the use of adenoviral vectors relative to other vectors decreases year on year.

3.2 Adeno-associated viruses (AAV)

Adeno-associated viruses (AAV) are small non-enveloped DNA viruses belonging to the parvovirus family. The single-strand DNA genome of about 4.8 kb comprises two open reading frames (*rep* and *cap*) flanked by inverted terminal repeats (ITRs). Twelve serotypes have been isolated from primate or human tissues (Schmidt et al., 2008). Advantages of AAVs include an apparent lack of pathogenicity, an ability to infect both non-dividing and dividing cells, and stable integration into the host genome at a specific site of the human

chromosome 19 when the vector includes the *rep* gene. In the absence of the *rep* gene, chromosomal integration occurs infrequently and at random sites (Huser et al., 2010).

The AAV infection cycle is initiated by the binding of the viral capsid to cell surface receptors. One of the main receptors involved is heparan sulfate proteoglycan (HSPG); moreover, several co-receptors contribute to transduction (Asokan et al., 2006). Receptor binding mediates endocytosis, endosomal escape and, finally, transport to the nucleus.

AAV vectors are constructed by replacing the viral DNA with an expression cassette encoding the gene of interest under transcriptional control of a suitable promoter. Vector production is achieved by transfection of a cell line with three plasmids: one contains the expression cassette flanked by the ITRs; another contains *rep cap* helper sequences, and the third is an adenoviral helper plasmid encoding the adenoviral E2a, E4, and VA helper genes (Grimm & Kleinschmidt, 1999).

AAVs have become very popular as gene therapy vectors because of both their ability to mediate stable and efficient gene expression and their good safety profile. The major drawbacks of AAVs are the small amount of DNA that the virus can carry, which results in low capacity; and the difficulty of producing the vector in high titers (Michelfelder & Trepel, 2009). AAVs have been used in at least 80 clinical trials (as of 2011), in strategies based on the delivery of cytotoxic genes, tumor suppressor genes, and other types of genes.

3.3 Lentiviruses

Lentiviruses are retroviruses that include the human immunodeficiency virus 1 (HIV-1). They have a lipid envelope and two identical single-stranded genomic RNA molecules that require a reverse transcriptase for conversion to DNA. The HIV genome is composed of two

	Adenovirus	Adenovirus-associated virus	Retrovirus	Lentivirus
Genome integration	Rarely	No (in absence of <i>rep</i> gene) Yes (in presence of <i>rep</i> gene)	Yes	Yes
Transgene expression	Transient	Stable	Stable	Stable
Immune response	Marked	According to conditions (animal, transgene, injection conditions,...)	Absent to moderate	Absent to moderate
Target cells	Quiescent or dividing	Quiescent or dividing	Dividing	Quiescent or dividing
Transgene size	up to 8 kb	limited	8-9 kb	8-9 kb
Main use in gene therapy	<i>in vivo</i>	<i>in vivo</i>	<i>ex vivo</i> - <i>in vivo</i>	<i>ex vivo</i>
Titer	>10 ¹¹	>10 ¹¹	>10 ⁸	>10 ⁸
Genotoxicity	No	No	Mutagenesis-related risks	No

Table 2. Characteristics of four viral vectors: adenovirus, adenovirus-associated virus, retrovirus, lentivirus.

regulatory genes, *tat* and *rev*, which are necessary for viral replication; and four accessory genes, *vif*, *vpr*, *vpu*, and *nef*, which are not required for *in vitro* replication or growth but are crucial for *in vivo* replication. The *tat* and *rev* proteins are involved in regulating HIV gene expression at the transcriptional and post-transcriptional levels, respectively (Pauwels et al., 2009).

Lentiviral particle production involves co-transfection by calcium phosphate precipitation of gag-pol, env, and vector plasmids into HEK 293T cells. Viral particles are then recovered from the cell medium, concentrated, and filtered. Finally, the viral titer is determined (Dull et al., 1998) (Kutner et al., 2009). The transgene present in recombinant lentiviruses is integrated into the host genome via an integrase and is therefore expressed in a stable manner over time. Among retroviruses, lentiviruses efficiently infect both dividing and non-dividing cells (Naldini et al., 1996) without inducing genotoxicity with insertional mutagenesis (Montini et al., 2009), since they are integrated mainly within intragenic or intronic regions. Lentiviruses (e.g., the HIV) use cell receptors such as CD4 and the co-receptors CCR5 or CXCR4 to penetrate the cells. Lentiviral vectors express various types of proteins that are recognized by cell receptors and co-receptors, leading to a very broad tropism.

Since these vectors were first introduced, they have been modified in several ways with the goal of improving their safety profile. Now, these viral vectors are being increasingly used. However, their lack of tissue specificity may limit their use, and several methods have been developed to improve their ability to target the desired site.

4. Current strategies for viral vector targeting

Today, the major goal in cancer gene therapy is to improve tumor targeting, thus preventing transgene expression by normal cells and therefore diminishing the risk of toxic side effects. Initially, the vector was injected directly into the tumor. However, vectors are now available that target the tumor after being administered systemically.

Efforts to improve viral vector targeting can modify the binding of the virus to the cell and entry of the virus into the cell (entry targeting/transductional regulation) or the events that occur once the virus is in the cell (post-entry targeting/transcriptional regulation). Several approaches have been devised such as envelope or capsid modifications, the use of various adapters, placement of transgene expression under specific promoter control, and modifications of the transgene sequence.

4.1 Pseudotyping: Envelope or capsid modification

Viral vectors infect their natural host-cell populations preferentially and with the greatest efficiency. Viral infection occurs when host-cell receptors recognize the viral envelope proteins. Pseudotyping consists in changing the plasmid encoding the expression of envelope proteins. The result is a shift in the range of host cells and, consequently, in the tissue tropism of the viral vector. The vector surface is modified via the incorporation of foreign envelope glycoproteins that have a restricted natural population of host-cell receptors (Frecha et al., 2008). This technique was the first to be used for modifying viral tropism, particularly in retroviruses such as lentiviruses, which have an envelope. Adenoviral vectors have no envelope, and the viral attachment protein must therefore be incorporated into a protein capsid instead of a lipid bilayer.

Lentiviral vector pseudotyping is usually achieved using the vesicular stomatitis virus G (VSV-G) protein, which exhibits a broad tropism for various cell types. Additional advantages of VSV-G-pseudotyped lentivirus are the higher viral titers compared to those obtained with other envelope proteins and the improved vector particle purification due to increased stability of the virus. However, when used in high concentrations, lentiviral vectors bearing VSV-G may exert cytotoxic effects (Chen et al., 1996). Fortunately, this drawback can be overcome either by improving purification of the lentiviral particles using gradient centrifugation to eliminate unincorporated transgene particles (Ricks et al., 2008) or by using other proteins for pseudotyping. VSV-G-pseudotyped particles are convenient to use *ex vivo* to express a transgene in a broad spectrum of cell lines. However, VSV-G-pseudotyped viruses can be inactivated by human serum (DePolo et al., 2000). In clinical trials of cancer gene therapy, the objective is to limit the tropism of the vector to the cancer cells.

Miletic et al., worked on a gene therapy strategy for malignant gliomas, which are the most common primary brain tumors and carry a poor prognosis due to their infiltrative growth (Miletic et al., 2004). Miletic and co-workers compared the expression of various pseudotyped lentiviruses in normal brain cells and malignant glioma cells. VSV-G pseudotyped lentiviruses infected the neurons and astrocytes, whereas the tropism of lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) pseudotypes was virtually confined to the astrocytes. LCMV-G-pseudotyped lentivirus was specifically and efficiently transduced in rat gliomas, whereas VSV-G-pseudotyped lentivirus was considerably less efficient in transducing glioma cells.

Another protein often used to target cancer cells is the modified sindbis virus envelope. Pariente et al., (2007) used it successfully to target prostate cancer cells.

Transduction efficiency is low after tumor cell infection with adenoviruses. One reason is the limited expression of the coxsackievirus-adenovirus receptor (CAR) in tumor cells. To overcome this obstacle, the adenovirus fiber can be modified by removing interactions with both CAR and integrins, the main components involved in adenovirus transduction (Einfeld et al., 2001). This modification diminishes the native tropism and enhances the efficacy of specific targeting ligands in redirecting the adenovirus to the target tissues.

Malignant gliomas are refractory to adenovirus-mediated gene therapy, chiefly because CAR is not expressed by the tumor cells. Zheng et al. identified several receptors that were over-expressed in tumor cells, and they created a series of pseudotyped adenoviral vectors. Some of these vectors enhance gene transfer to tumors and warrant further development for glioma gene therapy. (Zheng et al., 2007)

Yu et al., (L. Yu et al., 2005) reported increased infection of esophageal and oral carcinoma cells with adenoviruses whose Ad5 fiber was substituted with fibers from Ad11 or Ad35, compared to unmodified adenoviruses. Similarly, attaching the Ad3 fiber to the Ad5 backbone was particularly effective for targeting ovarian cancer and squamous cell carcinoma of the head and neck.

The efficacy of pseudotyping may be limited by the lack of tissue specificity and ubiquitous expression of some of the receptors. Furthermore, the viral envelope modifications may diminish viral stability and limit viral production, leading to low titers.

4.2 Use of adapters: Antibody/ligand

Another technique consists in fusing special adapters or proteins to the envelope proteins. These adapters determine the affinity of the vector for the target.

4.2.1 Antibody

A protein can be specifically targeted by the use of specific antibodies, antibody fragments, or single-chain antibodies fused to the viral membrane. There are two main methods for using antibodies to improve targeting by vectors.

- The entire antibody or an antibody fragment directed against both a viral envelope protein and a tumor cell membrane receptor can be used as a bridge to attach the virus to specific cells.
- An antibody fragment (usually the fragment crystallizable region Fc) can be expressed at the viral envelope and the rest of the antibody can be directed against a specific antigen of the target cells.

For prostate cancer gene therapy, Kraaij et al. developed a targeted method based on bi-specific antibodies constructed as conjugates between an anti-adenovirus fiber knob Fab' fragment and an anti-prostate specific membrane antigen (PSMA) (Kraaij et al., 2005). These bi-functional antibodies, used as a bridge between capsid proteins and cell surface receptors, were selective for the prostate cancer cell lines. They may hold promise for gene therapy of prostate cancer.

Another strategy, developed by Zhang et al., consists in binding trastuzumab (or Herceptin®, a monoclonal antibody directed against the human epidermal growth factor receptor (HER-2)) to the lentivirus envelope. Thus, the vector targets cells that overexpress HER-2, such as prostate cancer cells, to which it delivers the transgene. Zhang et al. engineered these lentiviruses to express thymidine kinase and showed that prostate cancer cell lines infected by these lentiviruses became vulnerable to ganciclovir. (Zhang et al., 2009) Poulin et al. worked on a new adenoviral vector and investigated the usefulness of capsid protein IX (a minor protein of the adenoviral capsid) as a platform for presenting single-chain variable-fragment antibodies and single-domain antibodies for virus targeting. Given the ability of this protein to fuse to large polypeptides, Poulin et al. decided to test large targeting ligands such as antibodies. Presence in the vector of single-chain variable-fragment antibodies was not sufficient to ensure accurate targeting, contrary to the presence of single-domain antibodies (Poulin et al., 2010).

However, this method is still complicated to use, as it requires the production of monoclonal antibodies, which is both time-consuming and costly. In addition, a specific tumor cell antigen must be obtained, which may be difficult. Finally, the titer of vectors that express the antibody in their envelope is sometimes low.

4.2.2 Ligand

The first attempts at inserting a ligand into the viral membrane used various types of ligand such as growth factors, hormones, and peptides, which were inserted at various sites of the viral surface.

Morizono et al., (Morizono et al., 2009) used a strategy based on a lentiviral vector bearing the biotin-adapter-peptide. In earlier studies of adenoviral or AAV vectors, peptides that were biotinylation substrates were inserted and associated with biotinylated sites, bound avidin, neutravidin, or streptavidin. (Parrott et al., 2003; Pereboeva et al., 2007; Stachler et al., 2008)

Similarly, Liu and colleagues (Liu et al., 2011) used a serotype 5 adenoviral vector (Ad5) whose fiber knob was deleted and replaced by a biotin-acceptor peptide. The advantage of this new adenoviral vector is that no CAR-dependent cell uptake and transduction occurs; moreover when the vector is biotinylated, biotinylated antibodies can be used to achieve targeting. AAV vectors can also be biotinylated.

A hybrid approach using an antibody and a protein ligand has been described in two papers by a group working at the University of California, Los Angeles. (Joo & Wang, 2008), (L. Yang et al., 2006). This group of researchers engineered a lentiviral vector whose surface bears two distinct molecules, an antibody conferring target specificity to the engineered vector and a pH-dependent fusogenic protein that allows the engineered vector to penetrate the target cells. Evaluation by image processing showed highly specific incorporation of this lentivirus into the cells.

Hajitou et al. (Hajitou et al., 2006) developed an AAV vector combined with a double cyclic peptide (RGD-4C) of an fd-tet phage. Their aim was to target αV integrins, a cell surface receptor that is overexpressed in tumors and interacts with the RGD peptide. The native tropism of AAV for mammalian cells is eliminated, since there is no AAV capsid formation and the ligand peptides allow homing to tissue specific receptors. To obtain chimeric viruses, Hajitou et al. inserted an eukaryotic gene cassette from the AAV into an intergenomic region of the RGD-4C phage. The vector was functional and efficiently targeted human Kaposi sarcoma (KS 1767 cells) grafted in nude mice *in vivo*. Using a ganciclovir cytotoxicity strategy, Hajitou et al. obtained a decrease in tumor volume in mice receiving this vector compared with those given a non-specific vector. Using the same strategy, Bauerschmitz et al. (Bauerschmitz et al., 2002) used an adenovirus modified with a RGD domain to target ovarian cancer cells. As seen with the other approaches involving transductional targeting, limited viral production and stability may occur when the viral envelope is modified.

4.3 Tissue-specific promoter

A promoter is a DNA region that is located upstream of the gene and plays a key role in regulating gene expression. The insertion of a cell-specific regulated promoter upstream from the transgene may limit the expression of the promoter to the targeted cells. Several cancer-specific promoters have been found effective in cancer gene therapy, including prostate stem cell antigen (PSCA) promoter in prostate cancer (Petrigliano et al., 2009), carcinoembryonic antigen (CEA) promoter in gastric cancer (Tanaka et al., 2006), and alpha-fetoprotein (AFP) enhancer and albumin promoter in hepatocellular carcinoma (He et al., 2000).

These promoters are tissue-dependent, however. A universal tumor-specific promoter targeting tumor cells of any origin would be of considerable interest. For instance, given that hypoxia is a common physiological feature of tumor tissue, an optimized hypoxia-responsive promoter (OBHRE) may be effective in increasing the therapeutic window of cytotoxic cancer gene therapy (Binley et al., 2003). In a range of cell types, this promoter expresses high levels of transgene in hypoxic tissue but has minimal activity in normoxia. Moreover, the OBHRE promoter in a recombinant adenovirus allowed high-level expression of the transgene in tumor cells but was not expressed in normal tissues such as the liver, spleen, lung, and kidney. Binley et al. developed a GDEPT strategy using CYP2B6 or thymidine kinase as the transgene in combination with CPA and ganciclovir, respectively. Direct administration of the gene therapy vector containing OBHRE into established tumor models was effective, and this method limited the toxic effects due to hepatic sequestration of the adenovirus.

A characteristic promoter of cancer cells is the prostate stem cell antigen (PSCA) promoter. Petrigliano et al. (Petrigliano et al., 2009) used the PSCA promoter to develop a lentiviral

vector targeting prostate cells. PSCA is consistently expressed by high-grade prostate intraepithelial neoplasias and invasive prostate cancers (Watabe et al., 2002). The lentiviral vector carried a cytotoxic thymidine kinase gene and was combined with ganciclovir treatment. Lentiviral gene therapy vector driven by a short PSCA promoter induced prostate-specific cellular toxicity *in vivo* and *in vitro*. This strategy could be used to treat local and advanced metastatic prostate cancer.

However, one of the main problems with the specific promoter strategy is that faithful reconstitution of a complete gene sequence promoter can be difficult. Moreover, transcriptional targeting cannot prevent the sequestration of therapeutic viruses in normal tissues, which may result in toxicity and loss of efficacy.

5. A new strategy for viral vector targeting: micro RNAs (miRNA)

In addition to the above-mentioned methods, microRNAs (miRNAs) may hold potential for improving viral vector targeting, as they are involved in the post-transcriptional regulation of gene expression.

5.1 microRNAs (miRNAs)

The small non-coding RNAs (~20-25 nucleotides) known as miRNAs regulate gene expression at the post-transcriptional level. They are involved in a variety of biological processes including development, differentiation, apoptosis, and cell proliferation. They repress gene expression by binding to their complementary target sites in mRNAs, thereby increasing the degradation or preventing the translation of the transcripts. Thus, cells that express an miRNA complementary to an mRNA do not express the protein coded by this mRNA: miRNAs are endogenous negative gene regulators. (Figure 3).

In 1993, miRNAs were identified for the first time, in the nematode *Caenorhabditis elegans*, in which they were encoded by the *lin-4* and were complementary to mRNA for the *lin-14* gene (R. C. Lee et al., 1993). The *lin-4* gene product is a small RNA of 22 nucleotides (i.e., a miRNA) that is specific of the 3'UTR of the *lin-14* gene and therefore inhibits the production of the *lin-14* protein, thus preventing the transition from larval stage L1 to stage L2. Since the discovery of miRNAs, their mechanisms of action and biogenesis have been studied in detail, and they have been shown to play a major role in physiological processes, development, and disease.

Briefly, miRNA biogenesis involves four stages: transcription of pri-miRNA; cleavage by Drosha to release a precursor pre-miRNA; export of the precursor to the cytoplasm; and cleavage of the pre-miRNA precursor by Dicer. All miRNAs are processed from precursor molecules called pri-miRNAs (Y. Lee et al., 2002), which are transcribed from independent miRNA genes or are portions of introns of protein-coding RNA polymerase II transcripts. Typically, a single pri-miRNA often contains sequences of several different miRNAs.

These pri-miRNAs of about 100 nucleotides are folded into hairpin structures and characterized by imperfectly base-paired stems. These molecules are then processed by a multiprotein complex including the RNase III type endonuclease Drosha and DiGeorge syndrome critical region gene 8 (DGCR 8). The hairpin structures are recognized in the nucleus by DGCR 8, a double-stranded RNA-binding protein (dsRBP). DGCR8 and the Drosha complex process the pri-miRNAs to pre-miRNA hairpins composed of about 70 nucleotides. Pre-miRNAs are then transported from the nucleus to the cytoplasm by exportin 5. In the cytoplasm, they undergo a final maturation step consisting in cleavage by

Dicer, which is complexed with TAR RNA binding protein (TRBP). This cleavage step releases an miRNA duplex of about 20 nucleotides. Mature miRNAs are integrated into a ribonucleoprotein complex called RNA induced silencing complex (RISC) or miRNA-induced silencing complex (miRISC). The components of miRISC complexes are mature miRNAs, Dicer and TRBP proteins, and proteins of the Argonaute family (AGO).

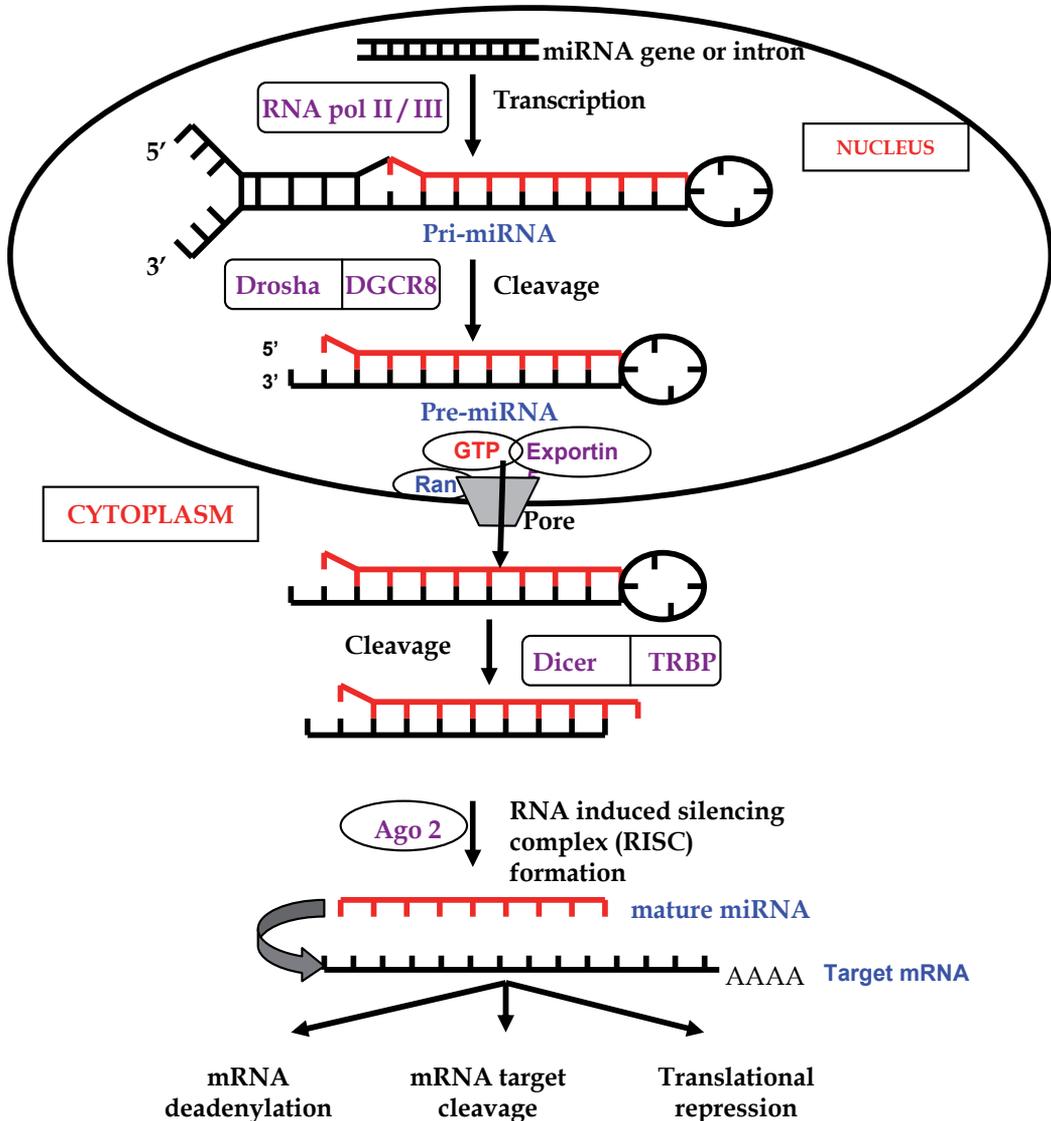


Fig. 3. Principle of miRNA biogenesis

AGO proteins represent the key components of miRISCs; in mammals, four AGO proteins (AGOs 1, 2, 3, and 4) have been identified. They are involved in the miRNA repression function via protein synthesis repression, whereas only AGO2 contributes to the RNA interference (RNAi) function. (Jaskiewicz & Filipowicz, 2008).

Binding of miRNAs to complementary target sites on mRNAs prevents the translation of the transcript or accelerates its decay. The regulation of miRNAs depends on the binding of the first 2 – 8 bases of their mature sequence to the 3'UTR of target genes. To date, 1048 human miRNA precursor sequences have been deposited in the miRBase (<http://www.mirbase.org>) (Kozomara & Griffiths-Jones)

There is now sound evidence that miRNAs are involved in the pathogenesis of conditions such as cancer and inflammatory responses. It has been shown that miRNA expression is deregulated in cancer cells. The differences in miRNA expression between normal and malignant cells may be related to the location of miRNA genes in cancer-associated regions, to epigenetic mechanisms, and to alterations in the miRNA processing machinery (Calin & Croce, 2006). Several studies suggest that miRNAs may contribute to oncogenesis by acting either as tumor suppressors (excessive regulation) or as oncogenes (insufficient regulation).

5.2 Targeting strategy using miRNA

Recently, researchers have started to evaluate endogenous miRNA-mediated regulation as a means of targeting the expression of exogenous genes. Naldini and co-workers demonstrated that endogenous miRNAs could be broadly exploited to regulate transgene expression in various cell lines. This very elegant approach to the control of protein expression relies on the potent regulatory properties of miRNAs. Several studies demonstrated that miRNA expression in cancer cells is deregulated compared to normal cells. The idea is to use this deregulation to modulate the expression of the transgene (B. D. Brown et al., 2007a) (Figure 4). Naldini and colleagues first developed a vector characterized by suppression of transgene expression in hematopoietic cells. The vector contains target sequences for the hematopoietic cell-specific miRNA miR 142-3p; thus, transgene expression is specifically suppressed in all hematopoietic cell lines but is not affected in other cell types. (B. D. Brown et al., 2007a)

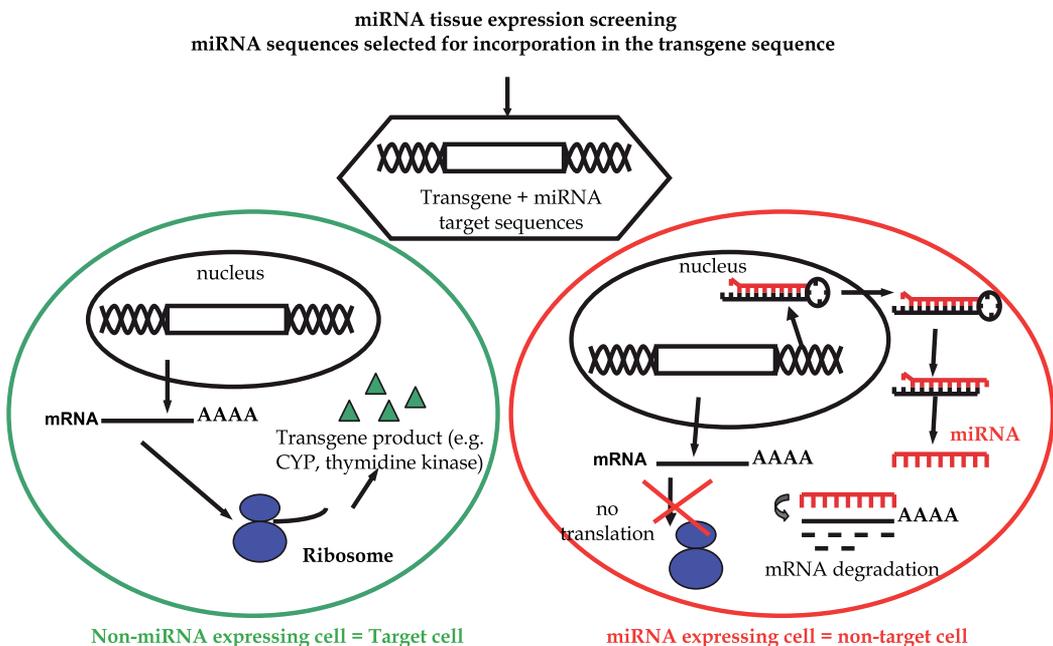


Fig. 4. Principle of miRNA targeting strategy

During the development of this technique, one issue was determination of the amount of endogenous miRNA needed to obtain effective target mRNA suppression. Brown et al., (B. D. Brown et al., 2007b) investigated this issue and concluded that target suppression depended on a threshold miRNA concentration.

Suzuki et al., (Suzuki et al., 2008) worked on a suicide gene therapy strategy based on the herpes simplex virus thymidine kinase (HSVtk) gene and ganciclovir (GCV), with adenoviral vectors. Based on the literature and their experiments, they showed that intratumorally injected adenoviral vectors were disseminated into the systemic circulation and transduced in the liver, resulting in hepatotoxicity. They therefore decided to produce a vector capable of preventing the hepatotoxicity of adenoviruses without altering the antitumor effects of suicide gene therapy. They hypothesized that insertion of sequences complementary to miR122a (which is highly expressed in the liver) into the 3'-UTR of a transgene expression cassette in adenoviral vectors would reduce hepatic transduction without affecting transgene expression in the tumor.

They constructed several vectors; among them, one had four tandem copies of sequences with perfect complementarities to miR122a. The copy number of miRNA target sequences is expected to play an important role in the regulation of transgene expression. An increase in the number of miRNA sequences leads to greater suppression of transgene expression (Doench et al., 2003); thus, four copies are better than two (B. D. Brown et al., 2007b). However, considerable work remains needed to determine the best number of copies and the best spacing elements between tandem copies of miRNA.

Simultaneously, Ylosmaki et al. have developed an adenoviral vector containing sequences complementary to miR 122. They tested the expression of a protein encoded by the vector in Huh7 cells. Huh7 cells resemble normal hepatocytes in that they have a high level of miR 122 expression. As mentioned previously, this strategy prevented transgene expression in the liver, thus avoiding adenovirus-induced hepatotoxicity.

An increasing number of studies combine tissue promoter regulation with miRNA regulation. For instance, Wu C et al. (Wu et al., 2009) developed a baculoviral vector, a strategy that could be extended to other viral vectors. To target glioblastoma cells, they used thymidine kinase/ganciclovir, and a glial fibrillary acidic protein (GFAP) gene promoter. Expression of the herpes simplex virus thymidine kinase gene was controlled by adding the repeated target sequences of three miRNAs that are enriched in astrocytes but downregulated in glioblastoma cells. To determine which miRNA sequences should be used, they reviewed the literature on miRNA expression in gliomas and normal brain tissues.

Downregulated miRNAs are miR 128, 137, 299, 31, 107, 132, 133a, 133b, 154, 323, 330, 127, 134, 181a, and 181b (Ciafre et al., 2005) (Silber et al., 2008); there is only one upregulated miRNA, namely, miR 10b. Wu and colleagues used these results to construct targeting vectors. Suicide gene expression controlled by specific miRNA sequences exerted selective cellular effects *in vitro* and *in vivo*. Glioma cells were specifically targeted, and ganciclovir was toxic in these cells. Wu et al. concluded that incorporating miRNA regulation into a transcriptional targeting vector provided a high level of control over transgene expression. The crucial steps in developing an efficient system include selection of a relevant tissue-specific promoter and determination of relative miRNA expressions in tumor cells and their normal counterparts. The next step is selection of miRNAs that are downregulated in tumor cells and expressed at high levels in normal cells.

This approach has also been studied in another cancer treatment strategy based on oncolytic viruses. Thus, Leja et al., (Leja et al., 2010) worked on an oncolytic adenovirus. Their aim was to abolish the hepatic tropism of the adenovirus, and therefore the occurrence of hepatotoxicity, without altering the antitumoral effects in neuroendocrine cells. They used not only a specific promoter but also miR 122 sequences. Similar to Suzuki et al. (Suzuki et al., 2008) and Ylosmaki et al. (Ylosmaki et al., 2008), Leja et al. found that hepatic tropism and expression were abolished.

Edge et al. (Edge et al., 2008) used another oncolytic virus, the vesicular stomatitis virus (VSV). They incorporated let-7 miRNA complementary sequences within the VSV to eliminate toxicity for normal cells without preventing expression in cancer cells *in vitro* and *in vivo*.

This approach has also been found effective in diseases other than cancer. Thus, an miR 142-3p regulated lentiviral vector has been used in hemophilia B (B. D. Brown et al., 2007a); miR 122 regulated transgene expression improved targeting to the heart (Geisler et al.); and a lentiviral vector containing miR 142 sequences regulated UGT1A1 expression in the liver (Schmitt et al., 2010)).

6. Conclusion

Cancer gene therapy and, in particular, suicide gene therapy holds considerable promise as a substitute for conventional chemotherapy. However, several aspects of gene therapy remain to be improved. In particular, there is a need for developing enzymes such as mutant forms of human enzymes that are more efficient than the wild-type enzyme regarding specificity and kinetics for the prodrugs, as exemplified by our CYP2B6TM-RED and CPA combination.

The viral vectors used to achieve gene transfer may have a broad tropism and may therefore infect healthy tissue. An insufficient ability of vectors to target tumors has contributed to slow the development of cancer gene therapy. Researchers have therefore expended considerable effort to improve viral vector targeting, as discussed in this chapter. Moreover, the accumulation of knowledge about miRNAs has opened up a new field of gene regulation. Using miRNA properties to regulate transgene expression, and therefore targeting, in cancer gene therapy is both extremely elegant and quite simple. Future strategies should combine several targeting methods (Figure 5). Several groups have already constructed vectors characterized by a double targeting system consisting of specific promoters and miRNA. Today, the development of vectors characterized by both transductional and transcriptional targeting is within reach. It is reasonable to hope that safe vectors capable of specifically targeting cancer cells will be available soon and will open up new horizons for cancer gene therapy.

Last, new prodrugs with greater effectiveness are needed. Given that hypoxia is a common environmental feature in solid tumors, prodrugs specifically activated by hypoxia should be designed. For example, our previously described fusion gene expresses both CYP2B6 and RED catalytic activities, and we plan to use CPA treatment in combination with additional prodrugs known to be activated to cytotoxic metabolites under hypoxic conditions, such as AQ4N by CYP 2B6 or mitomycin C and tirapazamine by RED (J. M. Brown & Wang, 1998; Cavazzana-Calvo et al., 2010; Friery et al., 2000; McErlane et al., 2005).

Recent clinical trials confirmed the usefulness of cancer gene therapy and its potential for application in the clinical setting, as a substitute for conventional chemotherapy or, if the

result is only a decrease in tumor size, in combination with surgery and radiotherapy. We hope that the expected improvements in cancer gene therapy outlined above will further facilitate the use of this strategy for treating solid tumors.

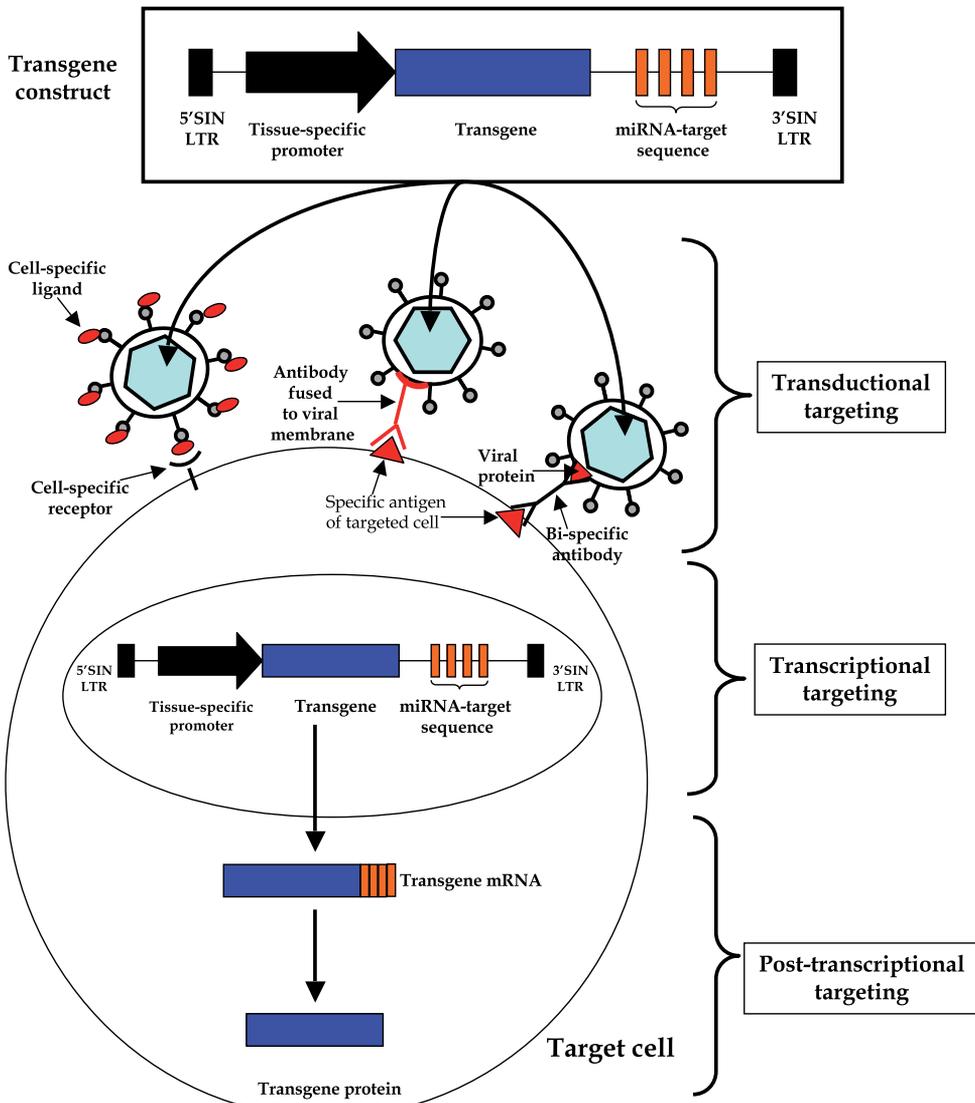


Fig. 5. Summary of various strategies for targeting lentiviral expression to cancer cells

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Epigenetic Therapies for Cancer

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1. Introduction

At the cellular level, cancers originate from the monoclonal expansion of a mutant cell leading to accumulation of aberrant cells that continue to lose differentiated features and acquire different biological properties in their progression toward disseminated or metastatic disease. The onset and progression of cancer involves genomic derangements that can be manifested in two ways: 1) Genetic and gross structural defects (e.g. single nucleotide polymorphism (SNP), classic deletion, insertion mutation, chromosomal deletion/inversion/translocation, allelic loss/gain, gene amplification/ deletion), and 2) Aberrant epigenetic covalent modifications (e.g. DNA methylation, histone acetylation, methylation, phosphorylation, citrullination, sumoylation, and ADP ribosylation).

Genomic instability can be triggered by chemical carcinogens, radiation, stress, oncogenic DNA viruses and the aging process. In almost all cancers, genomic instability in the form of genetic alterations or epigenetic modifications affects four classes of genes: oncogenes, tumor suppressor genes, apoptotic genes and/or DNA repair genes. Oncogenes encode proteins that function as positive proliferative signals for tumors. Tumor suppressor genes negatively regulate cell proliferation and are inactivated in many tumors. Apoptotic genes encode proteins that instruct the cell to commit suicide, while DNA repair genes encode proteins that maintain the fidelity of DNA sequences during transcription and replication. The uncontrolled expression of oncogenes or the silencing of tumor suppressor genes can lead to immortalization of cells. For example, in neuroblastoma, the overexpression of N-myc oncogene correlates with aggressive tumor behavior (Seeger et al., 1985). The ras oncogene is activated in more than half of the tumors studied in humans (Barbacid et al., 1987), and both relapse and decreased survival in breast cancer patients have been associated with overexpression of Her-2 oncogene (Slamon et al., 1987). The tumor suppressor and cell cycle regulator gene, p53, is mutated or deleted in more than 50% of human tumors (Hollstein M et al., 1991). p53 gene is described as the guardian of the genome because it can activate DNA repair genes when DNA is damaged, or induce apoptosis when DNA damage is sensed to be irreparable.

Despite the presence of defective genes in tumors, tumors actually arise through many different combinations of genetic alterations. The phenotypic diversity observed between normal and cancer cells cannot be explained simply by structural and genetic alterations. Epigenetic mechanisms have been shown to activate or inactivate genes. Conrad Waddington first coined the term epigenetic to mean changes above and beyond (epi) the primary DNA sequence (Waddington, 1939). The term epigenetic refers to heritable genetic

variations that give rise to distinct patterns of terminal differentiation phenotypes (Waddington, 1952; Ruden et al., 2005; Goldberg et al., 2007). These heritable variations are independent of the DNA sequence, can be reversible, and often are self-perpetuating (Bonasia et al 2010). Epigenetic modifications can include DNA methylation, histone acetylation, histone methylation, histone phosphorylation, citrullination, sumoylation, and ADP ribosylation. A classic example of epigenetic control is seen as cells undergo differentiation during development (Figure 1). While every cell in the human body has identical DNA sequence (except for T and B cells), epigenetic patterns lead the same cells to differentiate into a wide array of cell types and the formation of different tissues or organs (Figure 1). Also, animals cloned from the same donor DNA are not identical and develop diseases with different penetrance from the donor parent (Esteller, 2008; Rideout 3rd et al., 2001). The methylation patterns (Fraga et al., 2005b; Kaminsky et al., 2009), and histone modification profiles (Kaminsky et al., 2009), are different in monozygote twins, indicating that while epigenetic patterns are stable and heritable, they are also dynamic in the sense that genes can be silenced or activated due to changes in cellular environment (Figure 1). In normal cells, epigenetic patterns are in dynamic equilibrium (Szyf, 2007). Many diseases, of which cancer is no exception, arise when different types of epigenetic patterns are introduced at the wrong time, and/or the wrong place. For example, the hypermethylation of tumor suppressor genes, *t16^{INK4a}*, *p14^{ARF}* and *MGMT*, has been reported as an early event

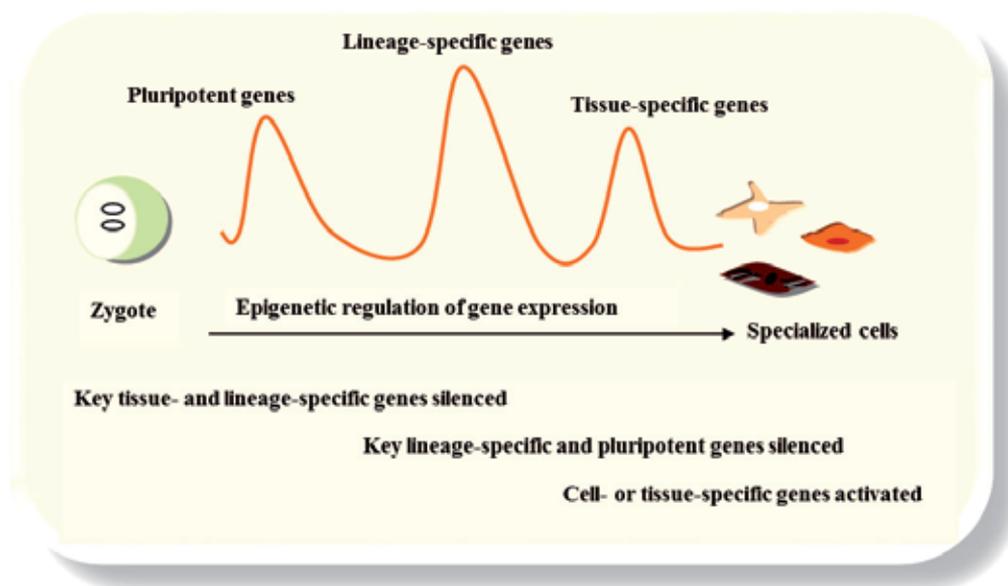


Fig. 1. Epigenetic changes leading to activation or silencing of genes during development. Depicted in the figure is the time dependent activation or inactivation of key genes during development (i.e. from zygote to specialized cells). While every cell has exactly the same DNA composition, the interplay between epigenetic modifications such as DNA methylation or histone modifications (e.g. histone methylation, acetylation, phosphorylation, ubiquitination and sumoylation) can lead to the expression or silencing of pluripotent genes, lineage specific genes or tissue specific genes during development leading to the formation of specialized cells or organs. Peaks depict activation and troughs denote silencing.

in tumorigenesis (Esteller et al., 2007). Because such epigenetic changes are reversible, the epigenome of cancer cells represents an ideal target for cancer treatments. Tumor suppressor genes turned off can be switched back on, and oncogenes turned on can be switched off to restore the epigenetic balance of cancer cells. How epigenetic modifications occur, and how epigenetic profiles define the genomic landscape of cancerous cells and their response to treatment will be the focus of this chapter.

2. Fundamentals of epigenetics

Epigenetic modifications can broadly be classified into three categories: DNA methylation, histone modification and nucleosome positioning. Epigenetic phenotypes result from the interplay between these three categories. Within the microenvironment of the cell, epigenetic patterns are established by transiently activated, and/or stably expressed factors that respond to environmental stimuli, developmental cues, or internal events. Of particular importance within the context of cancer, is the impact of exogenous substances on epigenetic modifications of gene regulation. Since the treatment of many cancers involves exposure to toxic substances, it is important to understand how some of these substances regulate epigenetic modifications. Hence the trajectory of cancer treatment is leaning toward a treatment regimen that includes epigenetic drugs capable of modifying the neoplastic phenotypes to induce shifts in phenotypic expression rather than cytotoxic alterations.

2.1 DNA methylation

DNA methylation was the first identified epigenetic regulation of gene expression in mammals (Holliday and Pugh, 1975; Riggs, 1975). It occurs by enzymatic transfer of a methyl group to carbon 5 of the pyrimidine base, cytosine, in the 5'-3' cytosine guanine (CpG) dinucleotide sequence. Mainly, S-adenosylmethionine (SAM) acts as methyl donor in this reaction. DNA methylation can be classified into four categories based on the region of the genome where methylation occurs. The first identified DNA methylation occurs exclusively in CpG dinucleotides. CpG dinucleotides normally cluster in regions of DNA called CpG islands. They are regions of DNA approximately 200-500 bases long with a G + C content greater than 50% and CpG to GC ratio of at least 0.6 (Bird, 1986; Gardiner-Garden and Frommer, 1987). CpG dinucleotides constitute 1% of the genome and are normally found in the promoter region of genes (Figures 2a and 2b). For example, the promoters of 50-70% of known human genes contain CpG dinucleotides (Bird et al., 1987; Larson et al., 1982; Wang and Lung, 2004). The second kind of DNA methylation is called gene body methylation (Hellman and Chess, 2007). This type of methylation occurs in the open reading frame of genes (Figure 2b) and functions to prevent spurious transcriptional initiation (Hellman and Chess, 2007), or alternative splicing of mainly ubiquitously expressed genes (Zilberman et al., 2007). Recently it has been suggested that gene body methylation also occurs at non CpG dinucleotides (i.e. CHG or CHH sites where H = A, C or T) (Lister et al., 2009; Laurent et al., 2010). A third kind of DNA methylation occurs at CpG island shores (Irizarry et al., 2009; Doi et al., 2009). The term CpG island shores refers to regions of the genome with lower density of CpG dinucleotides that lay approximately 2000 bases away from the CpG islands (Figure 2d). The CpG island shores determines the methylation pattern of tissues (Irizarry et al., 2009; Doi et al., 2009), and the reprogramming of stem cells (Doi et al., 2009; Ji et al., 2010). A fourth kind of DNA methylation occurs at repetitive sequences (e.g. transposable elements and microsatellite regions) (Figure 2e and 2f). The

transposable sequences constitute about 47% of the entire genome (Babushok and Kazazian, 2007). Examples of transposable elements are the DNA transposons (e.g. Mer1/2) and retrotransposons (e.g. non-terminal repeat retrotransposon, such as Lines and Sines and long terminal repeat retrotransposon, such as HERVs and IAPs). When activated, these repetitive sequences can move from one region of the genome to another through a so called “cut and paste” or “copy and paste” mechanisms, respectively. Microsatellites are tandem repeats of DNA embedded in various regions of the genome, and their methylation can result in either gene silencing or chromosome instability if it occurs in centromeric regions of the chromosome.

All four types of DNA methylation are catalyzed by a class of enzymes called DNA methyltransferases (DNMT). In mammals, there are five DNMT isoforms: DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L (Siedlecki and Zielenkiewicz, 2006). The catalytic domain is conserved between the DNMTs (except DNMT3L), while the regulatory domain responsible for a protein-protein interaction is variable. DNMT1 is a maintenance methyltransferase that ensures the pattern of DNA methylation is transferred from daughter to parent. DNMT1 and proliferating cell nuclear antigen (PCNA) colocalize to DNA replication foci in early S phase (Chuang et al., 1997). Loss of DNMT1 causes cell cycle arrest and apoptosis (Chen et al., 2007). DNMT2 has little DNA methylation activity and DNMT2 knockout mice display no aberrant DNA methylation patterns (Okano et al., 1998). Studies by Goll et al. (2006) have shown that the main function of DNMT2 is to methylate tRNA outside the nucleus. DNMT3a and DNMT3b are *de novo* methyltransferases responsible for establishing methylation patterns during early development. These *de novo* DNMTs are highly expressed in embryonic stem cells and their expression is downregulated after differentiation (Esteller et al., 2007). DNMT3L lacks the catalytic domain and is involved in establishing maternal genomic imprinting by acting as a stimulatory factor for DNMT3a and DNMT3b (Bourc'his et al., 2001). DNMT3L has been shown to interact and colocalize with both DNMT3a and DNMT3b in the nucleus (Chen et al 2005; Holt-Schietinger et al., 2010).

In general, DNA methylations (except gene body methylation) inhibit gene expression. DNA methylation at CpG islands, CpG island shores and repetitive sequence alters the conformation of the DNA in such a way that it prevents recruitment of transcription factors and positive regulators. In addition, methylated DNA promotes the recruitment of Methyl-CpG binding (MBD) proteins (Lopez-Serra and Esteller, 2008). Five classes of MBDs have been identified: MeCP2, MBD1, MBD2, MBD3, and MBD4. MBDs recruit histone deacetylases (HDACs) and histone methyltransferase (HMTs). HMTs methylate histone, with methylated histones being recognized and bound by heterochromatin complexes, such as heterochromatin protein 1 (HP1). These conditions combine to create a chromatin structure (heterochromatin) that favors transcriptional repression. Conversely, unmethylated DNA favors the formation of active chromatin (euchromatin). The formation of active chromatin results in recruitment of histone acetyltransferases and methyltransferases which create domains characterized by high levels of acetylation and trimethylation at H3K4, H3K36 and H3K79 leading to unwinding of chromatin and binding of transcriptional factors that lead to gene expression.

Compared to normal cells, cancer cells are characterized by global hypomethylation (overall 20-60 less CpG methylation) (Goel et al., 1985). Hypomethylation in cancer cells results in the induction of oncogenic genes, loss of imprinting, activation of transposable elements and microsatellite instability (Dunn, 2003; Esteller, 2008) (Figure 2). Hypomethylation at specific promoters can lead to aberrant expression of oncogenes and loss of imprinting (Figure 2a).

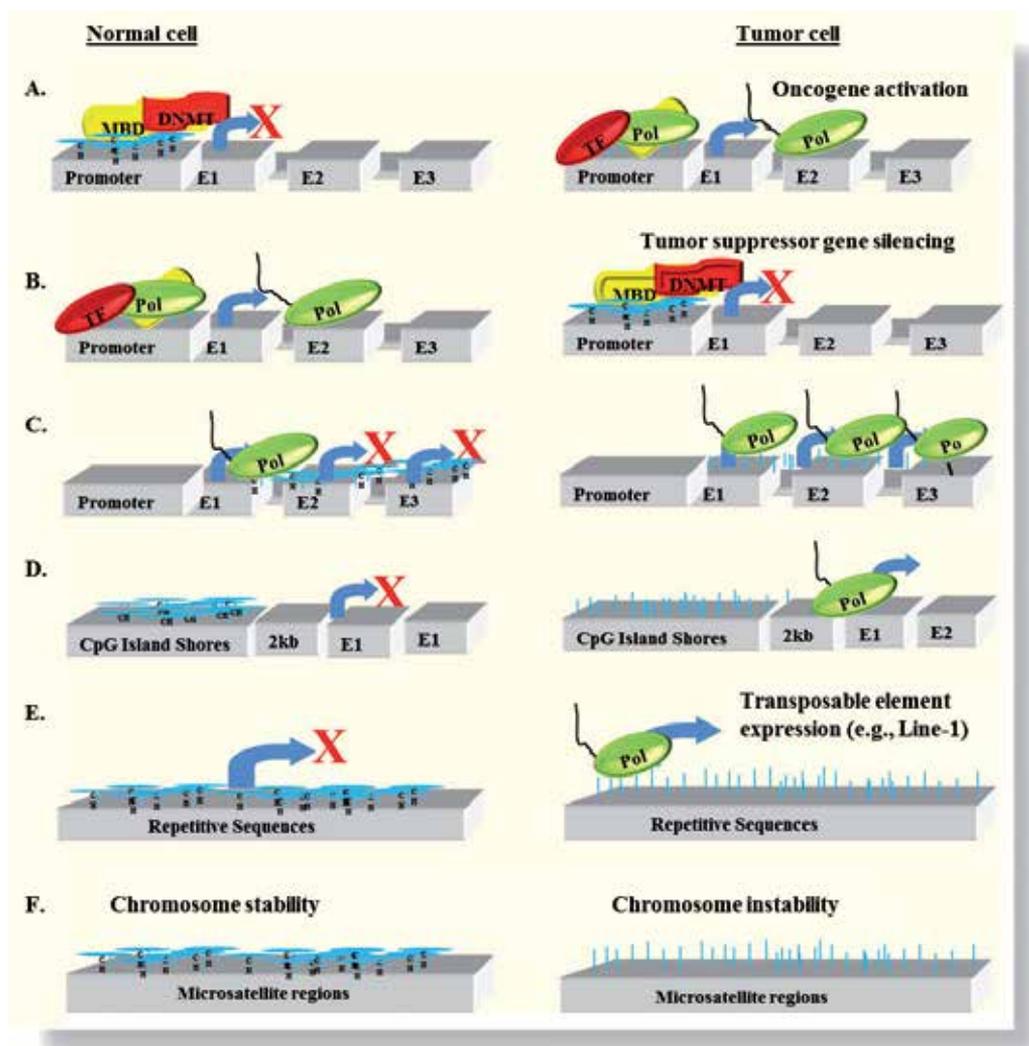


Fig. 2. Methylation patterns in normal versus cancer cells. A. Hypomethylation of oncogene leads to their activation in cancer cells. B. Hypermethylation at the promoter region of a tumor suppressor gene leads to their silencing in cancer cells. C. Gene body hypomethylation leads to spurious transcription initiation in ubiquitously expressed genes. D. Hypomethylation at CpG island shores leads to ubiquitous expression of key regulatory genes. E & F. Hypomethylation at repetitive or microsatellite sequences leads to expression of transposable elements and chromosomal instability, respectively.

Oncogenes such as S100P, SNCG, melanoma-associated gene (MAGE) and dipeptidyl peptidase 6 (DPP6) are hypomethylated in pancreatic cancer, breast cancer and melanomas, respectively (Wilson et al., 2007; Irizarry et al., 2009). This hypomethylation converts the expression of these genes into aberrantly expressed genes leading to increase growth and metastatic advantage. Loss of imprinting due to hypomethylation has been reported for insulin-like growth factor 2 (IGF2) gene in breast, liver, lung and colon cancers (Ito et al.,

2008). Repetitive sequences are hypomethylated and reactivated in many cancers (Goel et al., 1985; Gaudet et al., 2003; Futscher et al., 2004), as documented for lung, breast, bladder, and liver cancers (Wilson et al., 2007). Lastly, hypomethylation of microsatellite regions at the pericentromeric region leads to genomic instability (Kuismanen et al., 1999).

While the genomes of cancer cells are globally hypomethylated, some aberrant DNA methylation occurs at specific regions of the genome (Figure 2b). These aberrant methylations affect the expression of genes involved in cell cycle control (e.g. p53, Rb, p16^{INK4a}, p15^{INK4b}), apoptosis (TMSI, DAPKI, WIF-1 and SERP1) and DNA repair (e.g. BRAC1, WRN, MGMT, hMLH1). For example, hypermethylation of the CpG dinucleotide in the promoter region of the tumor suppressor gene p16 occurs in 20% of human cancers (Merlo et al., 1995). In addition, the guardian of the genome, p53, is epigenetically silenced in a large proportion of human cancers (Hollstein et al 1991; Jirtle, 1999, Jirtle and Skinner 2007; Jones and Baylin, 2004). Hypermethylation of the promoter region of *MASPIN* gene was reported as an early event in breast cancer (Futcher et al., 2004). Hypermethylation at CpG regions is also prone to spontaneous point mutations. Methylated cytosines can undergo spontaneous deamination and a subsequent conversion to uracil leading to C-T transition. This results in a rate of mutation at methylated CpG regions that is 42 times higher than predicted for random mutation (Cooper and Youssoufian, 1998). Point mutation (C-T) is frequently seen in p53 and Rb genes leading to loss of function of these proteins (Cooper and Youssoufian, 1988; Magewu and Jone, 1994; Tornaletti and Pfeifer, 1995; Manici et al., 1997). In addition, CpG sites are favored binding sites for carcinogens which also lead to increase rates of CpG mutations (Magewu and Jone, 1994; Yoon et al., 2001).

The question of why some regions of the genome are hypermethylated and others hypomethylated in cancer is poorly understood. However, most of the hypomethylated regions in tumors lie outside the so called CpG islands. In normal cells, these non CpG regions are methylated. Also the patterns of hypermethylation are tumor-specific leading to the idea that selection pressures in favor of growth for clonal cells might lead to different patterns of methylation. Another explanation for the site-specific hypermethylation could be due to recruitment of DNMTs by accessory proteins in cancer cells. The identity of these accessory proteins may be unique to the cancer cell phenotype, and also exhibit specificity for different cancer subtypes. Finally, epigenetic profiles in cancer may involve dysregulation of DNMT expression. This suggestion is consistent with the finding that DNMT1 and DNMT3b are overexpressed in many tumors (Miremedi et al., 2007). In addition, many tumors are characterized by downregulation of miRNAs due to methylation at their promoters (Saito et al., 2006; Melo et al., 2009). Conversely, miRNAs have also been shown to target DNMTs. In fact, miR-29 has been shown to target and downregulate both DNMT3a and DNMT3b, and indirectly DNMT3L (Garzon et al., 2009). Compared to normal cells, it is possible that regulation of DNMTs by miRNAs is dysregulated in cancer cells resulting in unique patterns of DNMT expression.

2.2 Nucleosome positioning

Total eukaryotic DNA is 2m long and must fit into the nucleus which has an approximate size of 2 μ m³. This is accomplished by an elaborate interaction between DNA, four core histone proteins (i.e. H2A, H2B, H3 and H4) and one linker histone. The core histone proteins are arranged into two sets of H3/H4 and H2A/H2B heterodimers. This arrangement forms into an octamer shaped structure, called the nucleosome, around which ~146bp of DNA is wrapped (Kornberg 1974). The interaction between the nucleosome and

DNA is facilitated by the positively charged amino acids of the histone proteins and the negatively charged phosphate backbone of the DNA. Nucleosomes are separated from each other by 20-100 bp linker regions. The linker region is bound by another histone protein called histone 1 (H1). H1 and its variants function to promote the coiling of nucleosomes into fiber like structures in cells (Bedner et al., 1998). Linker histones are distinguished from other histones because of special modifications at key amino acids, or in their tail regions or domain structures (Li et al., 2007). Nucleosomes, DNA and linker histones are packaged to form chromatin. The conformation of chromatin is determined by the positioning of the nucleosome and its level of modification. For example, loss of nucleosomes at the transcription start site is directly correlated to gene expression (Figure 3), and occlusion of the nucleosome at transcription start sites is correlated with transcriptional repression (Schomes et al., 2008; Cairns et al., 2009). At this level, nucleosomes act as barriers to transcription because they block access to binding sites for transcription factors and regulators (Figure 3), or block elongation by sterically hindering the movement of RNA polymerase II. The precise position of the nucleosome is influenced by linker histones (Zilberman et al., 2007), and chromatin remodeling complexes (Clapier et al., 2009). Incorporation of different histone variants can influence transcription and the methylation landscape in eukaryotic cells (Chodavarapu et al., 2010). For example, incorporation of histone linker H2A.Z protects genes against DNA methylation (Zilberman et al., 2008), thereby playing a role in epigenetic activation of transcription.

The chromatin remodeling complexes are classified into four families: mating type switch/sucrose non-fermenting (SWI/SNF) family, chromodomain helicase DNA-binding (CHD) family, Imitation SWItch (ISWI) family and Inositol/choline responsive element dependent gene activation mutant-80 (INO80) family. These four families are distinguished by unique features within their catalytic subunits that allow them to read specific histone post-translational modifications that stabilize their interaction with chromatin. They also differ in the composition of the other subunits (Ho and Crabtree, 2010). Chromatin remodeling complexes function by moving, ejecting, destabilizing or restructuring the nucleosome in an ATP dependent manner. The remodeling machinery is also influenced by both DNA methylation (Harikrisnan et al., 2005), and histone modifications (Wysoca et al., 2006).

Mammalian SWI/SNF enzymes are multisubunit complexes of 1–2MDa and consist of 9–12 subunits, one of which is an ATPase (De la Serna et al., 2006). The ATPase subunit is identified as either Brahma (BRM) or brama/swi2-related gene-1 (BRGI) which have been recognized as human homologs. The BRGI subunit is 75% identical between SWI/SNF family members. Functionally, SWI/SNFs are master regulators of gene expression. For example, SWI/SNFs are involved in regulating the expression of FOS, CRYAB, MIM-1, p21 and CSF-1. These complexes are also involved in alternative splicing (Reisman et al., 2009).

The CHD family of chromatin remodeling complexes is distinguished by having two chromodomains that have affinity for methylated histones (Marfella et al., 2007). There are nine CHD proteins that can be divided into three subfamilies based on the presence of other conserved domains and interacting factors: I. (CHD1 and 2), II. (CHD3 and 4), III. (CHD 5–9). Some CHD family members are involved in the sliding and ejection of the nucleosome thereby promoting transcriptional activation. Others like Mi-2/NuRD have HDAC activity and can also act as methyl binding protein (Clapier et al., 2009). These family members at this role act as transcriptional repressors.

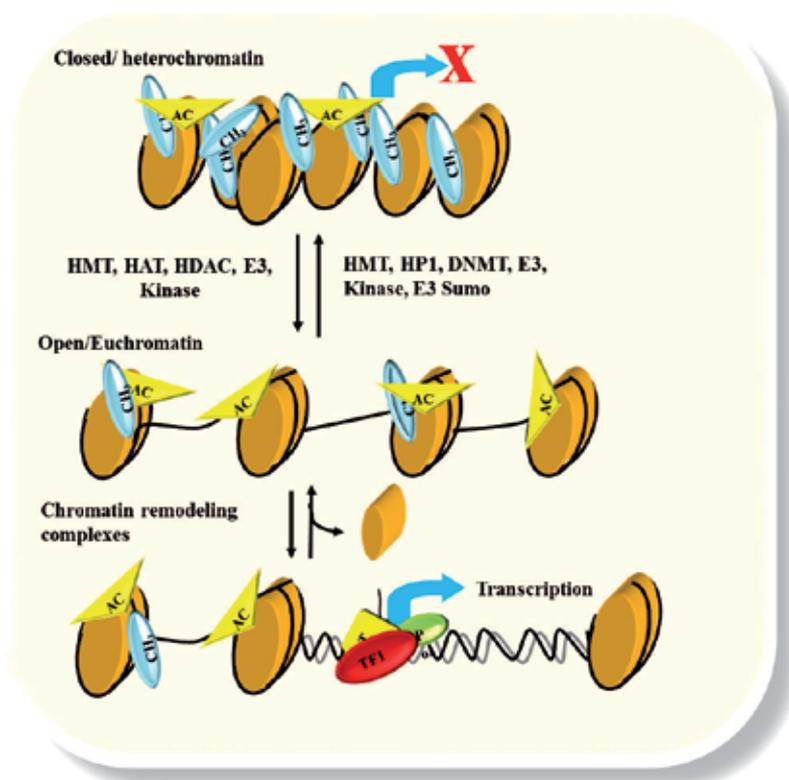


Fig. 3. Chromatin exists in a transcriptionally repressed (heterochromatin) or transcriptionally active (Euchromatin) states. Epigenetic modifications such as DNA methylation, histone methylation, phosphorylation, sumoylation and ubiquitination favor the formation of heterochromatin and lead to transcriptional repression. Conversely, histone acetylation, methylation, phosphorylation and ubiquitination favor the formation of euchromatin and transcriptional activation. Note that epigenetic modifications, such as histone methylation, phosphorylation and ubiquitination, favor both the formation of euchromatin and heterochromatin. This depends on the composition of trans-acting factors, cross talk between histone marks, microdomain created by the histone marks and the chromatin remodeling complexes. Additional details can be found in the text. HMT: Histone methyltransferase, HAT: Histone acetyltransferase; DNMT: DNA methyltransferase; E3: E3 ubiquitin ligase; E3 Sumo: E3 sumo ligase; HP1: heterochromatin protein 1; TF: transcription factor; Pol: Polymerase; Ac: Acetylation mark; CH₃: Methylation mark.

Imitation SWItch (ISWI) complexes were initially identified and purified from *Drosophila* embryo extracts as NUCleosome Remodeling Factor (NURF), ATP dependent Chromatin assembly and remodeling Factor (ACF), and CHRomatin Accessibility Complex (CHRAC) complexes. Currently ISWI complexes have been found in a variety of organisms from yeast to humans (Tsukiyama and Wu, 1995; Tsukiyama et al., 1995; Ito et al., 1997; Varga-Weisz et al., 1997). The ISWI family members such as ACT and CHRAC are involved in promoting chromatin assembly, thus acting as transcriptional repressors. However, the NURF complex has been shown to activate RNA polymerase II, thus acting as a transcriptional activator.

INO80 was identified as a gene encoding an ATPase that is incorporated into a large multisubunit complex (Ebbert et al., 1999). Biochemical characterization of the yeast INO80 complex has revealed the presence of chromatin remodeling activity and 3'-5' helicase activity (Shen et al., 2000). Yeast cells that lack either INO80 or one of its core subunits (Arp5 and Arp8) exhibit hypersensitivity to DNA damaging agents, suggesting that the INO80 complex is involved in DNA repair (Morrison et al., 2004; van Attikum et al., 2004). In accord with these findings, the INO80 complex is recruited to sites of double strand DNA breaks through interaction with phosphorylated H2A. From these findings and others, members of the INO80 family are believed to be involved in DNA repair, chromosomal segregation, DNA replication, telomere regulation and transcriptional activation (Ho et al., 2010). However, SWRI functions to restructure the nucleosome by removing the H2A-H2B dimers and replacing them with H2A.Z-H2B dimers (Clapier et al., 2009).

All four families of chromatin remodeling complexes are believed to be involved in tumorigenesis. For example, the BRG1 subunit of the SWI/SNF families has been characterized as a tumor suppressor, and is silenced in about 20% of non-small lung cancer (Medina et al., 2008). BRG1 subunit also functions to destabilize p53 hence acting as a tumor suppressor (Naidu et al., 2009). SWI/SNF complexes have been shown to interact with oncogenes, such as Rb, Myc, p53, breast cancer 1 (BRCA1) and myeloid/lymphoid or mixed-lineage leukemia (MLL) (Roberts et al., 2004). Point mutations in the SNF subunit of the SWI/SNF complex have been implicated in renal tumors, choroid plexus carcinomas, medulloblastoma and neuroectodermal tumors (Robert et al., 2004). In colon cancer, promoter hypermethylation of the *MLH1* gene results in occlusion of the transcription start site by nucleosomes (Lin et al., 2007). The CHD5 complexes are targets of CpG hypermethylation (Mulero-Navarro and Esteller, 2008) resulting in downregulation of these complexes. In addition, crosstalk between chromatin remodeling complexes and histone modifications is vital to transcription regulation and tumorigenesis. For example, members of the CHD family are components of the NRD deacetylating and the SAGA acetyltransferase complexes (Tong et al., 1998). In accord with these findings, The H3K4 methyltransferase MLL has been shown to interact with SNF5/BRG1-associated factors (BAF) 47 (Rozenblatt-Rosen et al., 1998), and H3K4 methylation has been shown to recruit and mediate association of ISWI with chromatin to initiate transcription (Santos-Rosa et al., 2003). Acetylation of H3K56 leads to recruitment of SWI/SNF complexes (Xu et al., 2005). Linker histones have also been shown to play a role in tumorigenesis, with increased expression of linker histone macroH2A in senescent lung cells (Sporn et al., 2009), indicating that lung tumors with high expression of macroH2A have a better prognosis.

2.3 Histone modifications

As noted, chromatin is made of DNA and its associated proteins which are in turn classified into histone and non-histone proteins. Non-histone proteins transiently interact with the DNA to regulate function after which they dropout or are removed. For example, non-histone proteins transiently interact with DNA during transcription, replication, and DNA repair mechanisms. Such proteins include polymerases, co-activators, co-repressors, chromatin remodeling complexes, structural proteins and histone like proteins (e.g. CENPS). On the other hand, histone proteins stably interact with DNA except during transcription, replication or DNA repair during which they are temporally displaced from the DNA as nucleosomes. Each histone can be divided into three segments: a basic N-terminal histone tail, a globular histone fold and a C-terminal tail. The conformation of chromatin is

dependent on posttranslational modifications at the tails of all histones which normally protrude from the nucleosome. Posttranslational modifications function by contouring the secondary structures of chromatin so as to allow or disallow accessibility to transcription and regulation sites. Posttranslational modification on histone can be classified as transient or stable. Transient posttranslational modifications include: phosphorylation, sumoylation and ubiquitination, and represent modifications that correlate with transient changes in gene regulation. Acetylation and methylation are fairly stable modifications that reflect the conformation of chromatin (e.g. closed/heterochromatin and open/euchromatin).

2.3.1 Histone acetylation

All histones are subjected to acetylation, and the process is catalyzed by histone acetyltransferase (HAT) complexes (Allfrey et al., 1964). Histone acetylation involves the transfer of acetyl groups from acetyl Coenzyme A to the imino group of lysine. HATs do not acetylate lysine moieties on histone randomly, a potential recognition motif (GKxxP) was revealed by crystal structure analysis (Rojas et al., 1999; Bannister et al., 2000). However, this motif is not a predictor of non-histone protein acetylation as a proteomic survey has identified different sets of preferentially acetylated amino acid stretches (Kim et al., 2006). Functionally, histone acetylation induces the so called open/euchromatin conformation via steric hinderance, changes in the positive charges of histones (Hong et al., 1993), and/or recruitment of regulatory proteins (Grant and Berger, 1999; Roth et al., 2001). The formation of open chromatin favors transcriptional activation. At the same time, the acetyl group of histones can be removed by histone deacetylase complexes (HDACs) which favors formation of closed chromatin and transcriptional repression (Yang and Seto, 2003). HATs are categorized into two groups based on their cellular localization. Type-A HATs are nuclear and acetylate histones and other chromatin-associated proteins. Type-B HATs are localized in the cytoplasm and have no direct influence on transcription. The latter are believed to function mainly to acetylate newly synthesized histones in the cytoplasm. Type-A HAT are further categorized into three groups: GNAT [GCN5 (general control of nuclear-5)-related N-acetyltransferase], p300/CBP [CREB (cAMP response element binding protein)-binding protein] and MYST [MOZ (Monocytic leukaemia zinc-finger protein), YBF2 (Yeast binding factor2)/SAS (something silencing), Tip60 (Tat interactive protein -60)] (Table 1). In addition some transcription factors and nuclear receptor coactivators have been shown to acetylate histones. For example, the nuclear receptor coactivator, Amplified in breast cancer-1 (AIB1), and transcription factor, TATA-box binding protein associated factor-250 (TAF250). Both of these proteins have been shown to acetylate histones H3 and H4.

The mechanism by which HAT complexes are recruited to the chromatin to acetylate histones is poorly understood. One possible mechanism involves the recruitment of HAT proteins as part of coactivator complexes. For example, p300/CBP HAT complexes are found to associate with polymerase II during transcription (Nakajima et al., 1997). Another plausible mechanism is through association with bromodomains containing proteins which recognize and bind to acetylated lysines (Dhalluin et al., 1999; Mujtaba et al., 2002). For example, the SWI/SNF complexes are recruited to the chromatin via their bromodomains (Hassan et al., 2002). In this case the HAT families of proteins are believed to complex with SWI/SNF complexes to acetylate histones.

The primary site for histone acetylation is the tail region which generally protrudes from the nucleosome. For example, core histones H3 and H4 can be acetylated at lysines 9, 14, 18, 23 and 5, 8, 12, 16 respectively (Roth et al., 2001) (Table 1). Histone acetylation can occur at

different sites giving rise to the possibility of functional crosstalk between different acetylation marks. In fact, communication is known to occur between the same histone marks (Wang et al., 2008), between marks within the same histone tail (Duan et al., 2008), or between marks in different histone tails (Nakanishi et al., 2008). These data suggest that a single acetylation mark does not determine the state of the chromatin. Indeed, the notion of a strictly closed and open chromatin conformation has been challenged by a recent study showing that up to 51 chromatin states are possible based on the level or combination of acetylation marks (Ernst et al., 2010). For example, an active H3K4me mark and a repressive H3K27me mark have been found to co-exist in embryonic stem cells, suggesting a chromatin state that is neither open nor closed (Bernstein et al., 2006; Mikkelsen et al., 2007).

HATs	Histone Substrates
GNAT family <ul style="list-style-type: none"> • GCN5 • PCAF 	H2B, H3-K9/14/23/27, H4-K8/16 H3-K14, H4-K8
P300/CBP family <ul style="list-style-type: none"> • p300 • CBP 	H2A-K5, H2B-K12/15/20, H3-K14/18/23, H4-K5/8/12 H2A-K5, H2B-K12/15/20, H3-K14/18/23, H4-K5/8
MYST family <ul style="list-style-type: none"> • Tip60 • MOZ 	H2A-K5, H3-K14, H4-K5/8/12 H3, H4

Table 1. HATs families and substrate specifications

As noted earlier, acetylation is a reversible process and the removal of acetyl groups is catalyzed by a class of enzymes called histone deacetylases (HDACs). Histone deacetylation is correlated with transcriptional repression. In humans, 18 isoenzymes of HDACs have been identified to date, and grouped into four classes based on their homology to yeast HDACs (Table 2). Class I (HDAC1, 2, 3 and 8) are related to yeast *RPD3* gene and are mostly located in the nuclei, except HDACs 3 and 8 which can also be cytoplasmic. Class II (HDAC 4, 5, 6, 7, 9 and 10) are related to yeast *Hda1* gene and are primarily located in the cytoplasm, but can shuttle to nucleus. Class II HDACs are further divided into two subclasses, IIa (HDAC 4, 5, 7, 9) and IIb (HDAC 6, 10), based on their sequence homology and domain organization. Class III, also known as the sirtuins (SIRTIUNS (SIRT) 1–7), are related to the yeast *Sir2* gene, and are virtually unaffected by class I and II HDAC inhibitors. They are localized in the cytoplasm, mitochondria and nucleus (Table 2). Class IV (HDAC11) has a conserved domain similar to the catalytic region of Class I HDACs. Class IV is a fairly new class and needs further characterization. Classes I, II and IV share similar structural organization and a common cofactor, Zn^{2+} . Class III HDACs are structurally different from the rest and their active site is occupied by the nicotinamide adenine dinucleotide (NAD). Functionally, class II HDACs are regulated by class I HDACs and together class I and II are involved in transcriptional silencing and genomic organization during development. Class III HDACs are involved in maintenance of acetylation, as well as specific gene silencing (Denu et al., 2003).

HDAC classes	Cofactor	Localization
I		
<ul style="list-style-type: none"> • HDAC 1 & 2 • HDAC 3 & 8 	Zn ²⁺	Nucleus
	Zn ²⁺	Nucleus/cytoplasm
IIa		
<ul style="list-style-type: none"> • HDAC 4, 5, 7 & 9 	Zn ²⁺	Nucleus/cytoplasm
IIb		
<ul style="list-style-type: none"> • HDAC 6 & 10 	Zn ²⁺	Nucleus/cytoplasm
III		
<ul style="list-style-type: none"> • SIRT 1, & 2 • SIRT 3 • SIRT 4 & 5 • SIRT 6 & 7 	NAD ⁺	Nucleus/cytoplasm
	NAD ⁺	Mitochondria/Nucleus
	NAD ⁺	Mitochondria
	NAD ⁺	Nucleus
IV		
<ul style="list-style-type: none"> • HDAC 11 	Zn ²⁺	Nucleus

NAD: Nicotinamide adenine dinucleotide; SIRT: Sirtuin; Zn: Zinc

Table 2. Classes of HDACs, Cofactors and Subcellular Localization

Compared to normal cells, the interplay between acetylation and deacetylation is dysregulated in almost all cancers. HDACs have been shown to be overexpressed or mutated in many cancers (Zhu et al., 2004; Ropero et al., 2006). Under normal conditions, HDACs associate with and regulate transcription factors, tumor suppressor genes and oncogenes. HDAC1 has been shown to be in a complex with Rb and to suppress the transcriptional activation of E2F (Brehm et al., 1998). Also SIRT1 has been shown to regulate the inflammatory, stress, and survival responses of p53 (Vaziri et al., 2001). Thus, it stands to reason that loss of HDAC1 or SIRT1 could result in uncontrollable growth of cell leading to transformation. In accord with this interpretation, SIRT1 has been shown to interact with DNMT1 to affect DNA methylation patterns (Espada et al., 2007). In acute myelogenous leukemia (AML), fusion of eight-twenty-one zinc-finger nuclear protein (ETO) to AML, a transcription activator, converts the AML protein into a dominant transcriptional repressor. The AML-ETO fusion protein permanently binds to HDAC corepressor complexes and/or blocks the recruitment of coactivator complexes leading to transformation (Scandura et al., 2002). HDAC2 overexpression is a hallmark of familial-adenomatosis-polyposis-induced tumors (Zhu et al., 2004) and truncation of HDAC 2 is reported in sporadic tumors (Ropero et al., 2006). In addition, HDAC6 expression is correlated with better prognosis for breast cancer patients (Zhang et al., 2004), suggesting aberrant acetylation in breast tumors. In addition to aberrant levels of HDACs, several cancers also bear aberrant fusion proteins, mutations, or deletion of HATs and HAT related genes (Bryan et al., 2002; Moore et al., 2004). For example, in cancer cells there is global reduction in monoacetylation at H4K16 (Fraga et al., 2005a), and HATs, such as p300 and CBP, are characterized as tumor suppressor genes since they can regulate the activity of oncoproteins, such as Jun, Fos, Myb and Rb (Yang et al., 2004). Likewise, p300 has been shown to be an interacting partner of the oncoprotein adenovirus E1A (Stein et al., 1999). CBP mutations have been shown to be involved in the initial steps of leukaemogenesis (Patrij et al., 1995). For the MYST family of HATs, mutations leading to loss of Tip60 acetyltransferase activity lead to apoptosis-resistant phenotypes and rampant cell proliferation (Ikura et al., 2000). In AML, a chimera

protein is created by fusion of CPB to MOZ protein creating a protein with both p300/CBP and MYST domains (Yang et al., 2004; Borrow et al., 1996). The CBP-MOZ chimera protein exhibits gain-of-function characteristics leading to hyperacetylation and aberrant transcriptional activation. This leads to global imbalance of histone acetylation in cancer cells. Table 3 summarizes predicted impacts of acetylation and deacetylation in cancer.

Gene	Histone Mark	Genetic Defect	Tumor Type	Function	Reference
Histone acetyltransferases (HATs)					
CBP (KAT3A)	H2AK5, H2BK12, H2BK15, H3K14, H3K18, H4K5, H4K8	Deletion	ALL; lung	Loss	Shigeno et al., 2004 & Kishimoto et al., 2005
p300 (KAT3B)	H2AK5, H2BK12, H2BK15	Deletion	cervix; ALL	Loss	Ohshima et al., 2001 & Shigeno et al., 2004
p300 (KAT3B)	H2AK5, H2BK12, H2BK15	Mutation	Breast; CRC	Loss	Gayther et al. 2000
p300 (KAT3B)	H2AK5, H2BK12, H2BK15	Translocation	AML	Loss	Ida et al., 1997 & Chaffanet et al., 2000
MOZ (KAT6A)	H3K14; H4K16	Translocation	AML	Loss	Chaffanet et al., 2000 & Panagopoulos et al., 2003
Histone deacetylases (HDACs)					
HDAC2	Many acetyl residues (except H4K16)	Mutation	MSI+	Loss	Ropero et al., 2006 & Hanigan et al., 2008

ALL: Acute Lymphoblastic Leukemia; AML: Acute Myeloid Leukemia; CRC: Colorectal cancer; MSI+: Colorectal cancer with microsatellite instability.

Table 3. Aberrant Acetylation and Deacetylation Marks in Cancer

2.3.2 Histone methylations

Histone methylation occurs at either arginine or lysine residues in the tails of all histones. Two classes of enzymes catalyze the addition of methyl groups to histones: those that catalyze the addition of methyl group to arginine residues, called protein arginine methyltransferase (RHMT), and those that catalyze the addition of methyl groups to lysine residues, called histone lysine methyltransferase (HKMTs). S-adenosyl methionine (SAM) donates the methyl group in both cases of histone methylation (Pluemsampant et al., 2008). Histone methylation at arginine residues occurs at mono- or di-methylated states, while methylation at lysine residues occurs at mono-, di, and tri-methylated states. A total of 24 arginine and lysine methylation sites has been identified in all the core histones so far.

While DNA methylation generally represses transcription (except for gene body methylation), the functional impact of histone methylation is highly dependent on cellular context (Jenuwein and Allis, 2001). For example, histone methylation has been shown to cause both transcriptional activation and repression (Table 4). An emerging theme for these dual roles is explained by the fact that histone methylation creates motifs or domains that are recognized and bound by different proteins. The composition of these protein complexes

determines whether the modification results in gene activation or repression. Another explanation for this phenomenon is that histone modifier genes and regulatory genes have a tissue-specific expression pattern. The composition and recruitment of tissue specific proteins determines transcriptional states. Also, there is crosstalk between histone methylation and other epigenetic modifications. For example, several histone methyltransferases have been shown to direct DNA methylation (Tachibana et al., 2008; Zhao et al., 2009) by recruiting DNMTs. DNMT3L specifically interacts with H3 tails and induces recruitment of DNMT3a, however this interaction is strongly inhibited by H3K4me (Ooi et al., 2007). Thus, the transcription state of the chromatin is also an interplay between different epigenetic modifications. In addition, the domain composition of HMTs is a determining factor of the transcriptional state. Most HMTs contain a conserved SET (Suppressor of variegation 3-9), Enhancer or zeste, Trithorax) domain which in combination with other domains/complexes has the ability to confer either transcriptional activation or repression. SET domain containing proteins are divided into five families: SET1, SET2, SUV39, RIZ (retinoblastoma protein interacting zinc-finger) and SMYD3 (SET and MYND-domain containing protein 3) (Table 4). The mixed lineage leukemia (MLL), SMYD3, Nuclear receptor-binding SET domain protein-1 (NSD1) and CARM1 are HMTs that activate transcription. For example, MLL-specific methylation at H3K4 is followed by recruitment of bromo domain-containing trithorax complexes which result in formation of an open chromatin (Milne et al., 2002). SMYD3 in complex with RNA polymerase II and HELZ, a helicase, is recruited to the promoters of target genes where it methylates H3K4 leading to

Enzymatic Activity	Histone/Lysine	Biological Impact on Transcription
Lysine Histone Methyltransferase (KHMT)		
SET 1 family		
• SET1	H3K4	Activation
• EZH2	H3K27	Repression
• MLL 1 & 2	H3K4	Activation
SET 2 family		
• NSD1	H2K36	Activation
SUV39 family		
• SUV39H 1 & 2	H3K9 H4K20	Repression Repression
RIZ family		
• RIZ1	H3K9	Repression
SMYD3 family		
• SMYD3	H3K4	Activation
Arginine Histone Methyltransferase (RHMT)		
• CARM1	H3R2	Repression
• PRMT 5	H3R17	Activation
	H2A/H4	Repression

Note that only a few of the HMTs are cited to illustrate their role as transcriptional activation or repression.

Table 4. Histone Methylation at Specific Lysine and Arginine Residues

transcriptional activation. NSD1 methylation at H3K36 is responsible for activation of Hox genes (Wang et al., 2007). CARM1 methylation at H3R17 has also been shown to activate transcription by complexing with hormone receptor co-activator complexes (Hong et al., 2004). On the other hand, SUV39H1, EZH2, RIZ1 and PRMT5 participate in transcriptional repression. For example, EZH2 methylation at H3K27 leads to recruitment of chromo domain-containing polycomb complexes resulting in silencing of homeotic genes (Valk-Lingbeek et al., 2004). SUV39h1 trimethylation at H3K9 of the promoters of cell cycle control genes leads to recruitment of chromo domain-containing heterochromatin protein 1 (HP1) which in turn leads to transcriptional repression (Bannister et al., 2001; Lachner et al., 2001). RIZ1 methylation at H3K9 of the promoters of cell cycle control genes leads to apoptosis in breast cancer cells (He et al., 1998). Finally, methylation at H2A/H3 by PRMT negatively regulates cyclin E transcription resulting in cell cycle arrest (Fabbri et al., 2002). Table 4 summarizes the influence of histone methylation on transcription.

As described for histone acetylation, histone methylation is also reversible. Two classes of enzymes are responsible for removing methyl groups from histones: lysine-specific methyltransferase (KMAT) and arginine-specific methyltransferase (RMAT). KMAT includes amine oxidase domain-containing demethylases (Forneris et al., 2005) and Jumonji C (JmjC) domain containing demethylases (Tsukada et al., 2006) while RMAT includes peptidylarginine deiminase 4 (PAD4) (Cuthbert et al., 2004; Wang et al., 2004). PAD4 is the only RMAT known so far and it does not convert methylarginine to arginine, instead it converts methylarginine to citrulline. Citrullination is described as yet a unique histone modification. Several KMAT exist and the first one discovered is the lysine-specific

Gene Name	Histone Mark	Genetic Defect	Tumor Type	Function	Reference
Histone Methyltransferases (HMTs)					
DOT1L (KMT4)	H3K79	Aranslocation	AML	Loss	Okada et al., 2005
EZH2 (KMT6)	H3K27	Amplification	Prostate	Gain	Bracken et al., 2003
EZH2 (KMT6)	H3K27	Mutation	Lymphoma	Loss	Morin et al., 2010
NSD3	H3K4, H3K27	Amplification	Breast	Gain	Angrand et al., 2001
RIZ1 (KMT8)	H3K9	CpG hypermethylation	Breast, Liver	Loss	Du et al., 2001
SMYD2 (KMT3C)	H3K36	Amplification	ESCS	Gain	Komatsu et al., 2009 & Li et al., 2007
SUZ12 (HMT complex)	H3K9, H3K27	Translocation	ESS	Loss	Panagopoulos et al., 2008
Histone demethylase (HDMTs)					
LSD1	H3K4, H3K9	Amplification	Prostate, Lung, Bladder	Gain	Kahl et al., 2006 & Hayami et al., 2010
UTX	H3K4, H3K9	Mutation	Multiple cancers	Loss	Van Haften et al., 2009
GASC	H3K9, H3K36	Amplification	Breast, Lung	Gain	Cloos et al., 2006 & Italiano et al., 2006 & Liu et al., 2009

Table 5. Aberrant histone methylation and demethylation in cancer

demethylase-1 (LSD1). LSD1 is a typical H3K4 demethylase, but can change its substrate specificity when in complex with different accessory proteins. For example, LSD1 in complex with androgen receptor is able to demethylate H3K9 (Mwtzger et al., 2005). LSD1 is also able to synergistically work with HDACs and HATs to affect transcription of target genes. For example, over expression of LSD1 in HEK293 cells leads to decreased H3K4 methylation which is in turn followed by H3 deacetylation and transcriptional repression (Lee et al., 2006). At the same time, inhibiting HDAC1 activity increases both H3 acetylation and H3K4 methylation (Lee et al., 2006).

In cancer, there is aberrant expression and composition of histone-modifier and -regulator genes. For example, silencing of the nuclear receptor SET domain protein I (NSD1) results in decreased H3K36 and H4K20 methylation, which is believed to play a role in tumors of the nervous system (Berdasco et al., 2009). CpG Island hypermethylation by the histone methyltransferase ,RIZ1, has been described in many cancers (Du et al., 2001). Suppressor of the zeste 12 homolog (SUZ12) which is a component of the PCR2/EED/EZH2 complex that methylates H3K9 and H3K27 is involved in cell proliferation and survival in tumors (Li et al., 2007). In leukemias, the presence of mixed lineage leukemia (MLL) fusion oncoproteins leads to aberrant patterns of H3K79 and H3K4 methylation and altered gene expression in these tumors (Krivtsov et al., 2008; Wang et al., 2009). Some histone demethylases have also been found to be overexpressed in prostate cancer and squamous carcinomas (Shi et al., 2007). For example, LSD1 overexpression is a predictive biomarker for prostate cancer (Kahl et al., 2006)

2.3.3 Histone phosphorylation

Histone phosphorylation is described as the addition of a phosphate group (PO_3^-) to histone. So far, a small number of kinases has been shown to phosphorylate histones, and these include protein kinase B (PKB/AKT), ribosomal S6 kinase-2 (Rsk-2), mitogen- and stress-activated protein kinases 1 and 2 (Msk1/2), mixed lineage triple kinase-alpha (MLTK- α), and aurora kinases. The most interesting of the histone kinases are the aurora kinases. In normal cells, aurora kinases are involved in chromosomal segregation, condensation and orientation (Katayama et al., 2003). For example, aurora-phosphorylates both H3S10 and H3S28 during mitosis and meiosis (Andrews et al., 2003). Aurora kinases are serine/threonine kinases that include auroras A, B and C. These proteins share similar carboxyl terminal catalytic domains, but divergent amino terminals of variable length. In cancer cells, aurora kinases are frequently overexpressed and their overexpression is implicated in oncogenic transformation, as evidenced by chromosomal instability and derangement of multiple tumor suppressor and oncoprotein-regulated pathways. The mechanism through which these kinases are activated is dependent on the microenvironment of the cell. These mechanisms include, but are not limited to, activation by mitogens, cytokines, stress, signaling pathways (e.g. Ras-mitogen-activated protein kinase pathway (MAPK)) and chemical and environmental toxicants.

Functionally, histone phosphorylation alone, or when synergistically-coupled to other histone modifications (e.g. acetylation and methylation), can either facilitate or repress transcription (Cheung et al., 2000). This dichotomy is explained by the fact that histone phosphorylation can create domains that are recognized and bound by transacting factors, the composition of which determines the transcriptional state. Also histone phosphorylation can facilitate or repress further acetylation or methylation thereby regulating the transcriptional state of chromatin. For example, phosphorylation at threonine 11 has been shown to hasten removal of repressive H3K9 methylation by recruitment of the histone

demethylase Jumonji C domain containing protein (JMJD2C) (Metzger et al., 2008). At this capacity, phosphorylation facilitates transcription by recruitment of histone demethylases. In addition, phosphorylation at H3 serine 10 inhibits recruitment of heterochromatin protein 1 (HP1) (Fischle et al., 2005; Hirota et al., 2005), which in turn prevents recruitment of DNMTs (DNMT1 and DNMT3a) thereby leaving chromatin in the open conformation and ready for transcription. At this level, phosphorylation prevents methylation of DNA by preventing recruitment of DNMTs. Phosphorylation can also facilitate transcription by allowing recruitment of histone acetyltransferase (HAT). For example, mutation at H3 serine 10 ablates recruitment of HATs (Chuang et al., 2000; Lo et al., 2000). Phosphorylation can also activate genes which in turn activate signaling pathways responsible for gene activation. Stimulation of inflammatory cytokine signaling activates I κ B kinase α which phosphorylates histone H3 at serine 10 in the promoters of multiple nuclear factor responsive genes (Anest et al., 2003; Yamamoto et al., 2003). These phosphorylations result in expression of several inflammatory responsive genes. Lastly, it is been shown that phosphorylation of core H3 at serine 10 (cH3S10) and threonine 11 (cHT11) occur during active transcription (Chuang et al., 2000; Nowak and Cores, 2000, 2004, Metzger et al., 2008), suggesting that the negative phosphate groups added to histones might neutralize the positive charges on DNA, thus causing chromatin to unwind and allow transcription to continue. Conversely, evidence for transcriptional repression due to histone phosphorylation is mounting. For example, during mitosis, H3S10 phosphorylation is associated with condensed chromosome (Goto et al., 1999). Aurora-B kinase-mediated phosphorylation of H3S28 is also associated with condensed chromosome. Constitutive phosphorylation of H1 through the Ras-MAPK pathway leads to chromatin condensation (Chadee et al., 1995). In addition, mammalian Sterile20-like 1 (Mst1) phosphorylation of H2B-14 is associated with condensed chromatin leading to apoptosis (Cheung et al., 2003).

Gene name	Histone Mark	Genetic Defect	Tumor Type	Function	Reference
Histone phosphorylation					
Jak2	H3Y41	Point mutation	Hematological tumors	Loss	(Dowson et al., 2009)
ATM/ATR	H2AXS139	Double stranded breaks	Melanomas	Loss	(Fernandez-Capetillo et al., 2004) and (Bassing, C.H. et al. 2003)
Aurora-kinase-B	H3S10	Chromosomal instability	Aneuploidy and Colorectal cancer	Loss	(Fischle, W. et al. 2005) and (Hirota, T. et al. 2005)
Mst1	H2BS14	Apoptosis resistance		Loss	(Hanahan and Weinberg, 2000) and (Ahn, S.H. et al. 2005)

Table 6. Aberrant Histone Phosphorylation in Cancer

Like other histone modifications, histone phosphorylation is counteracted by dephosphorylation. Phosphatases catalyze the removal of phosphate groups from histones. For example, phosphatase type 1 (PP1) interacts with aurora-B during mitosis as a feedback mechanism (Katayama et al., 2001). Also PP1 regulates Aurora-B and H3 phosphorylations

during cells division (Murnion et al., 2001). In cancer cells, the balance between phosphorylation and dephosphorylation is dysregulated. For example, histone phosphorylation has been shown to play a role in cancer through modulation of the DNA repair response, chromosome instability and apoptosis. Recently JAK2, a nonreceptor tyrosine kinase, has been shown to be activated by chromosomal translocation and point mutations in hematological malignancies (Dawson et al., 2009). Also, JAK2 has been shown to phosphorylate H3Y41 which prevents the recruitment of heterochromatin protein 1 α (HP1 α) leading to increased expression of genes in this region. Phosphorylation at serine 139 in the highly conserved C-terminal tail (-SQEY) of H2A.X has been shown to play an important role in DNA double-strand break (DSB) repair and tumor suppression (Fernandez-Capetillo, O. et al. 2004). In addition, H3S10 and H2BS14 phosphorylations play a role in chromosomal instability and apoptosis resistance, respectively which are both hallmarks of cancer (Fischle, W. et al. 2005; Hirota, T. et al. 2005; Hanahan and Weinberg, 2000; Ahn, S.H. et., 2005). Activation of MAPK pathway by environment carcinogens leads to phosphorylation of H3 which in turn results in the induction of immediate early genes. More specifically, ultraviolet light has been shown to activate MAPK pathways resulting in phosphorylation of H3S10 by p38 kinase.

2.3.4 Histone ubiquitination and sumoylation

Ubiquitination and sumoylation involve the transfer of a polypeptide to the histone tail. The polypeptide molecules for ubiquitination and sumoylation are ubiquitin and small ubiquitin related modifier (SUMO) (Takada et al., 2007), respectively. The enzymatic cascade responsible for ubiquitination and sumoylation are similar, with three classes of enzymes involved in both instances: E1 activating enzymes, E2 conjugating enzymes and E3 ligating enzymes. In the first step of this multistep cascade, E1 adds Ubiquitin/SOMO to the target substrate in an ATP dependent manner. E2 then transfers the Ubiquitin/SOMU to E3 which associates and ligates the Ubiquitin/SOMU to histones (Nathan et al., 2003). Histone ubiquitination involves mono-ubiquitination which is different from poly-ubiquitination in that it does not result in proteosomal degradation of the target histone. Depending on the lysines that are ubiquitinated, ubiquitination can result in transcriptional activation or repression (Table 7). For example, both H2A and H2B are targets of mono-ubiquitination, and mono-ubiquitination has been shown to be a precursor to histone methylation (Gerber and Shilatifid, 2003; Hampsey and Reinberg, 2003; Osley, 2004; Margueron et al., 2005). In particular, mono-ubiquitination at H2B lysine 120 (H2BK120) by E3 ligase (RNF20/RNF40) initiates methylation at H3 lysine 4 (H3K4) resulting in recruitment of homeobox genes (Zhu et al., 2005) and transcriptional activation. Conversely mono-ubiquitination at H3 lysine 119 (H3K119) by Bmi/Ring1A induces transcriptional repression (Wand et al., 2004).

It is not completely clear what role sumoylation plays on transcriptional regulation, however sumoylation has recently been shown to cause transcriptional repression (Shiio and Eisenman, 2003; Girdwood et al., 2003) (Table 1). For example, H4 sumoylation is associated with recruitment of HP1 and HDAC which is known to repress transcription. A number of oncogenes and tumor suppressor genes, including PML, Mdm2, c-Myb, c-Jun, Rb and p53, undergo SUMOylation (Muller et al., 1998; Bushmann et al., 2000; Bies et al., 2002; Schmidt et al., 2002; Huang et al., 2004; Besten et al., 2005; Ghioni et al., 2005; Ghost et al., 2005). SUMOylation of Mdm2 increases its E3 activity toward p53 tumor suppressor. SUMOylation negatively regulates c-Jun activity and thus restricts its oncogenic capacity. SUMOylation of

Histone Modification	Modifying Enzyme	Type of Modification	De-modifying Enzyme	Impact on transcription
Phosphorylation	Kinase e.g. JAK	Transient	Phosphatase	↑ ↓
Sumoylation	E3 Ligase	Transient	SUMO Protease e.g. SENP1	↓
Ubiquitination	E3 Ligase	Transient	De-ubiquitinase	↑ ↓
Methylation	HMT/PRMT	Stable	PADI, JMJD	↑ ↓
Acetylation	HAT	Stable	HDAC	↑

Red arrows indicate transcriptional activation, while black arrows indicate transcriptional repression.

Table 7. Summary, Impact of Posttranslational Modification of Histones on Transcription

c-Myb increases its stability, but negatively regulates its transactivation function of. Increasing evidence supports the notion that protein SUMOylation is important during the course of tumorigenesis and oncogenesis, and altered in human cancers, however further work is needed to determine the impact of sumoylation on cancer.

In summary, epigenetic modifications are not stand alone processes. Instead, considerable crosstalk exists among the different types of epigenetic marks. In accord with this principle, epigenetic marks by themselves, or synergistically with other epigenetic marks, function to either repress or activate transcription (Table 7). Of relevance to epigenomic regulation is that all epigenetic modifications identified to date have been shown to be reversible. Genes regulated by epigenetic modification remain intact and can therefore be returned to their original state. The reversibility of epigenetic mechanisms makes them highly susceptible to pharmacological intervention and therefore, ideal targets for cancer therapeutics.

3. Epigenetic therapies

Genetic mutations and gross structural defects permanently activate or inactivate genes; however genes modified by aberrant epigenetic modification remain structurally intact and subject to reversal of aberrant epigenetic modifications that can restore their original state. This phenomenon has made epigenetic modifications an ideal target for the treatment of many diseases, including cancer. As discussed in previous sections, cancers are plague with aberrant epigenetic modifications which have been shown to contribute to initiation and transformation. In fact, several exogenous chemicals used to treat cancers have been shown to cause unintended epigenetic modifications which in many cases have led to exacerbation of tumor progression. These factors, combined and our understanding of epigenetic modifying enzymes, pathways and accessory proteins pivotal to epigenetic modifications, have lead to the development of therapies targeting DNA methylation and DNMTs, histone modifications and histone modifying enzymes (i.e HAT, HDAC, kinases, HMT, SUMO ligase, ubiquitin ligase, etc.). Indeed, therapies targeting chromatin remodeling complexes have attracted significant interest in recent years as a means for cancer prevention, either alone or in combination with conventional cancer treatments.

3.1 DNA Methyltransferase inhibitors (DNMTis)

Inhibitors of DNA methylation (DNMTis) cause reactivation of silenced genes, inhibition of cell proliferation, apoptosis and enhancement of sensitivity to other cancer drugs. DNMTis can be grouped into nucleoside DNMTis and non-nucleoside DNMTis, based on their structure and mode of action. Nucleoside DNMTi are analogues or derivatives of the nucleoside cytidine and they include 5-azacytidine (5-Aza-CR), 5-Aza-2-deoxycytidine (5-Aza-CdR), zebularine, cytarabine and 5-Fluoro-2-deoxycytidine. The cytidine analogues (5-Aza-CR and 5-Aza-CdR) have been approved by FDA for the treatment of myeloid malignancies in the USA. The anticancer activity of these drugs is believed to be mediated by two mechanisms: (1) cytotoxicity which stems from incorporation of these drugs into DNA and/or RNA, and (2) reactivation of tumor suppressor genes by demethylation of their promoter regions (Jones and Liang, 2009). These drugs do not demethylate DNA *per se*, but rather with continued replication, cytidines are replaced by the cytidine analogues resulting in serial dilution of methylable cytidines. In addition, DNMTs are trapped in covalent adducts with DNA through the incorporated cytidine analogues. 5-Aza-CR and 5-Aza-CdR are taken into the cell through the concentrated nucleoside transporter 1 (hCNT1) (Rius et al., 2009). Once inside the cell, 5-Aza-CR is phosphorylated by uridine-cytidine and 5-Aza-CdR by diphosphate kinase (Stresemann et al., 2008; Issa et al., 2009) which in turn convert them into active triphosphates (i.e. 5-Aza-CTP and 5-Aza-dCTP). 5-Aza-CTP is incorporated into the DNA resulting in the formation of covalent adducts between DNMTs and DNA (Santi et al., 1984). This traps the DNMTs and prevents further methylation. In other studies, 5-Aza-dCTP was shown to be incorporated into RNA which interferes with ribosomal biogenesis and protein synthesis (Mompalmar et al., 1984; Stresemann et al., 2008). In accord with these findings, Ghoshal et al. 2005 and Kuo et al. 2007 have both shown that 5-Aza-CTP and 5-Aza-dCTP hypomethylate the genome through passive dilution of cytidine and not through active demethylation. Because of the cytotoxicity and instability of 5-Aza-CR and 5-Aza-CdR, DNMTis cannot be continually given to patients. For this reason, zebularine has been developed as an alternative. Although this drug works in a manner similar to 5-Aza-CR and 5-Aza-CdR, it is more stable and less toxic than 5-Aza-CR and 5-Aza-CdR DNMTis (Zhou et al., 2002; Cheng et al., 2003). In line with these findings, zebularine has been shown to reactivate tumor suppressor genes (Flotho et al., 2009; Billam et al., 2010), enhance tumor cells' chemotherapy and radiation sensitivity (Dote et al., 2005), exert angiostatic and antimetogenic activities (Balch et al., 2005; Hellebrekers et al., 2006) and to be stable enough for oral administration (Zhou et al., 2002; Cheng et al., 2003). In addition, at low doses, zebularine can be given to patients continuously without the overt cytotoxicity associated with 5-Aza-CR and 5-Aza-CdR. Another cytidine analogue, 5-fluoro-2-deoxycytidine (FdCyd) has been shown to cause demethylation in human breast and lung cancer cells (Beumer et al., 2008). In the case of FdCyd, the hydrogen atom at carbon-5 (C5) which is the methyl acceptor during the methylation reaction is replaced by a fluorine atom. When FdCyd is incorporated into DNA, the β -elimination step in which DNMT transfers the methyl group to the cytidine is inhibited. At the same time, the fluorine atom traps the DNMT to prevent elimination of the FdCyd moiety (Jones et al., 1980; Reither et al., 2003). FdCyd is currently in Phase I clinical trials for the treatment of breast and other solid tumors (Gowher et al., 2004) (Table 8). Moreover, FdCyd, in combination with other epigenetic drugs (i.e. tetrahydrouridine and dihydro-5-azacytidine (DHAC), is being evaluated in clinical studies for the treatment of malignant mesothelioma (Kratzke et al., 2008). Although nucleoside DNMTis have proven effective for the treatment of cancers, their cytotoxicity remains a significant limitation. To address this shortcoming, non-nucleoside

DNMTis are being evaluated. Non-nucleoside DNMTis include procaine, L-tryptophan derivatives, RG108, hydralazine, MG98, procainamide, and epigallocatechin-3-gallate (EGCG). Procaine is a local anesthetic drug that can also function as a DNMTi. For example, procaine has been shown to cause global demethylation and reactivation of tumor suppressor genes in human breast cancer cells (Jin et al., 2009). Unlike the nucleoside analogues, procaine competes with DNMTs for binding to CpG rich regions (Jin et al., 2001). Procainamide and hydralazine are antiarrhythmic drugs that can also function as DNMTis, and both agents have been shown to inhibit DNA methylation through interactions between the nitrogen atom of procainamide and hydralazine with the lys-162 and Arg-240 moities in the catalytic site of DNMTs (Song et al., 2009; Singh et al., 2009; Mund et al., 2006). In accord with these findings, procainamide has been shown to specifically inhibit DNMT1 (Lee et al., 2005). RG108 is a small molecule inhibitor of DNMTs that inhibits free DNMTs (Brueckner et al., 2005). This drug works by blocking the catalytic pocket of DNMTs without the formation of covalent adducts that cause cytotoxicity (Stressmann et al., 2006). Studies have also shown RG108 to cause demethylation and reactivation of tumor suppressor genes without affecting the methylation level of microsatellite regions in lung cancer cells (Suzuki et al., 2010), suggesting a specificity level in RG108 that has not been seen in other DNMTis. Table 8

DNMTi	Phase	Type of cancer	Clinical trial finding	Reference
NON-NUCLEOSIDE ANOLOGUES				
MG98	N/A	Cervical cancer	26% SD	Garzon et al., 2009
EGCG	Phase I		N/A	Brueckner et al., 2004
Procaine	Phase I	Solid Tumor	N/A	Villar-Garea et al., 2003
Procainamide	N/A	Colon cancer	N/A	Segura-Pacheco et al., 2003
Hydralazine	N/A	Cervical cancer	N/A	Song et al., 2009
RG108	N/A	Colon cancer	N/A	Suzuki et al., 2010
COMBINATION THERAPIES				
Aza-CR+Sodium Phenylbutyrate	Preclinical	Solid Tumors	50 24.2% CR; PR 11.2%	Soriano et al., 2007
Aza-CR + Valproic Acid	Phase II	MDS	62 30.7% CR; PR, 15.4%	Blum et al., 2011
Aza-CR + Lenalidomide	Phase I	MDS	44% CR, 17% HI, and 67% ORR	Jabbour et al., 2009
Aza-CR + Cytarabine	Phase I	MDS/AML	N/A	Plummer et al., 2009

DNMTI	Phase	Cancer Type	Clinical Trial Findings	Reference
NUCLEOSIDE ANOLOGUES				
5-Aza-CR	Phase II	MDS	7% CR, 16% PR, 37% HI	Yoo et al., 2008
		MDS	17% CR, 12% PR, 42% SD	Silverman et al., 2006
		MDS	10.8% CR, 9.5% PR, 20.3% HI	Santini et al., 2009
		MDS/AML	15.6% CR, 25% HI, 34.4% SD	Martin et al., 2009
5-Aza-CdR	Phase II	SM	N/A	Winqvist et al., 2006
		MDS	9% CR, 13% HI, 17% ORR	Muller-Thomas et al., 2009
		MDS	34% CR	Wijermans et al., 2008
		MDS	17% CR, 18% HI, 32% ORR	Kantarjian et al., 2007
		MDS/AML	26% SD	Schrump et al., 2006
Zebularine	Phase II	MDS	13.4% CR, and 7.5% PR	Gore et al., 2006
5-FdCyd	N/A	Breast and lung	N/A	Reither et al., 2003

AML: Acute Myeloid Leukemia; CR: Complete Remission; HI: Hematologic Improvement; MDS: Myelodysplastic Syndrome; PR: Partial response; ORR: Overall Response Rate; CR: Conventional Care Regimens; N/A: Data not available; SD: Stable Disease. Note that only few DNMTis are shown here to illustrate their role in the treatment of cancers.

Table 8. DNMTis and Their Impact in Cancer

summarizes the DNMTis in clinical trial and their efficacy for cancer treatment either alone or in combination with other regimens.

3.2 Histone acetyltransferase inhibitors (HATis)

HAT inhibitors can be classified into synthetic peptide CoA base bisubstrate HATis, natural product HATis and small molecule HATis (Table 9). The synthetic bisubstrate HATis were the first to be identified based on the observation that polyamine-CoA conjugates can inhibit HAT activity in cell extracts (Cullis et al., 1982). In particular, H3-CoA-20 and Lys-CoA specifically inhibit pCAF and p300 (Lau et al., 2000) rather weakly. Introduction of a phenyl or methyl group between lysine and CoA improves the inhibition fourfold (Sagar et al., 2004). Most of the synthetic bisubstrate HATis work by mimicking the acetyl CoA-lysine intermediate complex in the HAT reactions. Crystal structure information between GCN5 and these HATis shows that GCN5 interacts with the pyrophosphate moiety, the pantoic moiety and the phosphate group of CoA. The major deficiency for this class of HATis is their impermeability to cells. Unfortunately, most of the naturally occurring HATis also suffer from a similar problem. For example, anacardic acid isolated from the shell of cashewnuts displays permeability restriction in vitro. Nevertheless, garcinol and isogarcinol were both shown to inhibit p300 and pCAF (Balasubramnyam et al., 2004; Mantelingu et al., 2007). The derivative of isogarcinol, LTK14, was shown to selectively inhibit p300, but not

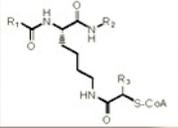
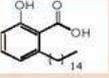
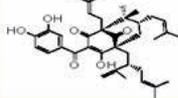
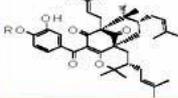
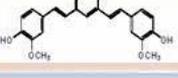
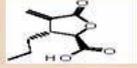
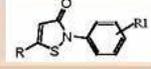
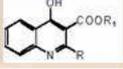
HATi compound	Parental and Derivative compounds	Specificity	Reference
SYNTHETIC HATIs			
	Lys-CoA: (R1 = CH ₃ ; R2 = R3 = H)	p300	Lau et al., 2000
	H3-CoA-20: (R1 = G-G-T-S-K-R-A-T-Q-K-T-R-A-NH-COCH ₃ ; R2 = A-P-R-K-Q-L; R3 = H)	PCAF	Lau et al., 2000
	H3-(Me)-CoA: (R1 = CH ₃ CO-NH-A-R-T-A-R-K-S-T-G-G; R2 = A-P-R-K-Q-L; R3 = Me)	p300	Sagar et al., 2004
	Lys-Phe-Coa: (R1 = Phe, R2 = R3 = H)	p300	Sagar et al., 2004
	Lys-CoA-3'dephospho: R1 = (R1 = Phe, R2 = R3 = H)	p300	Sagar et al., 2004
NATURAL HATIs			
	Anacardic Acid	p300, PCAF	Varier et al., 2004
	Garcinol	p300, pCAF	Balasubramanyam et al., 2004a
	Isogarcinol: (R = H) LTK14: (R = CH ₃)	p300 pCAF p300	Mantelingu et al., 2007
	Curcumin	p300 CBP	Balasubramanyam et al., 2004b
SMALL MOLECULES HATIs			
	γ -Butyrolactone	hGCN5	Biel et al., 2004
	Isothiazolones: (R = H, Cl; R1 = NO ₂ , Cl, CF ₃ , OCH ₃ , COOEt)	p300 PCAF	Mantelingu et al., 2007 & Stimson et al., 2005
	Quinolines	N/A	Mai et al., 2006

Table 9. HAT Inhibitors and Their Selectivity

pCAF (Mantelingu et al., 2007). The best characterized of the naturally occurring HATIs is Curcumin, which is isolated from the *Curcuma longa* rhizome. Curcumin has shown high efficacy in the prevention and treatment of colorectal, prostate, kidney, lung, ovarian, breast, cervical and liver cancers (Balasubramnyam et al., 2004). The last group of HATIs includes a number of small molecules designed to overcome the challenges in permeability of the first two groups. These include γ -butyrolactone MB-3, quinoline and isothiazolone and their

derivatives. Although in their infancy, isothiazolone has been shown to inhibit the enzymatic activity of both pCAF and p300 leading to reduction in cell proliferation of human ovarian and colon cancer cell lines (Stimson et al., 2005). γ -butyrolactone MB-3 inhibits GCN5 and contains an $\alpha\beta$ -unsaturated carbonyl group that is prone to covalently bind to the thiol group in the active site of GCN5 (Biel et al., 2004).

3.3 Histone deacetylase inhibitors (HDACis)

HDAC inhibitors (HDACis) have been classified into seven categories based on their chemical structures and mode of inhibition: short chain fatty acids, benzamides, cyclic peptides, electrophilic ketones, hydroxamine-acid-derived compounds (Espino et al., 2005; Rasheed et al., 2007), miscellaneous compounds (e.g. Depudecin and MGCD-0103) and sirtuin inhibitors (Table 10). Sirtuin or class III HDACis can be further classified structurally, but for simplicity, they are classified here into a single group. For details on class III HDACi subgroups, the reader is referred to Schemies et al., 2009. Class I, II and IV HDACis share a common metal binding domain that serves to block Zn^{+} chelation at the active site (Miller et al., 2003). Because of the presence of a different co-factor (nicotinamide (NAD)) at the active site of class III HDACs, zinc-dependent HDACis are ineffective against them. Class III

HDACi Class	Chemical Compound
Hydroxamic acid-derived compounds	TSA (trichostatin A), SAHA (suberoylanilide hydroxamic acid or Vorinostat) CBHA (<i>m</i> -carboxycinnamic acid bis-hydroxamide), ABHA (azelaic bis-hydroxamic acid), LAQ-824, LBH-589, oxamflatin, PXD-101, scriptaid, pyroxamide, SK-7041, SK-7068 and tubacin.
Cyclic peptides	Romidepsin (depsipeptide, FK-228/FR-901228), aplidin, CHAPS (cyclic hydroxamic acid-containing peptides) and trapoxin.
Short-chain fatty acids	Valproic acid, phenylbutyrate, phenylacetate and AN-9.
Benzamides	MS-275 and CI-994.
Ketones	Trifluoromethyl ketone.
Miscellaneous	Depudecin and MGCD-0103.
Sirtuin inhibitors	Nicotinamide (NAD), 2-Anilino-benzamide, Sirtinol, Dihydropyridine, Cambinol, etc.

Table 10. Histone Deacetylase Inhibitor Subgroups

HDACs are inhibited by nicotinamide, NAD⁺ analogues, indoles, hydroxynaphthaldehyde derivatives, Splitomicins, Suramins and kinase inhibitors (Schemies et al., 2009). NAD works by specifically blocking the entry of nicotinamide adenine dinucleotide into the active site of class III HDACs. The modes of action of other sirtuin inhibitors are still unknown. While zinc-dependent HDACs are established anticancer drugs, and two inhibitors (Vorinostat (SAHA), Romidepsin) have been approved for cancer treatment in the United States (Johnstone et al., 2002), much less is known about the biological consequences of sirtuin inhibitors (North et al., 2004; Wesphal et al., 2007; Fatkins et al., 2008). In fact, sirtuin inhibitors shown to be effective in lower organisms, do not work on human subtypes (Biel et al., 2005; Schafer et al., 2005). In general, HDACs have shown to induce cell cycle arrest and apoptosis in G1 or G2/M. For example, in response to HDACs, p21 gene is consistently upregulated in a p53-independent manner and p21 expression is correlated with cell cycle G1 arrest (Gui et al., 2004; Vrana et al., 1999). The upregulation of p21 gene is correlated with increased acetylation of histones H3 and H4 near the p21 promoter (Hirsch et al., 2004). In addition, HDACs, such as butyrate and trichostatin A, have been shown to stabilize p21 mRNA (Hirsch et al., 2004). Moreover, HDAC inhibition represses cyclins A and D, and activates p16 and p27 to induce cell cycle arrest (Sandor et al., 2000; Wharton et al., 2000). In other studies, HDACs upregulate the expression of pro-apoptotic genes (i.e., TRAIL, DR5, Bax, Apaf-1, Bmf, Bim and TP2) and/or downregulate the expression of anti-apoptotic genes (i.e., Bcl-2, Mcl1, and XIAP) (see review by Bolden et al., 2006). The biggest advantage for many HDACs is that they can induce their effect in the nano/micromolar range, as seen for SAHA and butyric acid, respectively (Espino et al., 2005; Kelly et al., 2003). Moreover,

Class	HDACs	Relevant HDACs
I	HDACs 1 and 2	SK-7041, SK-7068, MS-275, VPA, romidepsin butyrate, trapoxin, SAHA, TSA, PXD-101, LBH-589, LAQ-824 and MGCD-0103
	HDAC 3	MS-275, VPA, butyrate, trapoxin, SAHA, TSA, PXD-101, LBH-589, LAQ-824 and MGCD-0103
	HDAC 8	VPA, butyrate, trapoxin, SAHA, TSA, PXD-101, LBH-589 and LAQ-824
IIa	HDAC 4	Romidepsin, VPA, butyrate, trapoxin, SAHA, TSA, PXD-101, LBH-589 and LAQ-824
	HDACs 5, 7 and 9	VPA, butyrate, trapoxin, SAHA, TSA, PXD-101, LBH-589 and LAQ-824
IIb	HDAC 6	Romidepsin, tubacin, SAHA, TSA, PXD-101, LBH-589 and LAQ-824
	HDCA 10	Tubacin, SAHA, TSA, PXD-101, LBH-589 and LAQ-824
III	SIRT-1, -2, -3, -4, -5, -6 and -7	Nicotinamide (NAD), 2-Anilino-benzamide, Sirtinol, Dihydropyridine, Cambinol, etc.
IV	HDAC 11	SAHA, TSA, PXD-101, LBH-589, LAQ-824 and MGCD-0103

HDAC: Histone deacetylase; SAHA: Suberoylanilide hydroxamic acid; SIRT: Sirtuin; TSA: Trichostatin A; VPA: Valproic acid.

Table 11. HDAC Classes and Relevant Inhibitors

HDACis have been shown to suppress angiogenesis and to activate and enhance the host immune system in cancer patients (Bhalla et al., 2005; Dokmanovic et al., 2005; Bolden et al., 2006).

At the clinical level, HDACis function synergistically with a host of structurally and functionally diverse cancer drugs, chemotherapeutic agents and biologically active polypeptides. In this manner, HDACis can increase the efficacy of other drugs by increasing target susceptibility. For example, in breast cancer therapy, the effectiveness of topoisomerase II inhibitors can be increased by pretreatment with SAHA (Marchion et al., 2004). In addition, HDACis have been used in combination with DNA demethylating agents in an attempt to reactivate silenced genes involved in tumor suppression. For example, three Phase I/II trials combining 5-Aza-CR or decitabine with HDAC inhibitors (phenyl butyrate or valproic acid) in patients with AML and MDS showed both tolerability and promising efficacy (Gore et al., 2006; Maslak et al., 2006; Garcia-Manero et al., 2006). Of a total of 93 patients who were treated, 14 showed complete remissions (CR), two showed partial complete remission (pCR), four showed partial response (PRs) and 6 showed hematologic improvements (HI) (Table 12). These studies combined have an overall response rate of 28%. In another phase I clinical study, the efficacy of CI-994 and various chemotherapeutic agents was examined in 104 patients. The results from these studies showed that CI-994 at doses of 4-10mg/m²/day can be safely administered to patients for 7-21 days in a 3-4 week dosing regimen (Nemunaitis et al., 2003; Undevia et al., 2004; Paur et al., 2004). In this study, two patients with esophageal and bladder cancer showed complete remission (CR) and five (three with non-small lung cancer and two with colorectal cancer) demonstrated partial remission (PR). On a related note, some leukemia and breast cancers plagued with the expression of fusion proteins (RAR-PML, RAR-PLZF or AML-ETO chimeras) that inhibit differentiation have shown improvements when HDACis in combination therapy with transretinoic acid (ATRA) is used to inhibit the function of these fusion proteins (Johnstone et al., 2003) (Table 12). Another emerging area of combination therapy is the use of HDACis with tyrosine kinase inhibitors in cancers that overexpress antiapoptotic genes. For example, SAHA, LBH-589, LAQ-824 and romidepsin have demonstrated synergistic apoptotic activity in combination with imatinib and other tyrosine kinase inhibitors, such as AMN-107 in imatinib-sensitive, as well as imatinib-resistant *bcr-abl* leukemic cells (Nimmanapalli et al., 2003a, 2003b; Yu et al., 2003; Kawano et al., 2004; Fiskus et al., 2006).

HDACis	Phase	Tumor type	Patient number	Responses	Reference
Single Therapy					
Phenyl butyrate	I	Solid	75	CR(1) and SD (9)	Carducci et al., 2001; Gilbert et al., 2001; Phuphanich et al., 2005
Valporic acid	I/II	AML/MDS	18	OR(8) and PR (1)	Kuendgen et al., 2004
SAHA	I	Mesothelioma	37	PR (2)	Kelly et al., 2003
	I	Solid	13	TR (4)	Krug et al., 2006
Romidepsin	II	CTCL	28	CR(2), PR(8), SD(16)	Whittaker et al., 2006

Combination Therapy	Phase	Tumor Type	Patient number	Response	Reference
Phenyl butyrate (+)					
5-Aza	I/II	AML/MDS	29	CR (4), PR (1), HI (6)	Gore et al., 2006
5-Aza	II	AML/MDS	10	PR (3), SD (2)	Maslak et al., 2006
ATRA	I	APML	5	CR (1)	Zhou et al., 2002
Valproic acid (+)					
Decitabine	I/II	AML/MDS	54	CR (10) and CRp (2)	Garcia-Manero et al., 2006
ATRA	II	AML	11	CR (1) and CRi (2)	Raffoux et al., 2005
ATRA	II	AML/MDS	20	HI (6/11 evaluable)	Pilatrino et al., 2005
ATRA	II	AML	30	CR (1), CRi (1), PR (1) and SD (20)	Kuendgen et al., 2006
SAHA (+)					
Carboplatin + paclitaxel	I	Colorectal	9	PR (4) and SD (2)	Ramalingam et al., 2006
CI-994 (+)					
Capecitabine	I	Solid	4	PR (1) and SD (19)	Undervia et al., 2004
Carboplatin + paclitaxel	I	Pancreatic	30	CR (2) and PR (5)	Pauer et al., 2004

5-AZA: 5-Azacytidine; AML: Acute myeloid leukemia; APML: ATRA: All-trans-retinoic acid; CR: Complete response; CRi: Morphologic complete remission with incomplete count recovery; CRp: Complete response without complete platelet recovery; HDACi: Histone deacetylase inhibitors; MDS: Myelodysplastic syndrome; PR: Partial response; SAHA: Suberoylanilide hydroxamic acid; SD: Stable disease.

Table 12. HDACis in Clinical Trial and Combination Therapies

4. Concluding remarks

Only a handful of studies have been published examining the usefulness of drugs targeting ubiquitination, sumoylation and phosphorylation of histone as a means to combat the proliferative and differentiation deficits seen in cancer. The above discussion was not intended to provide complete coverage to a fast emerging field at rather as a means to highlight the most promising therapies investigated to date employing epigenetic-based approaches. Although these early successes establish the promise of epigenetic-based chemotherapeutic regimens in the treatment of various cancers, the degree to which gene-specific epigenetic modifications can be achieved, or the extent to which targeted therapies can be developed using epigenetic approaches remains to be fully investigated. Undeniably, the ultimate benefit to be realized from such strategies is based on the fact that the epigenetic modifications in cancer cells are reversible and subject to environmental control. Clearly, the jury is still out!

5. References

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Electrotherapy on Cancer: Experiment and Mathematical Modeling

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1. Introduction

In this chapter are discussed the use, antitumor mechanisms and potentialities of electrotherapy of low-level direct current in cancer. We make emphasis in one of the most stimulating problems in the theme of electrotherapy-cancer as is the propose of electrode arrays that efficiently distribute the electric current density (electric field) in the tumor and its surrounding healthy tissue in order to maximize the tumor destruction with the minimum damage to the organism. A mathematical theorem is intended to obtain the analytical expressions for three-dimensional electric current density (electric field) generated by arrays of electrodes with finite length from those obtained for a point electrodes array. The importance and application of these electrode arrays in therapeutic planning are also discussed.

Electrotherapy is based on principles developed during the nineteenth and twentieth centuries following the first demonstration of "animal electricity" by Luigi Galvani in the eighteenth century. In medicine, the term electrotherapy has been applied to a range of alternative medical devices and treatments. Reputable medical and therapy Journals report that the use of electrotherapy devices has been widely researched and the advantages have been well accepted in the field of rehabilitation and in the treatment of chronic wounds, pressure ulcers, pain (improves range of joint movement) and neuromuscular dysfunction (improvement of strength, improvement of motor control, retards muscle atrophy and improves local blood flow). Also, electrotherapy has been applied in tissue repair (enhances microcirculation and protein synthesis to heal wounds and restores integrity of connective and dermal tissues), acute and chronic edema (accelerates absorption rate, affects blood vessel permeability, and increases mobility of proteins, blood cells and lymphatic flow), peripheral blood flow (induces arterial, venous and lymphatic flow), iontophoresis (delivery of pharmacological agents), urine and fecal incontinence (affects pelvic floor musculature to reduce pelvic pain and strengthen musculature and treatment may lead to complete

continence). Yet some of the treatment effectiveness mechanisms are little understood. Therefore effectiveness and best practices for their use in some instances are still anecdotal [Joa, 2010].

On the other hand, the application of electrotherapy in cancerous tissue has been found to have a beneficial effect in some cases of cancer. Many different forms of electrical current with respect to frequencies, pulse-shapes and amplitudes have been employed in biomedicine with the aim of remodeling tissues by enhancing or suppressing cell proliferation. One of the scopes is also employment of direct current as an antitumor agent [Cabralles et al., 2001; Ciria et al., 2004; Jarque et al., 2007; Ren et al., 2001; Schaefer et al., 2008; Turler, et al., 2000; Vodovnik et al., 1992; Xin et al., 2004; Yoon et al., 2007].

2. Electrotherapy on cancer

Cancer is uncontrolled cell growth and its cause is not well understood. The tumor cells are aggressive (grow and divide without respect to normal limits), invasive (invade and destroy adjacent tissues) and metastatic (spread to other locations in the body) [Cohen & Arnold, 2008]. These malignant properties of cancer differentiate of the benign tumors, which are self-limited in the growth and do not invade or metastasize.

Tumor cells have some structural and physiological characteristics that reveal their electric properties, which differ from the ones of the surrounding healthy cells. One of these properties is a smaller transmembrane potential, which happens due to the fact that sodium and water present an inward flow, while potassium, zinc, calcium and magnesium flow outwards. Another of these properties is the accumulation of an excessive amount of negative charges in the outer area, which causes the reduction of intracellular potassium and the increase of intracellular sodium that lead to a carcinogenic state in the cell. Other characteristics are the electric field decrease through the membrane, a greater electrical conductivity and permittivity, the existence of abnormal electron-transference systems, the negative bioelectrical potentials, the alteration of the normal energy production process which uses electron transport and the hydrogen ion gradient through the mitochondrial membrane, and finally, the existence of areas with a relative electron deficit [Haltiwanger, 2008; Joa, 2010].

Surgery, chemotherapy, radiation therapy, immunotherapy (vaccines, monoclonal antibody therapy, among other) are the conventional therapies for treating cancer [Haltiwanger, 2008; Vinageras et al., 2008; Xiang et al., 2008; Xin et al., 2004]. Choice of therapy depends upon tumor characteristics (location, histological variety, size and stage) and state of patient. However, these conventional therapies have major side effects, have not given a complete solution to the cancer problem, and are costly too. Hence attractive alternative, affordable, effective treatments are sought and one of the upcoming treatments is the use of electrical therapies, as electrochemotherapy [Sadacharam et al., 2008] and electrotherapy [Cabralles et al., 2010]. The characteristics of tumor cells above mentioned may prevent the reparation and re-establishing of the normal metabolic functions of the tumor cell, but, on the other hand, they facilitate the anti-tumor action of the electrotherapy.

2.1 Preclinical and clinical studies

Electrotherapy consists in the application of a low-level direct current to the solid tumor by means of the electrodes (i.e., platinum, platinum-iridium 90/10, stainless steel). The needles are connected to an electrical device that produces a direct current, which is generated by an

applied voltage between two electrodes. The needles with a positive charge are named anodes, while the needles with a negative charge are the cathodes. Different shapes of needles are used for treatment of tumors in dependence of the size and constitution of the tumor type. Harder needles are used to treat superficial tumors (breast, skin, melanoma cancers) [Jarque et al., 2007; Xin et al., 2004] and more elastic needles are used to treat visceral tumors (lung, liver, esophageal, prostate and rectal cancers) [Chou et al., 1997; Vogl et al., 2007; Xin et al., 2004; Yoon et al., 2007]. The location of the tumor should be determined before treatment. It and the tumor size are determined by palpation with hand for the case of superficial tumors; however, in visceral tumors are determined by means of computer tomography, X ray, Imaging Nuclear Magnetic Resonance and/or ultrasound. Normally, the treatment with electrotherapy is carried out under local anesthetic and on an outpatient basis. The tumor size determines how many needle electrodes are required, which are introduced into the tumor through the skin.

Many physicians have successfully used electrotherapy, also known as electrochemical tumor therapy, Galvanotherapy and electro-cancer treatment, as a standalone treatment in thousands of cases, with some truly spectacular results. There are many potential advantages of electrotherapy over conventional treatments, such as: (1) Direct current is suitable for all types of superficial or visceral tumors, both malignant and benign. (2) This therapy is easy to perform, safe, effective, inexpensive, induces minimum damages to the organism, can be carried out on an out-patient basis and it can be applied when the conventional therapies fail or cannot be applied. (3) It may be best suitable for cancers near critical organs where surgery and/or radiation therapy have failed or could not be performed without damaging other normal parts. (4) This therapy not only reduces costs of chemotherapy, radiotherapy, hyperthermia and immunotherapy, but also improves compliance. (5) Electrotherapy may be suitable for nonresectable tumors and can save functional tissues [Jarque et al., 2007; Vogl et al., 2007; Xin et al., 2004; Yoon et al., 2007]. (6) The tumor and its surrounding healthy tissue have different electric and geometrical parameters [Aguilera et al., 2010; Cabrales et al., 2010; Foster, 2000; Foster & Schwan, 1996; Haemmerich et al., 2003; Haemmerich et al., 2009; Haltiwanger, 2008; Jiménez et al., 2011; Ng et al., 2008; Sekino et al., 2009; Seo et al., 2005; S.R. Smith et al., 1986; D.G. Smith et al., 2000], which enable electrically-mediated treatments to be more efficient for a given dose and the tumor tissue is more susceptible to damage from direct current than normal tissue, thus allowing the destruction of cancerous cells to occur when direct current is applied directly to the malignant tissue [Cabrales et al., 2001; Ciria et al., 2004; Jarque et al., 2007; Von Euler, 2003]. These are reasons for moving to direct current (electric field) method for treating cancer. Judging by the very positive therapy results, it can be assumed, that electrotherapy will become an important form of treatment for malignant diseases. In spite of these advantages, this therapy cannot be used on ascitic and hemolymphoetic system tumors [Xin et al., 2004].

The first time that the insertion of electrodes in the base of the tumor significantly increases its destruction rate and decreases the damages to the body after the electrotherapy is published in 1997 [Chou et al., 1997]. Their report claims that this way of inserting the electrodes and alternating the sequence of cathodes and anodes induces a uniform electric field in the whole tumor, which causes a significant destruction of it. They also report that the ratio of the number of electrodes to the size of the tumor, taking into account the effective area with necrosis around the electrodes (2 cm). Since that research is conducted, most scientists have been using this type of data configuration.

Electrotherapy antitumor effectiveness can be enhanced when it is combined with intratumor injection of a chemostatic drug (i.e., bleomycin, cisplatin) [Jarque et al., 2007; Xin et al., 2004], saline solution [Jarque et al., 2007; Lin et al., 2000] and/or immunotherapy [Serša et al., 1990; Serša et al., 1992; Serša et al., 1994; Serša et al., 1996]. It has been demonstrated that intratumor bleomycin (cisplatin) treatment is more effective than intravenous treatment at the same dose and direct current potentiates the antitumor effectiveness of bleomycin several-fold [Xin et al., 2004].

In vitro and in vivo studies have demonstrated that an increase of direct current (voltage) intensity leads to an increase of electrotherapy antitumor effectiveness, as shown in Figure 1. This figure shows the Ehrlich tumor growth kinetics for the control group (CG) and different treated groups: TG1 (treated group with electrical charge of 6.7 mA for 45 min), TG2 (treated group with 11.7 mA for 45 min), and TG3 (treated group with 17 mA for 45 min). The minimum of the amount of volumetric electric charge required for the tumor destruction must be 35 coulombs/cm³; however, high antitumor effectiveness is obtained when this physical magnitude is between 80 and 100 coulombs/cm³ [Jarque et al., 2007; Ren et al., 2001; Xin et al., 2004; Yoon et al., 2007], in agreement with 92 and 80 coulombs/cm³ for which the Ehrlich (TG3 in Figure 1) and fibrosarcoma Sa-37 tumors are completely destroyed, respectively [Ciria et al., 2004].

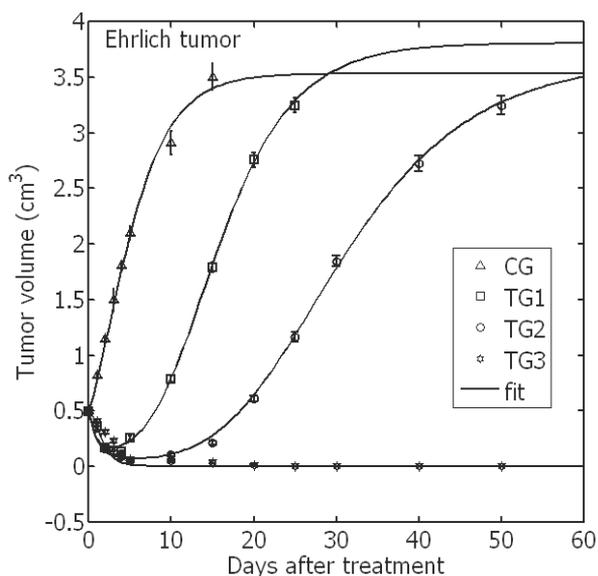


Fig. 1. Experimental data (mean \pm standard deviation) and modeled growth curves of Ehrlich tumor. Each experimental group is formed by 10 mice. CG (control group), TG1 (treated group with electrical charge of 6.7 mA for 45 min), TG2 (treated group with 11.7 mA for 45 min), and TG3 (treated group with 17 mA for 45 min). When the tumors reached approximately 0.5 cm³ in BALB/c mice, a single shot electrotherapy was supplied (zero day).

In 1978 the clinical use of direct current in the treatment of malignant tumors in humans is reported for the first time when Nordenström treated patients with lung cancer and explained that the anti-tumoral effects were due to the toxic products that came from the

electrochemical reactions induced on it because of the cytotoxic action of electrotherapy. Since Nordenström develops his work, the use of electrotherapy has been expanded for the treatment of patients with various histological types of cancer in other hospitals of Sweden and in different countries, as: China, Germany, Japan, Korea, Australia, Slovenia, the United States, Greece, Denmark, France, Brazil, Israel, Russia, Argentina and Cuba.

Since 1987 the electrotherapy has been used in China for the treatment of malignant and benign tumors, and so far it has been applied to over 20,000 patients. This is, therefore, the most complete clinical study to date. In the beginning, the group of researchers from China [Xin et al., 2004] changes the methodology for placing Nordenström's electrodes. Instead of placing one cathode in the tumor and one anode far from it, they insert several anodes in the center of the tumor and the same number of cathodes in the outer zone at the periphery of the tumor. Then, they modify again their electrode placing technique, and place the anodes and cathodes inside the tumor with the anodes in the center and the cathodes in the periphery. This change not only protects the normal tissue from destruction but also reinforces the effect of the therapy effect. The electrode placing technique is changed one more time by these researchers, when they place anodes and cathodes in an alternate way along the tumor volume, setting them 2 cm away from each other, just as is previously suggested by other authors [Chou et al., 1997].

In June 2005, electrotherapy is used for the first time in Cuba, under the supervision of Dr. Li Jing-Hong from the China-Japan Friendship hospital, located in China, for the treatment of patients with malignant and benign tumors [Jarque et al., 2007]. The study intends to test the electrode insertion procedures and the correct choice in electric charge amount in patients having advanced local tumors who are not recommended for conventional oncology treatment. The study also intends to evaluate the effectiveness and safety of the method. In 1997, Cabrales et al. conducted the first investigations in Cuba on the anti-tumoral effects of electrotherapy, using experimental murine tumors (Ehrlich and fibrosarcoma Sa-37 tumors) in BALB/c, NMRI and C57BL/6 mice. Studies carried out in rats are planned to evaluate the safety and the effects induced by the electrotherapy in tumors and in the body, taking into account the electric charge doses, as well as the number, polarity and orientation of the electrodes, the size and type of the murine tumor and the body characteristics [Cabrales et al., 2001; Ciria et al., 2004; Joa et al., 2010].

In clinical studies, the patient experiences a slight pressure pain or a slight tingling in the treated area during the electrotherapy application. Direct current brings about long lasting pain relief because it inhibits the activity of sensory nerve fibers. In the literature are reported different adverse events (effects), as: fever, wound infections, damages to the blood vessels when the electrodes are inserted close of these [Arsov et al., 2009; Haltiwanger, 2008; Jarque et al., 2007; Li et al., 2006; Salzberg et al., 2008; Vijn, 2006; Vogl et al., 2007; Xin et al., 2004; Yoon et al., 2007].

The underlying mechanisms more widely accepted are the toxic products from of the electrochemical reactions and change of pH. When low voltage (4 to 10 volts) and low amperage (40 to 100 mA) direct currents are administered the tumor area around the anode becomes highly acidic due to the attraction of negatively charged chloride ions and the formation of hydrochloric acid ($\text{pH} < 3$). The tumor areas around the cathode become highly basic ($\text{pH} > 10$) due to the attraction of positively charged sodium ions and the formation of sodium hydroxide. Also, chlorine gas and hydrogen gas emerge from the entry points of the anodes and cathodes, respectively. The pH change depolarizes cancer cell membranes and causes tumors to be gently destroyed [Li et al., 1997; Turjanski et al., 2009; Veiga et al., 2005;

Von Euler et al., 2003]. This suggests that the application of direct current (electric field) causes electrolysis, electrophoresis, electro-osmosis and electroporation in biological tissues, which create micro-environmental chemical changes and micro-electrical field changes [Haltiwanger, 2008; Li et al., 1997]. The chemistry of the microenvironment of healthy cells, injured cells and cancerous cells and the micro-electrical field of these cells are interrelated [Haltiwanger, 2008].

In a previous study [Li et al., 1997] is reported the existence of a group of biochemical alterations around the anode and cathode in tumors under treatment. Around the anode they find a pH of 2, acid hemoglobin, tissue hydration, hydrogen ions that are the result of water electrolysis, and oxygen and chlorine gas emissions. From these emissions they explain the formation of hydrochloric acid and the acid pH. In the cathode, meanwhile, they report a pH of 12, tissue dehydration, hydroxyl ions, which are the result of water electrolysis, and hydrogen gas emissions, from which they explain the formation of sodium hydroxide responsible for the basic pH. Halfway between the electrodes and far from them, no significant differences are observed between the pH and the water concentration in tumors treated with electrotherapy, and those in the untreated tumors. They conclude, then, that the electrochemical effects of this therapy happen around the electrodes.

Other antitumor mechanisms have been reported in the literature, such as: (1) immune system stimulation after treatment (the attraction of white blood cells to the tumor site) [Cabrales et al., 2001; Ciria et al., 2004; Jarque et al., 2007; Serša et al., 1996]; (2) loss of tissue water for electro-osmosis [Li et al., 1997; Vijh, 2004, 2006]; (3) change in the membrane potential of tumor cells, nutrient uptake by tumor cells and reduce deoxyribose nucleic acid production by tumor cells [Chou et al., 1997; Haltiwanger, 2008]; (4) both electrochemical reactions (fundamentally those in which reactive oxygen species are involved) and immune system stimulation induced by cytotoxic action of the direct current, could constitute the most important antitumor mechanisms [Cabrales et al., 2001]; (5) direct current treatment increases the expression of dihydronicotinamide adenine dinucleotide phosphate dehydrogenase (NADPH) oxidase subunits-derived reactive oxygen species which subsequently induces apoptosis of oral mucosa cancer cells [Wartenberg et al., 2008]. These authors also report that an increase of the reactive oxygen species brings about an increase of the expression of heat shock protein (Hsp 70) and Cu/Zn superoxide dismutase (antioxidative enzymes) and a decrease of intracellular concentration of reduced glutathione, whereas the expression of catalase remains unchanged.

Some authors evidence apoptosis as tumor death mechanism after direct electric application [Wartenberg et al., 2008]; however, others report apoptosis and necrosis around anode and necrosis around cathode [Von Euler et al., 2003; Haltiwanger, 2008]. Our experience reveals that the morphologic pattern of necrotic cell mass is the coagulative necrosis 24 hours after direct current application [Cabrales et al., 2001; Ciria et al., 2004; Jarque et al., 2007]. Also, we observe in preclinical and clinical studies vascular congestion, peritumoral neutrophil infiltration, an acute inflammatory response, and a moderate peritumoral monocyte (and macrophages) infiltration, in agreement with other authors [Chou et al., 1997; Li et al., 1997; Serša et al., 1996; Vijh, 2004; Xin et al., 2004]. We are of the opinion that apoptosis, necrosis and the electrochemical reactions into tumor (mainly around electrodes) may be explained from reactive oxygen species.

We do not reject the possibility that the electric current density induced into the tumor may affect (directly or indirectly) the cellular membrane, and intracellular and extracellular

spacing that lead to irreversible damages in it. This statement may be corroborated because it has been reported that direct electric current can have significant effects on the symmetry of surface charge, resulting in a change in membrane potential. Electric fields can produce a redistribution of cell surface receptors and influence the flow of specific ions through plasma membrane ion channels [Salzberg et al., 2008; Schaefer et al., 2008]. Any change in the flow of ions through cellular ion channels can have significant effects on cellular metabolism, proliferation rate, cytoplasmic pH, mobility, cell cycle transitions, and apoptosis. Also, research shows that direct electric current application can provide electrons, helping thus to reestablish the biocurrent flows in cancer tissues that are electrically resistant, which brings about the reduction of the resistance, the reestablishing of the transmembrane potential in cancer cells, and the concentration of the sodium, potassium, chlorine and magnesium ions through cell repolarization [Haltiwanger, 2008]. On the other hand, it has been proved that some cell membrane structures can be influenced by the action of the electrical current, the electric field or the accumulated charge. These findings are also found in vitro studies [Haltiwanger, 2008; Joa, 2010; Yen et al., 1999].

Electrotherapy is not implemented in the Clinical Oncology because it is not standardized and its antitumor mechanism is poorly understood. The first reason is explained because the dosage guideline is arbitrary and dose-response relationships are not established. Also, different electrode placements are used and optimal electrode distribution has not been determined [Aguilera et al., 2010; Cabrales et al., 2010; Jiménez et al., 2011; Joa et al., 2010]. The standardization of this therapy from experimental point of view is complex, cumbersome, requires excessive handling of animals, and expensive in resources and time. That is why the mathematical modeling constitutes the core of this chapter.

2.2 Mathematical modeling on electrotherapy: electrode arrays

Computer modeling and simulation keep growing in the more important fields of mathematics and physics applied to biophysics, biology, biochemistry and bioengineering. The reasons for this growing importance are manyfold. Among them, the mathematical modeling has been shown to be a substantial tool for the investigation of complex biophysical, as the cancer. The cancer phenomenon continues to challenge oncologists. The pace of progress has often been slow, in part because of the time required to evaluate new therapies. To reduce the time to approval, new paradigms for assessing therapeutic efficacy are needed. This requires the intellectual energy of scientists working in the field of mathematics and physics, collaborating closely with biologists and clinicians. This essentially means that the heuristic experimental approach, which is the traditional investigative method in the biological sciences, should be complemented by a mathematical modeling approach [Bellomo et al., 2008; Cabrales et al., 2010].

The mathematical modeling has been little explored in the electrotherapy-cancer topic. Some studies have been focused to propose theoretical models and computer simulations in order to describe the tumor growth kinetics [Cabrales et al., 2008; Cabrales et al., 2010; Miklavčič et al., 1995]. Predicting tumor growth is important in the planning and evaluation of screening programs, clinical trials, and epidemiological studies, as well as in the adequate selection of dose-response relationships regarding the proliferative potential of tumors. Thus, it is apparent that theoretical mathematical models are needed to study cancer [Bellomo et al., 2008; Brú et al., 2003; Jiang, 2009; Mohammadi et al., 2009; Stein et al., 2008]. A modification to the Gompertz equation, named modified Gompertz equation, is made to describe the experimental data of Ehrlich and fibrosarcoma Sa-37 tumor growth kinetics

treated with different direct current intensities [Cabrales et al., 2008]. Fitting the experimental data of CG, TG1, TG2 and TG3 with this modified equation (solid line in Figure 1) suggests that it is feasible to describe the data of untreated and direct current treated tumors. This is also sustained for the small values of the sum of squares of errors (SSE), standard error of the estimate (SE), adjusted coefficient of multiple determination (r_a^2), predicted residual error sum of squares (PRESS), multiple predicted residual sum error of squares (MPRESS) and the errors of each parameter of this equation. This modified Gompertz equation establishes the analytical conditions for which are reached the four tumor responses types after treatment (progressive disease, stable disease, partial response and complete response) and it theoretically corroborates that the electrotherapy antitumor effectiveness increases with the increase of the direct current intensity, as shown in Figure 2 [Cabrales et al., 2008]. Also, this equation theoretically reveals a new antitumor response, named stationary partial response and that these different tumor responses depend on the ratio between the electric current applied to the tumor (i) and that induced in it (i_0), named i/i_0 ratio, keeping constant the other parameters of this equation, such as: the initial volume (V_0), the intrinsic growth rate of the tumor (α), the growth deceleration factor (β) related to the antiangiogenic process the growth deceleration factor related to the antiangiogenic process and the duration of the net effect induced in the solid tumor after treatment ($1/\gamma$).

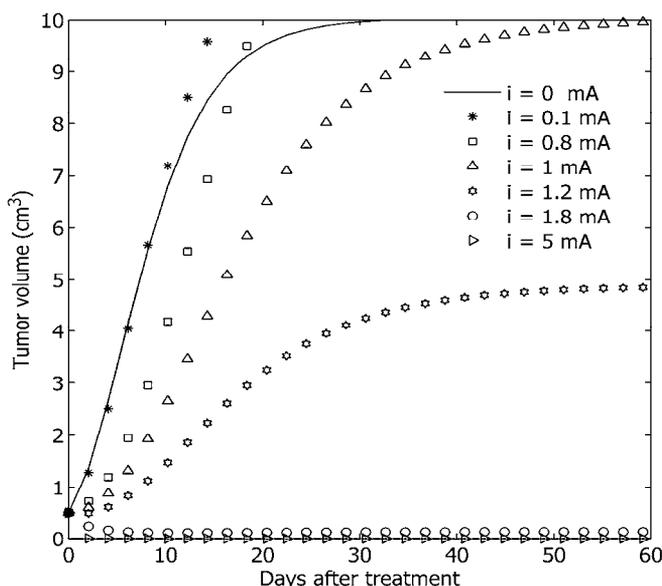


Fig. 2. Simulation of modified Gompertz equation for $\alpha = 0.6 \text{ days}^{-1}$, $\beta = 0.2 \text{ days}^{-1}$, $\gamma = 0.016 \text{ days}^{-1}$, $i_0 = 5 \text{ mA}$, $V_0 = 0.5 \text{ cm}^3$ and different magnitudes of i (mA) [Cabrales et al., 2008].

From modified Gompertz equation, it is easy to verify that the complete and stationary partial responses are reached for $i/i_0 > 2$ and $i/i_0 = 2$, respectively. This suggests the existence of a threshold value of i/i_0 ratio that may be related with the tumor reversibility condition. The tumor complete remission after direct current application suggests that the tumor growth kinetic is completely reversible, as is demonstrated in a previous study. The stationary partial response is characterized first by a significant decrease of the tumor

volume until a certain size, from which it remains constant in the time, fact that may be explained because the organism governs the equilibrium with this small tumor volume that survived to the direct current cytotoxic action. This tumor response type may suggest that the cancer may be a controllable chronic disease [Cabrales et al., 2010].

On the other hand, the mathematical modeling has been used to understand alterations on cellular membrane [Kotnik & Miklavčič, 2006], the role of pH in electrotherapy [Turjanski et al., 2009], the possible physicochemical reactions induced into the tumor during direct current application [Nilsson & Fontes, 2001] and the design of an one-probe two-electrode device in combination with a 3D gel model that contains the cathode and the anode very close to each other (0.1 cm) for studying pH spherical fronts and destroy a cancer cell spherical casket [Olaiz et al., 2010]. Also, the mathematical modeling constitutes a rapid way to propose an optimum electrodes array or close to it, in function of their parameters and those of tumor (localization, size, shape and consistency), using both analytical and numerical solutions. This allows the visualization of the potential, electric field intensity and electric current density distributions generated electrodes arrays in two-dimensional (2D) and three-dimensional (3D) tumors, in order to induce the highest electrotherapy effectiveness (higher tumor destruction with the minimum damage to the organism) [Aguilera et al., 2009; Aguilera et al., 2010; Čorović et al., 2007; Dev et al., 2003; Jiménez et al., 2011; Joa, 2010; Reberšek et al., 2008; Šel et al., 2003]. This later increases our understanding about the current flow inside tumor during direct current application. This is important because monitoring the current flow during aforementioned therapy is a challenging task due to the lack of available noninvasive electrical imaging techniques. We support that the direct current strength and its form of distribution, through electrodes, have potential biomedical applications and a decisive role in the electrotherapy effectiveness [Cabrales et al., 2010; Jiménez et al., 2011].

2D-electrode arrays are useful for planar tumors (basal cell carcinoma of the skin, cutaneous lymphoma, gastric cancer in its form of delinitis, and melanoma in clinical superficial extension) and the potential, electric field strength and electric current density distributions that these induce in the tumor are reported for electrodes circular array [Čorović et al., 2007; Dev et al., 2003; Šel et al., 2003] and electrodes elliptical array [Aguilera et al., 2009; Aguilera et al., 2010]. The explicit dependence of how electric current density distributions depend on the ellipse eccentricity, the ratio between the electric conductivities of the solid tumor (σ_1) and the surrounding healthy tissue (σ_2), named σ_1/σ_2 ratio, and positioning of the electrodes with respect to tumor-surrounding healthy tissue interface is shown in Figures 3 and 4 for an electrodes circular array (eccentricity = 0) and an electrodes elliptical array (eccentricity = 0.85), respectively [Aguilera et al., 2010]. In Figures 3a,d and Figures 4a,d, the electrodes are inserted in the tumor-surrounding healthy tissue interface. Electrodes inserted inside tumor are represented in Figures 3b,e and Figure 4b,e while those inserted in the surrounding healthy tissue are depicted in Figures 3c,f and Figures 4c,f. The influence of σ_1/σ_2 ratio on the electric current density distribution is evidenced for $\sigma_1/\sigma_2 = 1$ (Figures 3a-c and Figures 4a-c) and $\sigma_1/\sigma_2 = 10$ (Figures 3d-f and Figures 4d-f). The other parameters of the electrodes array are constant, such as: electrode radius (a), electrode potential (V_0), electrode polarity (red for the positive electrode, anode, and blue for the negative electrode, cathode), the angular separation between two adjacent-electrodes (θ), major radius (b_1) and minor radius (b_2) of the electrodes array, which are related by means of the eccentricity of it.

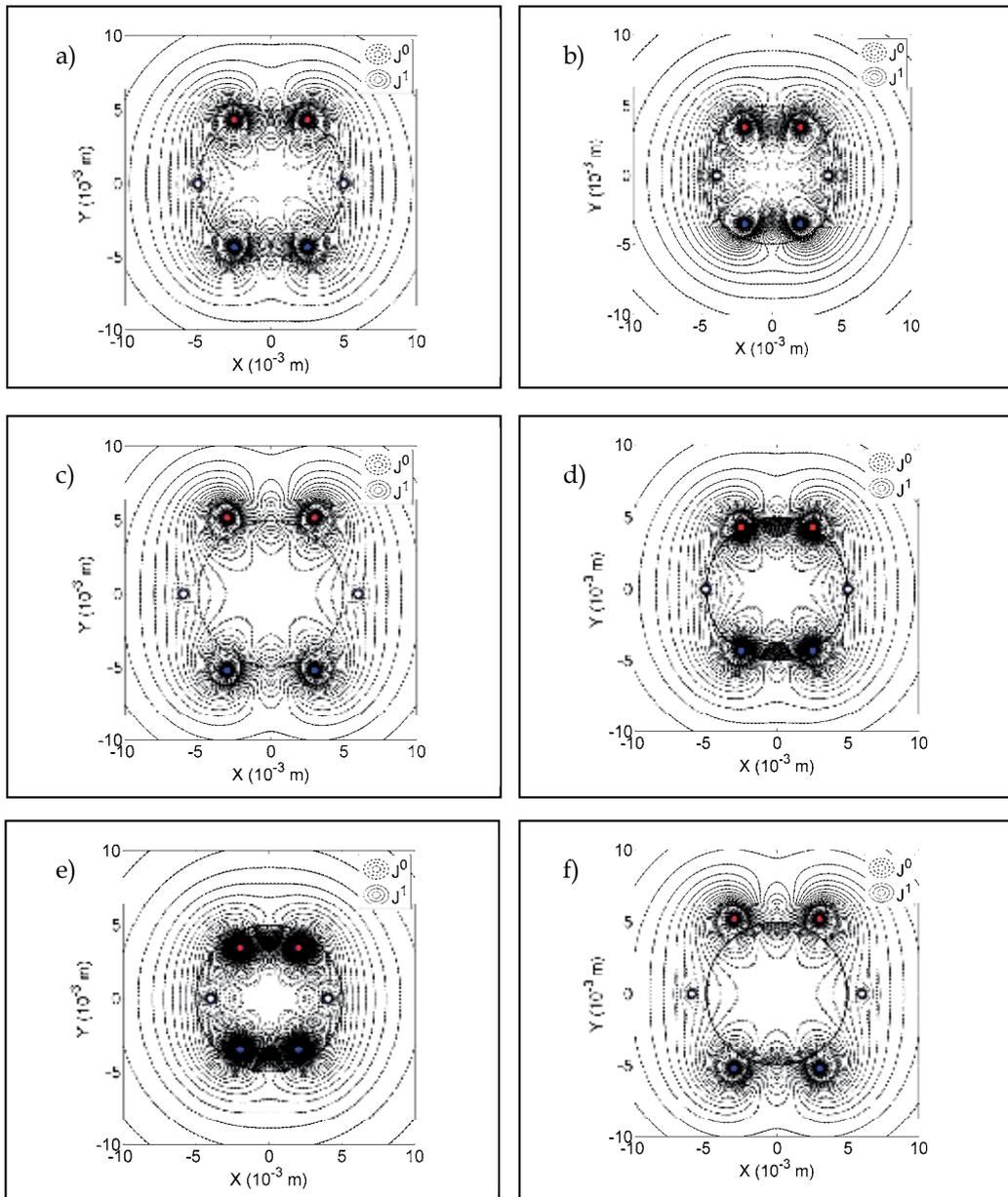


Fig. 3. Distributions of the electric current density, in leading-order, $J^0(x,y)$, and first-order term, $J^1(x,y)$, for an electrodes circular array (eccentricity = 0) for: (a) Configuration 1, $b_1 = b_2 = 0.5$ cm, and $\sigma_1/\sigma_2 = 1$; (b) Configuration 2, $b_1 = b_2 = 0.4$ cm, and $\sigma_1/\sigma_2 = 1$; (c) Configuration 3, $b_1 = b_2 = 0.6$ cm, and $\sigma_1/\sigma_2 = 1$; (d) Configuration 1, $b_1 = b_2 = 0.5$ cm, and $\sigma_1/\sigma_2 = 10$; (e) Configuration 2, $b_1 = b_2 = 0.4$ cm, and $\sigma_1/\sigma_2 = 10$; and (f) Configuration 3, $b_1 = b_2 = 0.6$ cm. These simulations are made for $\theta = 60^\circ$, $a = 0.0215$ cm, $V_o = +0,5$ V for the electrodes 2 and 3, $V_o = -0,5$ V for the electrodes 5 and 6, and $V_o = 0$ V for the electrodes 1 and 4. The parameters a, b_1, b_2 (in centimeter) are converted to meter.

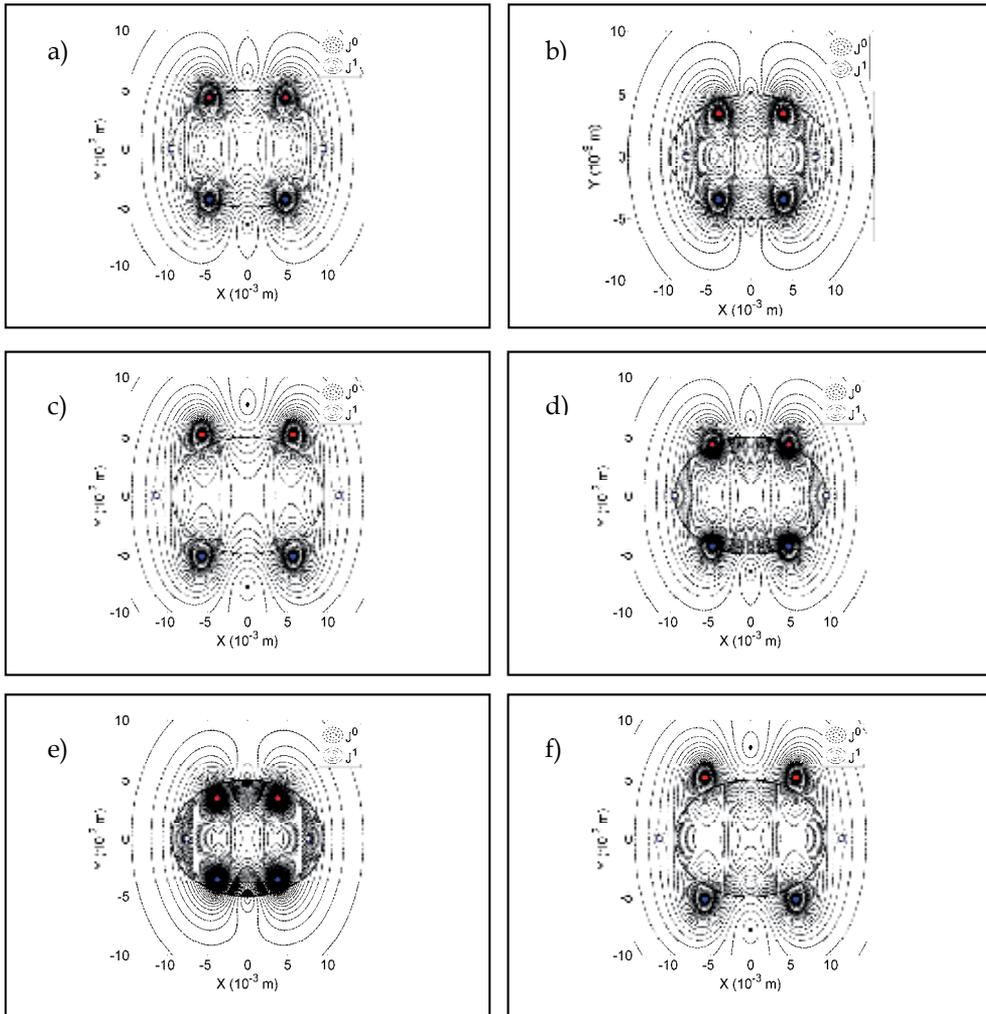


Fig. 4. Distributions of the electric current density, in leading-order, $J^0(x,y)$, and first-order term, $J^1(x,y)$, for an electrodes elliptical array with eccentricity = 0.85 for: (a) Configuration 1, $b_1 = 0.9492$ cm and $b_2 = 0.5$ cm, and $\sigma_1/\sigma_2 = 1$; (b) Configuration 2, $b_1 = 0.7593$ cm and $b_2 = 0.4$ cm, and $\sigma_1/\sigma_2 = 1$; (c) Configuration 3, $b_1 = 1.1390$ cm and $b_2 = 0.6$ cm, and $\sigma_1/\sigma_2 = 1$; (d) Configuration 1, $b_1 = 0.9492$ cm and $b_2 = 0.5$ cm, and $\sigma_1/\sigma_2 = 10$; (e) Configuration 2, $b_1 = 0.7593$ cm and $b_2 = 0.4$ cm, and $\sigma_1/\sigma_2 = 10$; and (f) Configuration 3, $b_1 = 1.1390$ cm and $b_2 = 0.6$ cm, and $\sigma_1/\sigma_2 = 10$. These simulations are made for $\theta = 60^\circ$, $a = 0.0215$ cm, $V_o = +0.5$ V for the electrodes 2 and 3, $V_o = -0.5$ V for the electrodes 5 and 6, and $V_o = 0$ V for the electrodes 1 and 4. The parameters a , b_1 , b_2 (in centimeter) are converted to meter.

In order to get more accurate insight of electric current density (potential and electric field intensity) distribution inside tumor with complex geometries, 3D modeling is studied because, in general, the solid tumors are volumetric. In extending both analytical solution and computational techniques for electric current density from 2D to 3D additional complexities arise, not only because of the 3D geometries but also from the physical nature of the field itself. Also, in this complexity is involved the biological characteristics of the

tissues. 3D solutions are very expensive and should only be undertaken after simpler models have been explored, e.g. the 2D cross-section for the end region for a solid tumor. There are different analytical ways to calculate the electric current density (electric field) distributions in tissues, as the method based on Green's theorem (to obtain solutions inside defined volumes in terms of surface values of potential and the normal derivative of potential) and the Clifford analysis (it allows the matching of the electric fields across boundaries separating different conductivity regions with the help of the Clifford product) [Krüger & Menzel, 1996]. We have recently published the analytic solutions that visualize 3D stationary electric current density as a function of the electrode length, tumor size and the conductivities of the tumor (spheroid) and the surrounding healthy tissue (infinite medium) generated by a radial electrode array [Jiménez et al., 2011]. This mathematical formalism is only valid for electrodes inserted along tumor diameters. This particular electrodes configuration may be obtained from a mathematical theorem that allows the calculus of 3D electric current density generated by an array of electrodes with arbitrary shape inserted in an arbitrary region from 3D electric current density induced by a point current source. This guarantees that the electrodes may be inserted in any place of the tumor.

2.2.1 3D stationary electric current density generated by a wire from a point current source

There is a three-dimensional, conductive, heterogeneous region consisting of two linear, homogeneous, isotropic media separated by an interface Σ . Medium 1 of constant mean conductivity σ_1 (in S/m) and Medium 2 of constant mean conductivity σ_2 (in S/m) are considered as homogeneous conducting media, as shown in Figure. 5a for the point current source and in Figures. 5b,c for a wire of length L , which are inserted inside the Medium 1.

2.2.2 Point current source

We consider that current is continuous and the magnetic field associated to it may be neglected (≤ 0.02 Gauss) then the calculus of the potential φ in the point \vec{r} generated by a point current source with current intensity I located inside tumor in the point \vec{r}_0 (Figure 5a) yield to the following boundary-value problem, named Problem 0

$$\begin{cases} \nabla^2 \varphi_1 = -\frac{I}{\sigma_1} \delta(\vec{r} - \vec{r}_0) \\ \nabla^2 \varphi_2 = 0 \end{cases} \quad (1)$$

$$\begin{cases} \varphi_1|_{\Sigma} = \varphi_2|_{\Sigma} \\ \sigma_1 \frac{\partial \varphi_1}{\partial \hat{n}}|_{\Sigma} = \sigma_2 \frac{\partial \varphi_2}{\partial \hat{n}}|_{\Sigma} \\ \lim_{r \rightarrow \infty} |\varphi_2| < \infty \end{cases} \quad (2)$$

where $\varphi_i|_{\Sigma}$ and $\partial \varphi_i / \partial \hat{n}|_{\Sigma}$ ($i = 1, 2$) are the potential and its normal derivative in the surface Σ that separate both mediums.

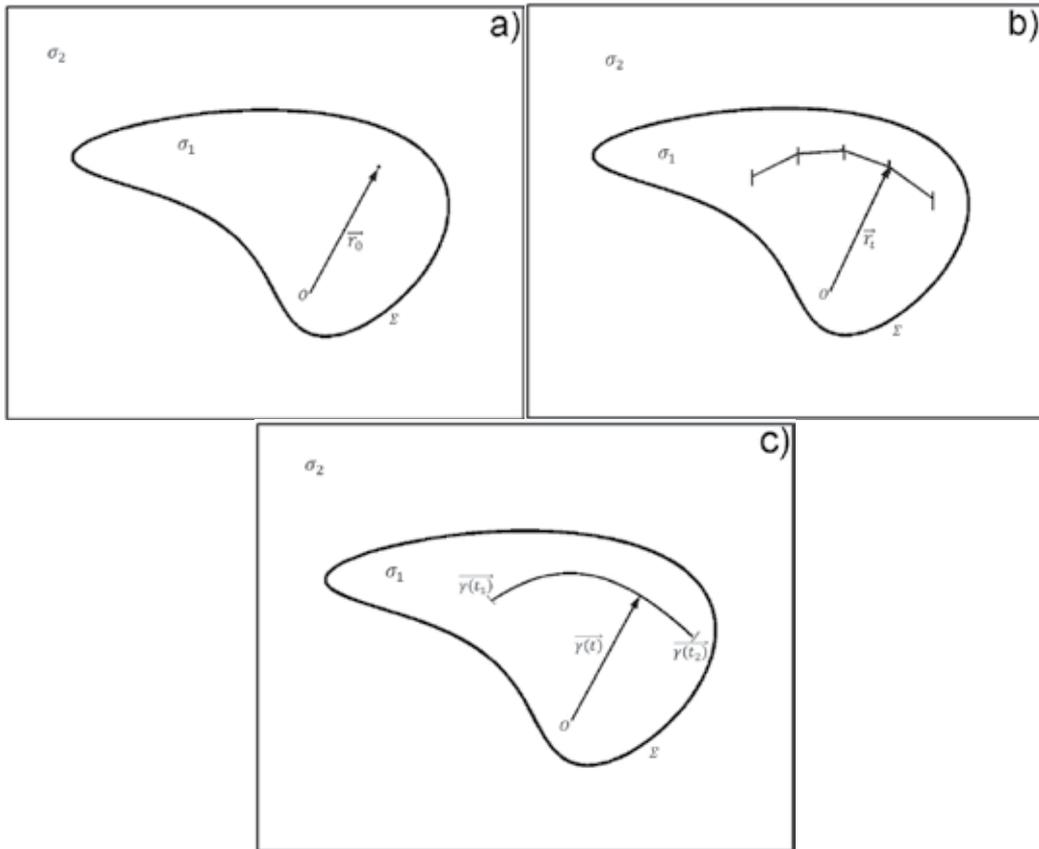


Fig. 5. Schematic representation of (a) a point electrode, (b) a wire subdivided in N small pieces of longitude δl and (c) a whole wire with ends $\vec{r}(t_1)$ and $\vec{r}(t_2)$ located in the Medium 1 of conductivity σ_1 surrounded of a Medium 2 of conductivity σ_2 . The axis y is perpendicular to the paper (toward us out of the page).

2.2.3 Electrode in form of wire of length finite

Instead of a point current source we now assume the case of a wire of length L (Figure 5c) analytically represented for the following parametric differentiable form of the curve $\vec{r}(t)$ of parameter t

$$\vec{r}(t) = (x(t), y(t), z(t)), \quad t \in [t_1, t_2] \tag{3}$$

The wire is subdivided in N small pieces of longitude δl (Figure 5b), so that the first equation of the system (1) can re-write as

$$\nabla^2 \psi_1 \approx -\frac{I}{\sigma_1 L} \sum_{i=1}^N \delta(\vec{r} - \vec{r}(t_i)) \delta l_i \tag{4}$$

with

$$L = \int_{t_1}^{t_2} \left\| \frac{d\vec{\gamma}}{dt} \right\| dt \tag{4a}$$

$$\left\| \frac{d\vec{\gamma}}{dt} \right\| = \sqrt{\left(\frac{dx}{dt}\right)^2 + \left(\frac{dy}{dt}\right)^2 + \left(\frac{dz}{dt}\right)^2} \tag{4b}$$

and

$$\delta l_i = \left\| \frac{d\vec{\gamma}}{dt} \right\|_{t=t_i} \Delta t \tag{4c}$$

where ψ_1 is the potential generated in the tumor by the wire electrode. $\left\| d\vec{\gamma}/dt \right\|$ is the modulus of the tangent to $\vec{\gamma}(t)$ in Cartesian coordinates, $\Delta t = (t_2-t_1)/N$ is the variation of t . When $\Delta t \rightarrow 0$ (in the limit $N \rightarrow \infty$) results the following boundary-value problem (named Problem 1)

$$\begin{cases} \nabla^2 \psi_1 = -\frac{I}{\sigma_1 L} \int_{t_1}^{t_2} \delta(\vec{r} - \vec{\gamma}(t_i)) \left\| \frac{d\vec{\gamma}}{dt} \right\| dt \\ \nabla^2 \psi_2 = 0 \end{cases} \tag{5}$$

$$\begin{cases} \psi_1|_{\Sigma} = \psi_2|_{\Sigma} \\ \sigma_1 \frac{\partial \psi_1}{\partial \hat{n}}|_{\Sigma} = \sigma_2 \frac{\partial \psi_2}{\partial \hat{n}}|_{\Sigma} \\ \lim_{r \rightarrow \infty} |\psi_2| < \infty \end{cases} \tag{6}$$

where ψ_1 and $\partial \psi_1 / \partial \hat{n}$ are the potential and normal derivative of the potential in Medium 1, respectively. ψ_2 and $\partial \psi_2 / \partial \hat{n}$ are these magnitudes but in Medium 2. \hat{n} is the unit normal vector to the surface Σ (directed from Medium 1 to Medium 2). δ is the Dirac delta. \vec{r} is the position of the spherical coordinate.

The solution of the Problem 1 may be expressed in a very simple way starting from the solution of the Problem 0 by means the following theorem

2.2.4 Theorem

Let be $\varphi_i(\vec{r}, \vec{r}_0)$, $i = 1, 2$, the solution of the Problem 0. Then, the solution of the Problem 1 is

$$\psi_i(\vec{r}) = \frac{1}{L} \int_{t_1}^{t_2} \varphi_i(\vec{r}, \vec{\gamma}(t)) \left\| \frac{d\vec{\gamma}}{dt} \right\| dt \tag{7}$$

The demonstration is immediate simply if we substitute (7) in (5) and (6). Substituting (7) in (5) results

$$\nabla^2 \psi_i(\vec{r}) = \nabla^2 \frac{1}{L} \int_{t_1}^{t_2} \varphi_i(\vec{r}, \vec{\gamma}(t)) \left\| \frac{d\vec{\gamma}}{dt} \right\| dt = \frac{1}{L} \int_{t_1}^{t_2} \nabla^2 \varphi_i(\vec{r}, \vec{\gamma}(t)) \left\| \frac{d\vec{\gamma}}{dt} \right\| dt \tag{8}$$

Making $\vec{r}_0 = \vec{\gamma}(t)$ in (1) and substituting in (8) result (5). Also, it can be demonstrated that (7) satisfies the boundary conditions (6).

To illustrate the theorem above mentioned, we use the particular case of a radial electrode array proposed in a previous study [Jiménez et al., 2011]. A three-dimensional, conductive, heterogeneous region consists of two linear, homogeneous, isotropic media (tumor and the surrounding healthy tissue) separated by an interface Σ . Solid tumor (Medium 1) is considered as a homogeneous conducting sphere of radius R (in m) and constant mean conductivity σ_1 (in S/m). The surrounding healthy tissue (Medium 2) is supposed to be a homogeneous infinite medium of constant mean conductivity σ_2 (in S/m), as shown in Figure 6a.

Point electrode and wire are inserted in plane $y = 0$ m along tumor diameters and in $(r_0,0,0)$, using the system of spherical coordinates with the origin in the center of the sphere, as shown in Figures 6b (one current source) and 6c (two current sources), respectively. In the particular case that both electrode types are located in the axis z (the radial coordinate in spherical coincides with axis z of the Cartesian coordinate or $(0,0,r_0)$).

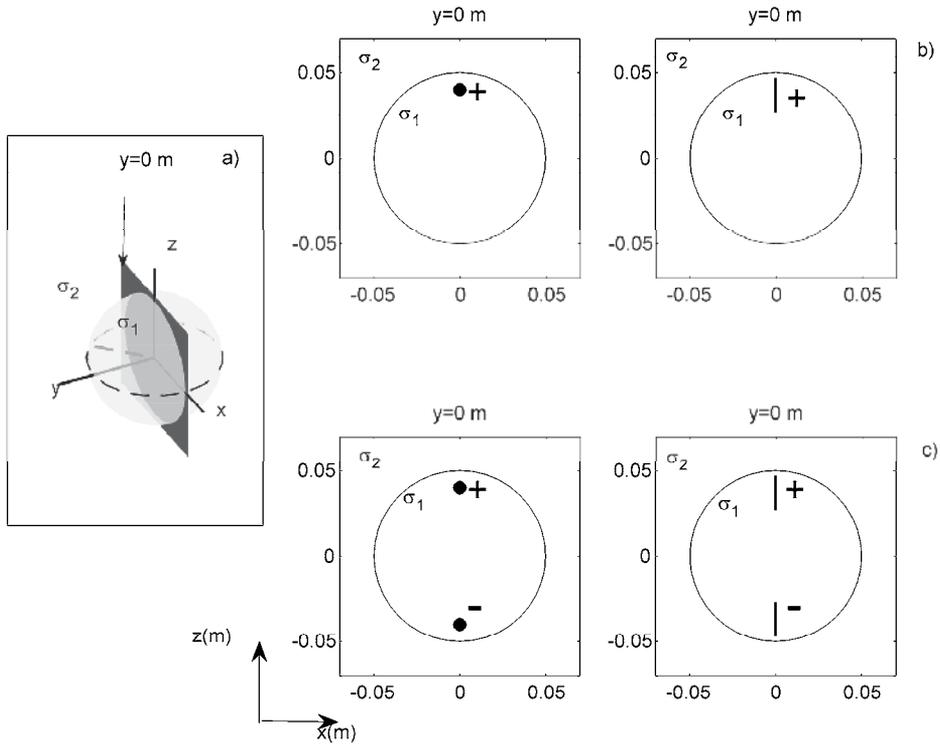


Fig. 6. (a) Spherical tumor of conductivity σ_1 (in S/m) and radius R (in m) surrounded by its healthy tissue of conductivity σ_2 (in S/m) and separated by the interface Σ . (b) A point electrode (positive) and an electrode with form of wire (positive) are inserted along tumor diameter (plane $y = 0$ cm). (c) Two point electrodes 1 (one positive and another negative) and two electrodes with form of wire (one positive and another negative) are inserted along tumor diameter (plane $y = 0$ cm).

As the point electrode is located in the tumor along the axis z (left picture in Figure 6a), the solution of the Problem 0 is given by

$$\varphi_1 = \frac{I}{4\pi\sigma_1} \frac{1}{\sqrt{r_0^2 + r^2 - 2rr_0 \cos \theta}} + \sum_{n=0}^{\infty} A_n r^n P_n(\cos \theta) \tag{9}$$

$$\varphi_2 = \sum_{n=0}^{\infty} B_n r^{-(n+1)} P_n(\cos \theta) \tag{10}$$

The coefficients A_n and B_n are obtained of the boundary conditions (2), resulting

$$\varphi_1 = \frac{I}{4\pi\sigma_1} \frac{1}{\sqrt{r_0^2 + r^2 - 2rr_0 \cos \theta}} + \frac{I}{4\pi} \left(\frac{\sigma_1 - \sigma_2}{\sigma_1} \right) \sum_{n=0}^{\infty} \frac{(n+1)r^n P_n(\cos \theta)}{R^{2n+1} [n\sigma_1 + (n+1)\sigma_2]} \tag{11}$$

$$\varphi_2 = \frac{I}{4\pi} \sum_{n=0}^{\infty} \frac{(2n+1)r^n P_n(\cos \theta)}{r^{(n+1)} [n\sigma_1 + (n+1)\sigma_2]} \tag{12}$$

Starting from (11) and (12), corresponding to a point electrode, we can pass to the solution of the potential generated by a wire with ends in a and b inserted in the tumor along the axis z (right picture in Figure 6b). In this particular case, the parametric equations of the wire are

$$\begin{cases} x = 0 \\ y = 0 \\ z = t \end{cases} \tag{13}$$

In this case, $L = b-a$ and $\|d\vec{y}/dt\| = 1$. Substituting (13), L and $\|d\vec{y}/dt\|$ in (7), we obtain the solutions for the wire, given by

$$\psi_1 = \frac{1}{(b-a)} \int_{t_1}^{t_2} \left[\frac{I}{4\pi\sigma_1} \frac{1}{\sqrt{t^2 + r^2 - 2rt \cos \theta}} + \frac{I}{4\pi} \left(\frac{\sigma_1 - \sigma_2}{\sigma_1} \right) \sum_{n=0}^{\infty} \frac{(n+1)t^n P_n(\cos \theta)}{R^{2n+1} [n\sigma_1 + (n+1)\sigma_2]} \right] dt \tag{14}$$

$$\psi_2 = \frac{1}{(b-a)} \int_{t_1}^{t_2} \frac{I}{4\pi} \sum_{n=0}^{\infty} \frac{(2n+1)t^n P_n(\cos \theta)}{r^{(n+1)} [n\sigma_1 + (n+1)\sigma_2]} dt \tag{15}$$

Integrating (14) and (15) from $t = a$ to $t = b$, we have

$$\begin{aligned} \psi_1 = \frac{I}{4\pi(b-a)} & \left\{ \frac{1}{\sigma_1} \ln \left[\frac{\sqrt{b^2 + r^2 - 2br \cos \theta} + b - r \cos \theta}{\sqrt{a^2 + r^2 - 2ar \cos \theta} + a - r \cos \theta} \right] \right. \\ & \left. + \left(\frac{\sigma_1 - \sigma_2}{\sigma_1} \right) \sum_{n=0}^{\infty} \frac{(b^{n+1} - a^{n+1}) r^n P_n(\cos \theta)}{R^{2n+1} [n\sigma_1 + (n+1)\sigma_2]} \right\} \end{aligned} \tag{16}$$

$$\psi_2 = \frac{I}{4\pi(b-a)} \sum_{n=0}^{\infty} \frac{(2n+1)(b^{n+1} - a^{n+1})P_n(\cos\theta)}{(n+1)r^{(n+1)}[n\sigma_1 + (n+1)\sigma_2]} \tag{17}$$

The superposition principle is used when two (Figs. 6c,d) or more current sources are inserted in the tumor. From (11), (12), (16) and (17) may be determined the electric field intensity ($\vec{E} = -\nabla\varphi$) and the electric current density ($\vec{J} = -\sigma\nabla\varphi$) inside and outside the tumor for a point current source (\vec{J}_{1p} and \vec{J}_{2p} are the current densities inside and outside, respectively) [Jiménez et al., 2011; Joa, 2010] and a wire of length L (\vec{J}_{1w}) and (\vec{J}_{2w} are the current densities inside and outside, respectively), which are given by

$$\left| \vec{J}_{ip} \right| = \sigma \sqrt{\left(\frac{\partial\varphi_i}{\partial r} \right)^2 + \left(\frac{\partial\varphi_i}{r\partial\theta} \right)^2} \quad (i = 1,2) \tag{18}$$

with

$$\frac{\partial\varphi_1}{\partial r} = \frac{I}{4\pi} \left\{ \frac{1}{\sigma_1} \frac{r - r_0 \cos\theta}{(r_0^2 + r^2 - 2rr_0 \cos\theta)^{3/2}} + \left(\frac{\sigma_1 - \sigma_2}{\sigma_1} \right) \sum_{n=0}^{\infty} \frac{(n+1)r_0^n r^{n-1} P_n(\cos\theta)}{R^{2n+1} [n\sigma_1 + (n+1)\sigma_2]} \right\} \tag{19}$$

$$\frac{\partial\varphi_1}{r\partial\theta} = \frac{I}{4\pi} \left\{ -\frac{1}{\sigma_1} \frac{r_0 \sin\theta}{(r_0^2 + r^2 - 2rr_0 \cos\theta)^{3/2}} + \left(\frac{\sigma_1 - \sigma_2}{\sigma_1} \right) \sum_{n=0}^{\infty} \frac{(n+1)r_0^n r^{n-1} T_n(\cos\theta)}{R^{2n+1} [n\sigma_1 + (n+1)\sigma_2]} \right\} \tag{20}$$

$$\frac{\partial\varphi_2}{\partial r} = -\frac{I}{4\pi} \sum_{n=0}^{\infty} \frac{(2n+1)r_0^n P_n(\cos\theta)}{r^{(n+2)} [n\sigma_1 + (n+1)\sigma_2]} \tag{21}$$

$$\frac{\partial\varphi_2}{r\partial\theta} = \frac{I}{4\pi} \sum_{n=0}^{\infty} \frac{(2n+1)r_0^n T_n(\cos\theta)}{r^{(n+2)} [n\sigma_1 + (n+1)\sigma_2]} \tag{22}$$

$$T_n = -\sin\theta \left[\frac{n \cos\theta P_n(\cos\theta) - n P_{n-1}(\cos\theta)}{\cos^2\theta - 1} \right] \tag{23}$$

where $\partial\varphi_i/\partial r$ and $\partial\varphi_i/r\partial\theta$ are the radial and angular components of electric field vector for inside ($i = 1$) and outside ($i = 2$) the tumor, respectively

$$\left| \vec{J}_{iw} \right| = \sigma \sqrt{\left(\frac{\partial\psi_i}{\partial r} \right)^2 + \left(\frac{\partial\psi_i}{r\partial\theta} \right)^2} \quad (i = 1,2) \tag{24}$$

with

$$\frac{\partial\psi_1}{\partial r} = \frac{I}{4\pi(b-a)} \left\{ \frac{A(a,b,r,\theta)}{\sigma_1} + \left(\frac{\sigma_1 - \sigma_2}{\sigma_1} \right) \sum_{n=0}^{\infty} \frac{n(b^{n+1} - a^{n+1})r^{n-1} P_n(\cos\theta)}{R^{2n+1} [n\sigma_1 + (n+1)\sigma_2]} \right\} \tag{25}$$

$$\frac{1}{r} \frac{\partial \psi_1}{\partial \theta} = \frac{I}{4\pi(b-a)} \left\{ \frac{B(a,b,r,\theta)}{\sigma_1} + \left(\frac{\sigma_1 - \sigma_2}{\sigma_1} \right) \sum_{n=0}^{\infty} \frac{(b^{n+1} - a^{n+1}) r^{n-1} T_n}{R^{2n+1} [n\sigma_1 + (n+1)\sigma_2]} \right\} \quad (26)$$

$$\frac{\partial \psi_2}{\partial r} = -\frac{I}{4\pi(b-a)} \sum_{n=0}^{\infty} \frac{(2n+1)(b^{n+1} - a^{n+1}) P_n(\cos \theta)}{r^{(n+2)} [n\sigma_1 + (n+1)\sigma_2]} \quad (27)$$

$$\frac{1}{r} \frac{\partial \psi_2}{\partial \theta} = \frac{I}{4\pi(b-a)} \sum_{n=0}^{\infty} \frac{(2n+1)(b^{n+1} - a^{n+1}) T_n}{(n+1)r^{(n+2)} [n\sigma_1 + (n+1)\sigma_2]} \quad (28)$$

where

$$A(a,b,r,\theta) = \frac{\frac{r - b \cos \theta}{\sqrt{b^2 + r^2 - 2br \cos \theta}} - \cos \theta}{\sqrt{b^2 + r^2 - 2br \cos \theta} + b - r \cos \theta} - \frac{\frac{r - a \cos \theta}{\sqrt{a^2 + r^2 - 2ar \cos \theta}} - \cos \theta}{\sqrt{a^2 + r^2 - 2ar \cos \theta} + a - r \cos \theta} \quad (29)$$

$$B(a,b,r,\theta) = \frac{\frac{b \sin \theta}{\sqrt{b^2 + r^2 - 2br \cos \theta}} + \sin \theta}{\sqrt{b^2 + r^2 - 2br \cos \theta} + b - r \cos \theta} - \frac{\frac{a \sin \theta}{\sqrt{a^2 + r^2 - 2ar \cos \theta}} + \sin \theta}{\sqrt{a^2 + r^2 - 2ar \cos \theta} + a - r \cos \theta} \quad (30)$$

T_n in (26) and (28) is given by (23). $\partial \psi_i / \partial r$ and $\partial \psi_i / r \partial \theta$ are the radial and angular components of electric field vector for inside ($i = 1$) and outside ($i = 2$) the tumor, respectively.

Figure 7 shows the isolines of $\left| \vec{J}_{1p} \right|$ and $\left| \vec{J}_{2p} \right|$ (Fig. 7a), and $\left| \vec{J}_{1w} \right|$ and $\left| \vec{J}_{2w} \right|$ (Figure 7b) in different planes of the sphere ($y = 0.1, 2$ and 4 cm) parallel to the plane that contains the electrodes ($y = 0$ cm) for a point current source and a wire, respectively. The point current source is located in $r_0 = 4$ cm (Figure 5a) whereas the wire with ends in $a = 1$ cm and $b = 4$ cm ($L = 3$ cm), as shown in Figure 5b. In both figures, we fix $\sigma_1 = 0.4$ S/m, $\sigma_2 = 0.2$ S/m, $I = 5$ mA and $R = 5$ cm. Figures 7a and 7b reveal that there are differences between the distributions of $\left| \vec{J}_{1p} \right|$ and $\left| \vec{J}_{1w} \right|$; however, non significant differences are observed between these distributions of $\left| \vec{J}_{1w} \right|$ and $\left| \vec{J}_{2w} \right|$ for different values of L . These differences are shown in Table 1 and are quantified by means of the maximum difference (D_{\max} , in A/m²) and the Root Means Square Error (RMSE, in A/m²), given by

$$D_{\max} = \max \left\| \left| \vec{J}_{wi} \right| - \left| \vec{J}_{pi} \right| \right\|, \quad (i = 1, 2) \quad (31)$$

$$RMSE = \sqrt{\sum_{i=1}^M \frac{\left(\left| \vec{J}_{wi} \right| - \left| \vec{J}_{pi} \right| \right)^2}{M}} \quad (i = 1, 2) \quad (32)$$

where J_{pi} are the i -th calculated values of $J_p(x,y)$ and J_{wi} are the i -th calculated values of $J_w(x,y)$ for different values of L (0.5, 1, 1.5, 2, 2.5 and 3 cm). M is the number of points where $\left| \vec{J}_{1p} \right|$ ($\left| \vec{J}_{1w} \right|$) and $\left| \vec{J}_{2p} \right|$ ($\left| \vec{J}_{2w} \right|$) are calculated.

\mathfrak{T}_1 ($\mathfrak{T}_1 = \sqrt{\sum_{k=1}^{m_1} \left| \vec{J}_{1k} \right|^2}$: sum of the local current density over all points in the tumor) and \mathfrak{T}_2

($\mathfrak{T}_2 = \sqrt{\sum_{k=1}^{m_2} \left| \vec{J}_{2k} \right|^2}$: sum of the local current density over all points in a region of the surrounding healthy tissue) are used in the figures to compare the overall effect of changing

of L on $\left| \vec{J}_{1p} \right|$ ($\left| \vec{J}_{1w} \right|$) and $\left| \vec{J}_{2p} \right|$ ($\left| \vec{J}_{2w} \right|$). \mathfrak{T}_1 and \mathfrak{T}_2 are evaluated in a set of discrete points m_1

and m_2 , respectively, for both point current source and wire. \mathfrak{T}_1 is calculated in all tumor volume ($m_1 = 84\ 050$ points) except in the points where the electrodes are inserted and in their vicinities. \mathfrak{T}_2 is also calculated in the surrounding healthy tissue ($m_2 = 35\ 301$ points) comprehended in a spherical cap (between R (5 cm) and $R + 2$ (7 cm)). Table 2 reveals that \mathfrak{T}_1 and \mathfrak{T}_2 for the point current source are higher than those for the wire for all value of L . For the wire, it is observed that an increase of L results in a decrease of \mathfrak{T}_2 whereas inside the tumor \mathfrak{T}_1 first decreases (up to $L = 2$ cm) and then increases. The behavior of \mathfrak{T}_1 with L is given in [Jiménez et al., 2011].

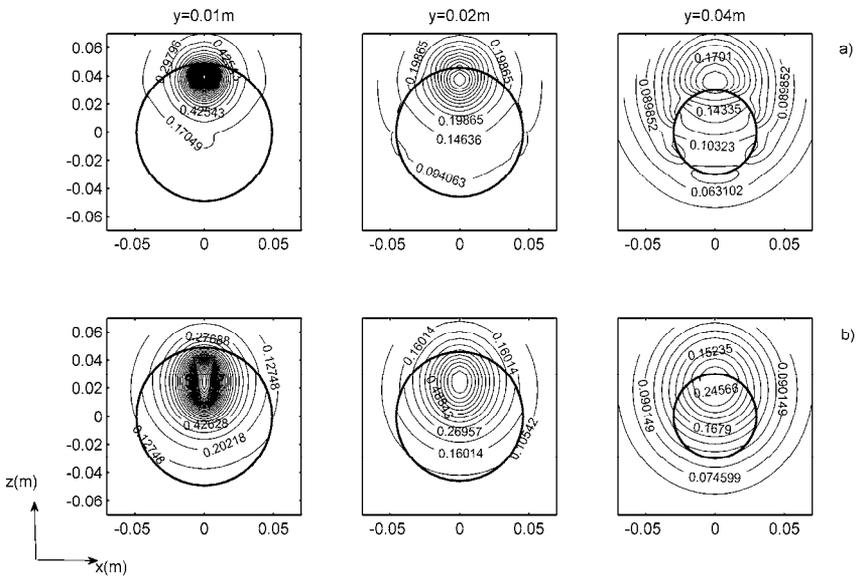


Fig. 7. Projections of $\left| \vec{J}_{1p} \right|$ (in A/m^2) and $\left| \vec{J}_{2p} \right|$ (in A/m^2) on planes $y = 0.1, 2$ and 4 cm for (a) one point electrode and (b) one electrode with form of wire ($\left| \vec{J}_{1w} \right|$, in A/m^2 , and $\left| \vec{J}_{2w} \right|$, in A/m^2). In each figure, the electrode polarity is positive (see Figure 6b).

Type of current source		Electric current density in the tumor (A/m ²)		Electric current density in the surrounding healthy tissue (A/m ²)	
		D _{max}	RMSE	D _{max}	RMSE
Point		0	0	0	0
Wire	L = 0.5 cm	39744.718	3.0283	0.537	0.0003
	L = 1 cm	39763.700	3.0296	0.877	0.0006
	L = 1.5 cm	39771.290	3.0302	1.103	0.0007
	L = 2 cm	39775.324	3.0305	1.262	0.0008
	L = 2.5 cm	39777.820	3.0307	1.379	0.0009
	L = 3 cm	39779.514	3.0309	1.468	0.0010

Table 1. D_{max} and RMSE of $\left| \vec{J}_{1w} \right|$ (in A/m²) and $\left| \vec{J}_{2w} \right|$ (in A/m²) for a wire of length L respect to $\left| \vec{J}_{1p} \right|$ (in A/m²) and $\left| \vec{J}_{2p} \right|$ (in A/m²) generated by a point current source. L varies from 0.5 to 3 cm.

Type of current source		\mathfrak{I}_1 (A/m ²)	\mathfrak{I}_2 (A/m ²)
Point		254800	63.080
Wire	L = 0.5 cm	1201.378	52.630
	L = 1 cm	1065.803	45.676
	L = 1.5 cm	1003.083	40.762
	L = 2 cm	996.252	37.128
	L = 2.5 cm	1045.207	34.344
	L = 3 cm	1182.477	32.155

Table 2. \mathfrak{I}_1 (norm of $\left| \vec{J}_{1p} \right|$ for a point electrode or $\left| \vec{J}_{1w} \right|$ for a wire) and \mathfrak{I}_2 (norm of $\left| \vec{J}_{2p} \right|$ for a point electrode or $\left| \vec{J}_{2w} \right|$ for a wire). The wire length is L (between 0.005 to 0.030 m). \mathfrak{I}_1 and \mathfrak{I}_2 are given in A/m².

The distributions of $\left| \vec{J}_{1p} \right|$, $\left| \vec{J}_{2p} \right|$, $\left| \vec{J}_{1w} \right|$ and $\left| \vec{J}_{2w} \right|$ for two point electrodes and two wires are shown in Figures 8a and 8b, respectively. The differences between these distributions are also quantified by means of D_{max} and RMSE (Table 3) and the values of \mathfrak{I}_1 and \mathfrak{I}_2 evaluated in the same discrete points m_1 and m_2 are given in Table 4. A comparison of Figures 7 and 8 reveals that an increase of the number of current sources (point or wire) results in a higher distribution of the electric current density lines in the tumor, being more evident for the electrodes in form of wire.

For the calculations of $\left| \vec{J}_{1p} \right|$, $\left| \vec{J}_{2p} \right|$, $\left| \vec{J}_{1w} \right|$, $\left| \vec{J}_{2w} \right|$, RMSE, D_{max}, \mathfrak{I}_1 and \mathfrak{I}_2 , the unities of y , a , b , L and R , given in cm, are converted to meter.

The 3D-analytical expressions shown in this chapter allow the visualization of the potential, electric field strength and electric current density distributions generated for point current

Type of current source		Electric current density in the tumor (A/m ²)		Electric current density in the surrounding healthy tissue (A/m ²)	
		D _{max}	RMSE	D _{max}	RMSE
Point		0	0	0	0
Wire	L = 0.5 cm	39744.681	4.2824	0.564	0.0005
	L = 1 cm	39763.672	4.2845	0.918	0.0008
	L = 1.5 cm	39771.267	4.2854	1.152	0.0011
	L = 2 cm	39775.306	4.2858	1.317	0.0012
	L = 2.5 cm	39777.807	4.2861	1.440	0.0014
	L = 3 cm	39779.507	4.2863	1.537	0.0015

Table 3. D_{max} and RMSE of $\left| \vec{J}_{1w} \right|$ (in A/m²) and $\left| \vec{J}_{2w} \right|$ (in A/m²) for an array of two equal wires with different lengths L (between 0.5 to 3 cm) respect to those generated by an array of two point electrodes ($\left| \vec{J}_{1p} \right|$, in A/m², and $\left| \vec{J}_{2p} \right|$, in A/m²).

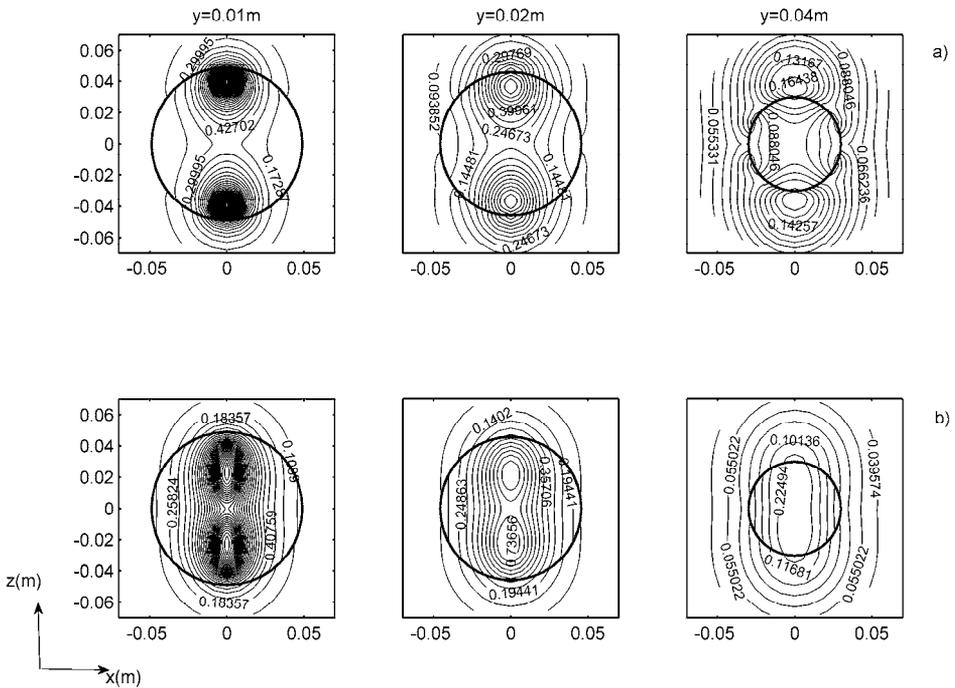


Fig. 8. Projections of $\left| \vec{J}_{1p} \right|$ (in A/m²) and $\left| \vec{J}_{2p} \right|$ (in A/m²) on planes $y = 0.1, 2$ and 4 cm for (a) two point electrodes and (b) two electrodes with forms of wire ($\left| \vec{J}_{1w} \right|$, in A/m², and $\left| \vec{J}_{2w} \right|$, in A/m²). In each figure, one electrode is positive and another is negative (Figure 6c).

Type of current source		\mathfrak{I}_1 (A/m ²)	\mathfrak{I}_2 (A/m ²)
Point		360340	82.912
Wire	L = 0.5 cm	1702.887	66.861
	L = 1 cm	1512.207	55.909
	L = 1.5 cm	1424.731	47.906
	L = 2 cm	1416.607	41.710
	L = 2.5 cm	1488.047	36.679
	L = 3 cm	1685.969	32.432

Table 4. \mathfrak{I}_1 (norm of $\left| \vec{J}_{1p} \right|$ for two point electrodes or $\left| \vec{J}_{1w} \right|$ for two wires) and \mathfrak{I}_2 (norm of $\left| \vec{J}_{2p} \right|$ for two point electrodes or $\left| \vec{J}_{2w} \right|$ for two wires). The wires are equals and have different lengths L (from 0.5 to 3 cm). \mathfrak{I}_1 and \mathfrak{I}_2 are given in A/m².

sources and radial arrays of electrodes with finite length. The results of the simulations reveal as these distributions in the tumor and its surrounding healthy tissue change in function of the tumor size, the positioning, number and polarity of the electrodes, and the difference of electrical conductivity between both tissues for, in agreement with previous theoretical studies [Aguilera et al., 2009; Aguilera et al. 2010; Ćorović et al., 2007; Jiménez et al., 2011; Joa, 2010; Reberšek et al., 2008; Šel et al., 2003] and experimental reports [Chou et al., 1997; Ren et al., 2001; Serša et al., 1997; Turler et al., 2000; Xin et al., 1994; Yoon et al., 2007].

3D-analytical expressions for the potential, electric field intensity and electric current density generated by wires completely inserted in the tumor along their diameters (plane $y = 0$ cm) are directly obtained from the application of this mathematical theorem and the parametric form of the curve given in equation (3). This justify that equations (16) and (17) are correct from the substitution of I for $\delta I = I/(b-a)dr_0$ in equations (14) and (15) and then integrating these expressions from $r_0 = a$ to $r_0 = b$, as is suggested in a previous study [Jiménez et al., 2011].

Non-uniform current density distributions are shown in a tumor (homogeneous conductor spheroid), as shown in Figures 7 (point electrodes) and 8 (electrodes with forms of wire). Normally, needles electrodes have highly non-homogeneous fields around their tips due to the sharp geometry. There are marked differences between the electric current density (potential and electric field) patterns generated by a point electrodes array and arrays of electrodes with length L, as is expected. Electric current density near the electrodes is also imaged for both point and wire arrays. Although the electric current density is maximum near electrodes, the magnitude of it fall even more rapidly towards the tumor edges in the perpendicular direction to the plane in which are the electrodes. Tumor regions unaffected by this electric current density re-grow after treatment. The singularities observed where the electrodes contact the tumor (large electric fields at the edges) can be avoided by grading the electric field near such edges. High current densities in the vicinity of the electrodes may result in tissue damage (example, coagulative necrosis), in agreement with our observations in mice [Cabrales et al., 2001; Ciria et al., 2004] and patients [Jarque et al., 2007]. Moreover, measurement of current density distribution near the current injecting electrodes provides information on the behavior of the electrode-tissue interface. Up to now, it has not discussed as depends on the electrotherapy antitumor effectiveness in function of the homogeneity

degree of the electric current density (electric field) induced in the tumor, essential aspect in the design of electrodes array and treatment planning.

The insertion of the electrodes along tumor diameters is a particular electrodes array that we have used in some patients whose tumor thickness (depth) is smaller respect to their other two dimensions (skin, breast and vulva cancers) when the conventional therapies fail or cannot be applied, as shown in Figure 9. A direct current of 10 mA for 60 min is delivered to this patient with vulva cancer through 19 electrodes inserted with alternate polarities and it is generated by ZAY-6B electric device (manufactured in Chinese). Cannulae with trocar are inserted into the tumor mass under local anesthesia and the number of these depends on the tumor size (20 cm in diameter). The cannulae are fixed with a distance (gap) between them of 1 cm and disposed along a semi-circumference because for this zone pass important blood vessels. This distance should not be further than 1.5 cm apart because the tumor killing area around the needle is about 2 cm in diameter. Then the trocar are withdrawn and electrodes are inserted into the tumor through the cannulae to ensure that the electric field will cover all the tumor mass when the direct current passes through electrodes. After insertion of the electrodes, the cannulae are withdrawn to the edge of normal tissue by palpation with hand. These cannulae in the edge are insulation tubes to protect the normal tissue from the injury due to electrolysis. This procedure guarantees that the electrodes are completely inserted into the solid tumor to maximize tumor destruction with the minimum damage in the organism. The electrodes are then connected to the cathode or anode of the ZAY-6B device to supply the direct current that pass through the solid tumor. This procedure guarantees that the electrodes are completely inserted into the tumor. We use platinum needles because these are resistant to erosion and have high electric conductivity. The diameter of the needles is 0.07 cm and length 15 cm, values that justify why we assume in this mathematical approach that the electrode cross section is neglected respect to its length [Jiménez et al., 2011]. Saline solution and bleomycin are intratumor injected before and immediately after direct current application, respectively. It is made with the aim to potentiate the electrotherapy antitumor effectiveness, fact that is theoretically verified when the tumor conductivity increases with respect to that of the surrounding healthy tissue because the electric current density lines mainly distribute inside tumor and its periphery.

We observe that as soon as direct current is connected to the electrodes, different electrochemical reactions influence the pH-value and can cause electrolysis of tumor tissue, which in turn, lead to the destruction of it. The tumor regression induced by this electrodes array is approximately 50 % one month after the application of this therapy. Minima adverse effects (events) are observed after direct current application, probably due to that the electrodes are inside tumor and this therapy is local. We are not observed immediate adverse events (first 24 hours after electrotherapy is applied); however, we have reported late adverse events (after 24 hours of applied electrotherapy), such as: necrosis on the ulcerated surface, erythema and slight edema at the area treated, inflammation because the cancerous tissue is being destroyed through this method of treatment. Immediately after treatment, we do not observe pain, fever, superinfections. The destroyed cancerous tissue is eliminated from the body and is replaced by scar tissue and then in the majority of the patients, we observe tissue granulation when the tumor is removed after this treatment [Jarque et al., 2007]. Similar results are reported in laboratory animal [Cabrales et al., 2001; Ciria et al., 2004; Haltiwanger, 2008; Mikhailovskaya et al., 2009; Sazgarnia et al., 2009; Vjih, 2006] and human [Arsov et al., 2009; Haltiwanger, 2008; Jarque et al., 2007; Li et al., 2006; Salzberg et al., 2008; Vjih, 2006; Vogl et al., 2007; Xin et al., 2004; Yoon et al., 2007].



Fig. 9. Patient with vulva cancer treated with electrotherapy.

The use of this electrodes array stops the bloody flux of this patient for the vulva immediately after the electrotherapy application due to the haemostatic effect of the cathode. This fact may be explained because the cathode produces a tissue desiccation and therefore a control of the hemorrhage, in agreement with other results that demonstrate that the tumor blood flow is reduced by direct current action, fact that can be exploited to improve therapeutic outcome. It is well known that reductions in tumor blood flow can lead to an increase in hypoxia and extracellular acidification and as a result a cascade of tumor cell death will occur, due to a lack of nutrients, oxygen and an accumulation of catabolite products [Griffin et al., 1994; Haltiwanger, 2008; Xin et al., 2004]. As a result of this, this patient does not receive more blood transfusions post-treatment. This patient dies one year after the electrotherapy application due to multiple metastases in brain, lung and liver.

In electrotherapy, the electrodes are generally inserted outside of the central plane [Cabralés et al., 2001; Cabralés et al., 2010; Ciria et al., 2004; Chou et al., 1997; Jarque et al., 2007; Ren et al., 2001; Turler et al., 2000], constituting a limitation of the use of this radial electrodes array. This mathematical theorem solves the Problem 1 from the Problem 0 both harder and elastic needles. The solution of this problem becomes difficult in dependence on the complexity of this parametric form of the curve since more arduous is to solve the integral that appears in Equation (7). The simulations clearly demonstrate that analytical model is reliable and useful to search new electrode arrays that induce the highest electrotherapy effectiveness. New 3D-mathematical formalisms are obtained in dependence of the parametric curve form (Equation 3), which allow the insertion of the electrodes (hard or flexible) in any place of the tumor with arbitrary shape. This leads to solve problems more complex than that shown in a previous study [Jiménez et al., 2011] and to compare their electric current densities with those generated by other electrode arrays [Cabralés et al., 2001; Ciria et al., 2004; Chou et al., 1997; Jarque et al., 2007; Jiménez et al., 2011; Joa, 2010; Xin et al., 2004; Yoon et al., 2007].

Different authors report that there is a good correlation between the electric current density spatial distributions observed with different imaging techniques, those obtained by means of analytical and numerical solutions and experimental results [Miklavčič et al., 1998; Serša et al., 1997]. Among these techniques may be mentioned the Electric Current Density Imaging [Halter et al., 2007; Serša et al., 1997], Electrical Impedance Tomography [Saulnier et al., 2001], Magnetic Resonance Electrical Impedance Tomography [Seo et al., 2005], Magnetic Induction Tomography, Magnetoacoustic Tomography and Magnetoacoustic Tomography with Magnetic induction [Li et al., 2007]. These imaging techniques are useful to map spatial distribution of electric currents generated for any electrodes array in the tumor and its surrounding healthy tissue and to visualize the changes on electric current density patterns when the electrodes array parameters above mentioned are modified. These imaging techniques provide information on electrical conductivity inside an electrically conducting domain such as the human body and evidence that electric current density strongly depends on the placing, polarity and geometry of the electrodes, in agreement with our simulations. The quantification of the differences between the electric current densities obtained theoretically and experimentally is possible by means of an element average error (e) that can be evaluated by computing the integral over the element of the difference between the current density determined directly from the analytical expression (J_a) and the nodally averaged (interpolated) current densities over the region of support V_s (J_s), given by $e = \int_{V_s} (j_a - j_s) dV$. For this, it should be used the information provided by neighboring nodes to evaluate the magnitude of the higher order terms in the solutions that have been neglected.

These facts indicate that these imaging techniques may be used to know as change the electrical conductivity and current density distribution before, during and after electrotherapy. As a result of this fact, we have an idea of the structural, functional and pathological conditions of the tissue and therefore provide valuable diagnostic information. For this reason, we include in the electric current density the information of the electrical conductivities of the tumor and surrounding healthy tissue, whose mean values may be measured by means of such imaging techniques above mentioned [Li et al., 2007; Saulnier et al., 2001; Halter et al., 2007; Seo et al., 2005; Serša et al., 1997]. This justifies why the bulk conductivities of both tissues are assumed constant in our mathematical approach. The bulk electrical conductivity values of heterogeneous and anisotropic tissues may also be calculated, with good approximation, by means of their electric conductivity tensor mean values [Sekino et al., 2009]. We believe that the higher electric conductivity of the tumor is along of the preferential direction of growth (major diameter of tumor with ellipsoidal shape); however, an experiment should be designed to demonstrate this hypothesis. Although the majority of the solid tumors are heterogeneous, all are homogeneous for volumes $\leq 3 \text{ cm}^3$. Also, there are very few types of tumors (adenomas, adenocarcinomas, breast ductal carcinomas and sarcomas) with volumes $> 3 \text{ cm}^3$ that are homogeneous, fact explained because is only observed tumor mass due to the equilibrium between the growth and the tumor cells angiogenesis. When this equilibrium is broken, the tumors make more heterogeneous due to the presence of necrosis, infiltration to tissues, among other alterations.

The fact that tumor conductivity is assumed higher than that its surrounding healthy tissue is justified because neoplastic tissues exhibit somewhat larger conductivity and permittivity values than homologous normal tissues due to that the water content is higher in the tumor

[Foster & Schwan, 1996; Haemmerich et al., 2003; Haemmerich et al., 2009; Miklavčič et al., 2006; Ng et al., 2008; S.R. Smith et al., 1986, D.G. Smith et al., 2000]. We believe that the presence of other charged particles (molecules, ions and electrons) and blood vessels (due to the angiogenic process) may also increase the tumor conductivity and therefore more current flows for the tumor, as we corroborate with the simulations shown in this chapter. These simulations have not included the effects that produce the direct current application on the tumor electric conductivity (permittivity); however, it should change during and after electrotherapy application. This may be due to that in the tumor are induced changes in the ions concentration in the intracellular and extracellular fluids [Griffin et al., 1994], structure and cellular density [Vijh, 2006; Von Euler et al., 2003], molecular composition [Von Euler et al., 2003], in the cellular membrane [Vodovnik et al., 1992; Yoon et al., 2007], among others. For instance, it has been demonstrated that the tumor conductivity changes before and after of the tumor thermal ablation [Haemmerich et al., 2009]. The changes of the electric conductivity may be one of the indicators of tissues conditions (anatomical and functional) [Seo et al., 2005]. We believe that for electric current densities (electric field intensities) below the reversible threshold value should not change significantly the tumor conductivity, not occurring thus above this threshold. This may be in correspondence with the tumor re-grow observed for $i/i_0 < 2$ and the stationary partial and complete responses for $i/i_0 \geq 2$, as shown in Figures 1 and 2 [Cabral et al., 2008]. Hence, a detailed study should be carried out to know the explicit dependence between the electrical conductivity and the physiological parameters of the tumor. This may be used to establish an index for the prediction of the possible evolution of the patient during and after the direct current application (alone or combined). Also, an improved understanding of the theoretical basis of this dependence will enable structural features of the tumor tissue to be deduced from the experimental measurements.

Although it is assumed that the fields and charges are non-time varying, and the magnetic field due to the current and the reaction of this field on the current, we do not discard that magnetic field due to the direct current intensity produce bioeffects in the tumor, mainly around electrodes, in agreement with other authors [Saulnier et al., 2001; Seo et al., 2005]. It is possible that this induced magnetic field induces mechanical forces and shear stresses in the tumor that in dependence of its strength and duration may also produce a wide variety of biological effects in cells and tumor tissue, such as: electrodiffusion/osmosis (various receptors, charged membrane molecules, can be transported along the cell surface) and change in transmembrane potential (voltage-gated channels may be opened to permit the transport of ions, such as calcium, into the cell) [Hart, 2008].

It is important to point out that the images obtained with these experimental techniques are important because reveal that the spatial distribution of electric currents do not depends only on electrode array but, also on their tissue contact, which is hard to control [Foster, 1995; Serša et al., 1997]. The interaction electrode-tumor is not considered; however, it may have an important role in the skin heating. This heating is determined both by palpation with the hand and skin erythema of the patients with tumors treated with direct current [Jarque et al., 2007]. The tissue near of electrodes is heated mainly by the absorbed electrical energy, while regions further away may be heated by thermal conduction and/or some biophysic (electrochemical processes) induced into the tumor. As a consequence, the preferential heating of the tumor is governed by the electrical parameters near the electrode, whereas thermal parameters become increasingly important further away. An analysis of the energy (heat) absorbed by the tumor and its surrounding healthy tissue may be another

way to select these optima parameters. The absorbed energy can be calculated by means of the expression $Q_{1,2} = \int (j_{1,2}^2 / \sigma_{1,2}) dV$, where Q , j and σ are the absorbed heat quantity, electric current density and electric conductivity in the medium, respectively. The sub-indexes 1 and 2 represent to the medium 1 (tumor) and medium 2 (surrounding healthy tissue). Similar analyses of the absorbed energy quantity are carried out in radiotherapy and hyperthermia [Hall, 1988; Sadadcharam et al., 2008; Schaefer et al., 2008]. On the other hand, as the difference in electrical parameters between the tumor and its surrounding healthy tissue is substantial that might result in preferential heating of the tumor. This heating in dependence of its duration and intensity may be a result of the increase of the intratumor temperature that may provoke changes either directly or indirectly in the tissue dielectric properties and therefore irreversible damages in it [Foster, 1995]. The temperature dependence of the electrical conductivity may be related with damages in the tumor. In order to correctly mimic the absorbed energy in the tumor and its surrounding healthy tissue during electrotherapy exposure, a three dimensional model must be used.

Model tumor system as a spheroid is assumed for the following reasons: 1) the spheroid system has been applied to a number of problems in cancer and it is a model of a solid tumor in vitro and in vivo. 2) This system mimics many of these tumor characteristics and provides a rapid, useful, and economical method for screening sensitizers and chemotherapeutic agents because it is intermediate in complexity between single-cell in vitro culture and tumors in experimental animals. 3) The spheroid system is simpler, more reproducible, more economical, and easier to manipulate than animal tumors, and yet the cells can be studied in an environment that includes the complexities of cell-to-cell contact and nutritional stress from diffusion limitations that are characteristic of a growing tumor. 4) Some cells, notably several rodent tumor cell lines, such as chinese hamster V79 lung cells, mouse EMT6 mammary and R1F fibrosarcoma cells, and rat 9L brain tumor cells grow as spheroids. At each successive division the daughter cells stick together, and the result is a spherical clump of cells that grows bigger and bigger with time. 5) Many types of human tumor cells can be cultured as spheroids with a wide spectrum of morphological appearance and growth rates. 6) Human tumor cell spheroids maintain many characteristics of the original tumor from the patient or of the some cells grown as xenografts. Human tumors successfully grown as spheroids include thyroid cancer, renal cancer, squamous carcinoma, colon carcinoma, neuroblastoma, human lung cancer, glioma, lymphoid tumors, melanoma, and osteosarcoma [Hall, 1988].

A feasible way to optimize 3D-electrode arrays is combining all the electrodes array parameters such that the electric current density in the tumor is the permissible maximum and that induced in the surrounding healthy tissue is smaller than 10 mA/m². For this, we suggest the following procedure: first, the parameters are automatically selected so that the electric current density in the surrounding healthy tissue is smaller than 10 mA/m². This guarantees the safety of the electrotherapy (Phase I of a Clinical Trial) and that the adverse effects in the organism are minima (Phase II of a Clinical Trial). Second, the selection of the optimum parameters depends on the electric current density (electric field strength) that induces the biggest tumor destruction (Phase III of a Clinical Trial), which may be experimentally verified by means of an experiment, in which is obtained the higher electrotherapy antitumor effectiveness, the maximum survival and life quality of the patient (laboratory animal). It evaluates the contributions of each parameter (alone or combined with other) and requires a significant consumption of calculation time for the parameters

quantity involved in these equations. For the implementation of this way, we should take into account that exposure of a biological cell to electric current density (electric field) can lead to a variety of biochemical and physiological responses. For this, it is required to know what electric current density values provoke significant biological effects: below 1 mA/m² (there are not biological effects); 1-10 mA/m² (minimum biological effects, which are not significant); 10-100 mA/m² (possible biological effects without risk to the health); 100-1000 mA/m² (biological effects without possible risk to the health) and above 1000 mA/m² (biological effects with proven risks to health) [International Commission on Non-Ionizing Radiation Protection, 1998]. This is very important because the electrotherapy effectiveness is highly depend on the magnitude and spatial distribution of electric currents flowing through the tumor and its surrounding healthy tissue, in agreement with other authors [Serša et al., 1997].

The knowledge of the optimum distributions of current density vector in the tumor and its surrounding healthy tissue allows the optimum design of noninvasive electromagnetic techniques for the cancer treatment. Several authors report that the ultralow-frequency extremely weak alternating component of combined magnetic fields exhibits a marked antitumor activity [Novikov et al., 2009]. These fields will avoid the insertion of electrodes in the tumor and therefore the little trauma that this provoke in the patients treated with electrotherapy. Weak magnetic fields activate the system of antitumor immunity (i.e., production of Tumor Necrosis Factor, activation of macrophages, among other) and produce reactive oxygen species, as is also observed on electrotherapy [Cabrales et al., 2001; Serša et al., 1994; Serša et al., 1996; Watenberg et al., 2008].

Different authors have experimentally evaluated the influence of direct current intensity [Ciria et al., 2004; Cabrales et al., 2010; Chou et al., 1997; Ren et al., 2001; Xing et al., 2004] and electromagnetic field [Novikov et al., 2009] on tumor growth kinetic; however, the weight of the different parameters of an electrodes array in it has not been widely discussed. Consequently, it is possible to simultaneously know, previous treatment, the possible tumor evolution in the time and the electric current density (potential, electric field intensity) distributions in the tumor and its surrounding healthy tissue and therefore the highest electrotherapy antitumor effectiveness. This improving therapy may be obtained when the tumor reaches its complete cure (complete remission or stationary partial response) [Cabrales et al., 2008; Cabrales et al., 2010] or the higher tumor growth delay (highest survival of the patients with good life quality and/or bigger disease free interval) [Ciria et al., 2004; Chou et al., 1997; Jarque et al., 2007; Ren et al., 2001; Xin et al., 2004; Yoon et al., 2007]. The evaluation of it requires to quantify different biological parameters, such as: tumor regression percentage, mean doubling time, survival rate, antibody responses, cellular responses, apoptosis, necrosis, histological examination, immune responses, and gene expressions, among others. This will contribute to elucidate the direct current antitumor mechanism.

The tumor complete response suggests that its growth kinetic is completely reversible, as shown in Figure 10 for fibrosarcoma Sa-37 tumor [Cabrales et al., 2010]. This behaviour is obtained from the experimental data [Ciria et al., 2004] and the analysis of the first and second parts of the tumor growth kinetic by means of the use of the modified Gompertz equation [Cabrales et al., 2010]. The first part comprehends the time that elapses from the initial moment at which tumor cells are inoculated in the host ($t = 0$ days) up to 15 days that is the moment of direct current application, when the tumor volume reaches $V_0 = 0.5 \text{ cm}^3$, whereas the second part is the time that elapses from V_0 up to the end of the experiment,

that is, time after direct current application. Both parts are obtained using the interpolation process and the time step is $\Delta t = 1/3$ days. The parameters i , α , β , γ and i_0 are above defined and obtained from fitting the experimental data [Cabral et al., 2008]. The analysis of these two parts reveals the existence of two tumor volumes that suggests the unavoidable tumor destruction: in the first, the tumor does not return to its state before direct current treatment, V_{id} , and therefore complete (stationary partial) response is reached. In the second, the small fraction tumor that survives to direct current action is completely destroyed by the organism, V_d , aspect that may suggest that the therapies for the cancer, including the electrotherapy, should be directed to that the tumor always reaches V_d . New investigations have been derived starting from this hypothesis.

In the case that complete remission (stationary partial response) of tumor is not reached after alone direct current stimulus, the electrotherapy should be directed to increase the survival and quality of life of patients (laboratory animals). First this, we should know the exact time in that electrotherapy may be repeated (time for which the tumor volume is between V_0 and the minimum volume observed in tumor growth, V_{min}), as shown in Figure 11 [Cabral et al., 2010]. When the tumor volume reaches this value there is a change of both slope and sign of the first derivate of the tumor volume (corresponding to minimum value of this first derivate), aspect that may indicate a tumor response (reorganization and/or activation of the growth and protection mechanisms) to the direct current action, whose intensity is not adequate to significantly perturb to it. This fact may be explained because the biological systems, as the tumors, respond to the external perturbations in order to reach their maxima survival. As a result, the electrotherapy should be repeated or combined with other therapies when the first derivate of the tumor volume changes of slope and sign because the tumor cannot be reorganized, in agreement with the current tendency of repeating weekly (every fifteen days) this therapy of 2 to 4 times. This constitutes a novel statement because establishes that this therapy should not be applied when the tumor volume reaches V_{min} , as is implemented for the treatment of patients at present [Jarque et al., 2007; Xin et al., 2004]. It is possible a fractionated therapy may lead to the complete (stationary partial) remission.

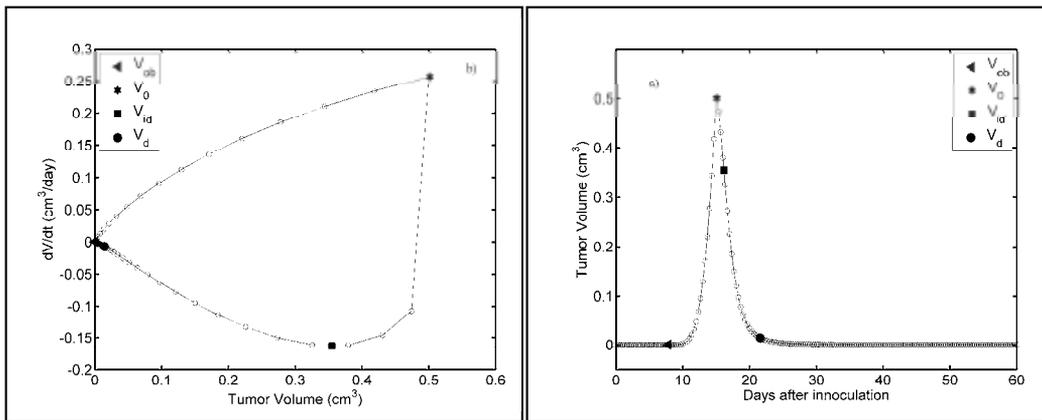


Fig. 10. Direct current-perturbed fibrosarcoma Sa-37 tumor growth kinetic for the parameters: $V_0 = 0.5 \text{ cm}^3$, $i = 14.8 \text{ mA}$, $\alpha = 0.006 \text{ days}^{-1}$, $\beta = 0.207 \text{ days}^{-1}$, $\gamma = 0.189 \text{ days}^{-1}$, $i_0 = 1.080 \text{ mA}$ and $\Delta t = 1/3$ days. Time dependence of tumor volume (left picture). First derivate of tumor volume versus tumor volume (right picture).

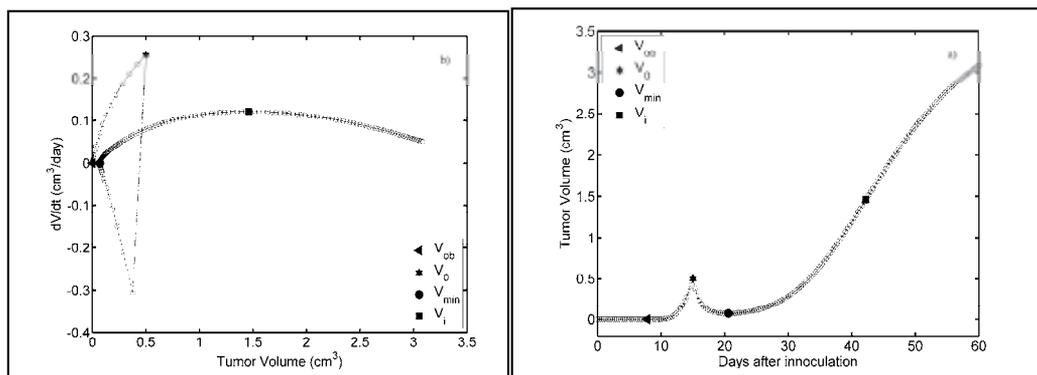


Fig. 11. Direct current-perturbed fibrosarcoma Sa-37 tumor growth kinetic for the parameters: $V_0 = 0.5 \text{ cm}^3$, $i = 11.7 \text{ mA}$, $\alpha = 1.584 \text{ days}^{-1}$, $\beta = 0.076 \text{ days}^{-1}$, $\gamma = 0.107 \text{ days}^{-1}$, $i_0 = 7.431 \text{ mA}$ and $\Delta t = 1/3 \text{ days}$. Time dependence of tumor volume (left picture). First derivate of tumor volume versus tumor volume (right picture).

The inclusion of the other electrodes array parameters, in addition to direct current intensity, in the tumor growth kinetic may efficiently lead to complete (stationary partial) response for smaller direct current intensities.

The knowledge of these two parts of the tumor growth kinetic is important to reveal further information of it and in the therapeutic planning, as is widely discussed in a previous study. Similar results for fibrosarcoma Sa-37 tumor are also found Ehrlich tumor (results not shown) [Cabralés et al., 2010].

By optimizing the electrodes array parameters and those of the tumor growth kinetic (for instance, modified Gompertz equation), the efficiency of electrotherapy might be improved further. This procedure will have to be tested in larger animals to assess the usefulness and safety of electrotherapy in vivo for the future application to humans.

In spite of the considerable progress of electrotherapy, a number of challenges remain for the future. The future strategies include (a) increasing the volume of destroyed tissue at a single treatment session (b) the integration of electrotherapy with the other in-site tumor antitumor techniques and (c) the necessity of to incorporate realistic geometric, conductivity, and, eventually anisotropic information in order to reach the highest electrotherapy effectiveness.

3. Conclusion

In conclusion, electrotherapy of low-level direct current is promissory for cancer treatment. The electric current density (potential and electric field strength) analysis results can be used for assessing effective treatment parameters of tumor. The use of this mathematical approach and the theorem provide a rapid way to propose different optimum electrode arrays in dependence of location, depth, shape and size of the solid tumors with the purpose of obtaining the higher antitumor effectiveness and as a result to implement the electrotherapy in the Clinical Oncology.

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Part 4

Complementary / Alternative Cancer Therapy Modalities

Antioxidants in Cancer Treatment

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1. Introduction

There are many different chemotherapeutic agents used in cancer treatment. Most of the chemotherapeutic drugs can be divided into alkylating agents, antimetabolites, anthracyclines, plant alkaloids, topoisomerase inhibitors, and other antitumour agents. All of these drugs affect cell division or DNA synthesis and function in some way. Several classes of chemotherapy work by producing a reactive oxygen compound or free radical.

Alkylating agents work to add alkyl groups to negatively-charged groups. They are known to stop tumor growth through cross-linking guanine nucleobases in strands of DNA, which directly damages the DNA by making it unable to uncoil and separate. The cell, when attacked in this way, is unable to replicate. While it may not die, it also cannot grow. Cyclophosphamide, a cytotoxic alkylating agent, is extensively used as an antineoplastic agent for the treatment of haematological malignancies and a variety of solid tumours, including leukaemia, ovarian cancer and small-cell lung cancer. Cyclophosphamide is bioactivated by hepatic cytochrome P450 enzymes resulting in the formation of phosphoramidate mustard and acrolein. The therapeutic effect of cyclophosphamide is attributed to phosphoramidate mustard, while the other metabolite, acrolein is associated with toxic side effects. The cellular mechanism of cyclophosphamide toxicity is due to the production of highly reactive oxygen free radicals by these metabolites. It is obvious that high levels of ROS within the body could culminate in oxidative stress.

Anthracyclines (or anthracycline antibiotics) are a class of drugs used in cancer chemotherapy derived from *Streptomyces* bacteria. Anthracycline has three mechanisms of action: inhibits DNA and RNA synthesis by intercalating between base pairs of the DNA/RNA strand, thus preventing the replication of rapidly-growing cancer cells; inhibits topoisomerase II enzyme, preventing the relaxing of supercoiled DNA and thus blocking DNA transcription and replication; creates iron-mediated free oxygen radicals that damage the DNA and cell membranes.

Radiation therapy is another type of cancer treatment that uses ionizing radiation to produce cell death through free radical formation. The cell death occurs by damaging the DNA of cancerous cells. This DNA damage is caused by one of two types of energy: photon or charged particle, directly or indirectly ionizing the atoms which make up the DNA chain. Indirect ionization happens as a result of the ionization of water, forming free radicals, notably hydroxyl radicals, which then damage the DNA.

The oxidative stress produced during cancer treatment induces a range of side effects such as hair loss, nausea or vomiting and cardiotoxicity. Several authors believe that the use of antioxidants during cancer treatment can reduce these side effects. However, there is a

concern that antioxidants might reduce oxidizing free radicals created by radiotherapy and some forms of chemotherapy, and thereby decrease the effectiveness of the therapy. The authors that support the idea that administration of oral antioxidants is contraindicated during cancer therapeutics, suggest that a drug's ability to destroy micrometastases may be impaired by the addition of antioxidants and, this may result in an improved short-term tolerance to treatment followed by an increased long-term chance for recurrence. On the other hand, there are several articles showing no evidence of significant decreases in the efficacy of chemotherapy with antioxidant supplementation and that supplementation of antioxidant vitamins during cancer treatment is effective, increasing quality and life expectancy.

Considering that the use of antioxidants during treatment is a very contentious issue, the purpose of this chapter is to review studies in humans to evaluate the use of these antioxidants as a therapeutic intervention in cancer patients, and their interactions with radiation therapy and chemotherapy.

2. Classes of agents used in cancer treatment that produce a reactive oxygen compound or free radical

The ultimate clinical effectiveness of any anti-cancer drug requires that it kill malignant tumor cells *in vivo* at doses that allow enough cells in the patient's critical tissues (e.g., bone marrow, gastrointestinal tract) to survive so that recovery can occur. This is difficult to accomplish because, in general, anticancer drugs are most useful against malignant tumor with a high proportion of dividing cells, and some normal tissues such as the bone marrow and GI tract also have a high cell-proliferation rate. Anticancer drugs used by themselves are primarily effective against high-growth-fraction tumors such as the leukemias and lymphomas. The most common malignant tumors, however, are "solid" tumors, including those of the colon, rectum, lung and breast. These tumors usually have a low proportion of dividing cells and therefore are less susceptible to treatment by drugs alone (Pratt, 1994).

There are some standard methods of cancer treatments: surgery, chemotherapy, radiation therapy, immunotherapy and biologic therapy. Undoubtedly, chemotherapy and radiotherapy are the treatments to fight cancer with more side effects.

Chemotherapy agents can be divided into several categories: alkylating agents (e.g., cyclophosphamide, ifosfamide), antibiotics which affect nucleic acids (e.g., doxorubicin, bleomycin), platinum compounds (e.g., cisplatin), mitotic inhibitors (e.g., vincristine), antimetabolites (e.g., 5-fluorouracil), camptothecin derivatives (e.g., topotecan), biological response modifiers (e.g., interferon), and hormone therapies (e.g., tamoxifen). The agents most noted for creating cellular damage by initiating free radical oxidants are the alkylating agents, the tumor antibiotics, and the platinum compounds (Lamson & Brignall, 1999).

2.1 Alkylating agents

Inhibiting DNA replication, therefore, affords a logical approach for retarding tumour growth. For this reason, DNA has become a critical target in cancer chemotherapy. Indeed, many of the antitumour agents currently in the cancer armamentarium are DNA-interactive. Among them, the DNA alkylators or cross-linkers, which include the platinum-based drugs, are the most active available for effective cancer management. By virtue of their high chemical reactivity, either intrinsic or acquired in a biological environment, all alkylating agents form covalent linkages with macromolecules having nucleophilic centres. They have

no specificity, but the chance reaction with DNA forms the basis for the antitumour effects. Bifunctional alkylating agents form covalent bonds at two nucleophilic sites on different DNA bases to induce interstrand (between two opposite strands) and/or intrastrand (on same strand) cross-links (Fig. 1). Monofunctional agents have only one alkylating group and, therefore, cannot form crosslinks (Siddik, 2002).

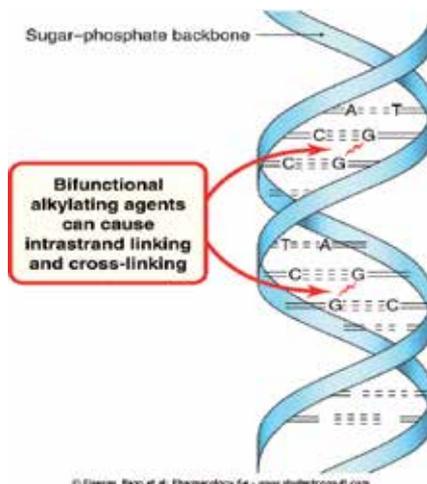


Fig. 1. The effects of bifunctional alkylating agents on DNA. Note the cross-linking of two guanines (www.studentconsult.com).

According to Siddik (2002), the end effect of these DNA-interactive agents is to inhibit DNA replication, which in turn may affect the production of RNA and protein. Such changes in the superhelical structure are then processed as distinct signals that determine whether a cell lives or dies.

The cyclophosphamide (CP) is a nitrogenous mustard pertaining to this group of substances named alkylating agents, which are effective against slow-growing tumors that damage cells at any phase of cellular growth. Cyclophosphamide is inactive per se and requires microsomal mixed function oxidase-mediated metabolism to activated metabolites capable of binding covalently to nucleic acids and proteins. The commonly accepted scheme of cyclophosphamide metabolism involves intermediate formation of 4-hydroxy-CP which undergoes ring-opening to form aldophosphamide, an isomer of 4-hydroxy-CP (Gurtoo et al., 1985). Aldophosphamide is metabolized to phosphoramidate mustard and acrolein (Murgo & Weinberger, 1993).

Phosphoramidate mustard forms DNA crosslinks between (interstrand crosslinkages) and within (intrastrand crosslinkages) DNA strands at guanine N-7 positions. This is irreversible and leads to cell death (Dong et al., 1995). According to Shanmugarajan et al. (2008), the therapeutic effect of cyclophosphamide is attributed to phosphoramidate mustard and acrolein is associated with toxic side effects.

Adams and Klaidman (1993) showed that acrolein and its glutathione adduct, glutathionylpropionaldehyde, induce oxygen radical formation. Acrolein was oxidized by xanthine oxidase to produce acroleinyl radical and $O_2^{\cdot-}$. Aldehyde dehydrogenase metabolized acrolein to form $O_2^{\cdot-}$ but not acroleinyl radical. The fact that glutathionylpropionaldehyde is a more potent stimulator of oxygen radical formation than

acrolein indicates that glutathionylpropionaldehyde is a toxic metabolite of acrolein and may be responsible for some of the *in vivo* toxicity of acrolein (Adams & Klaidman, 1993). In this regard, evidences reveal that oxidative stress plays a key role in the pathogenesis of cyclophosphamide induced cardiotoxicity (Shanmugarajan et al., 2008).

2.2 Anthracyclines (antibiotics)

Anthracyclines attack cancer cells by multiple mechanisms, inhibiting replication and cells damaging in ways that promote cell death. They work primarily by DNA intercalation. In order for a cell to divide, the DNA in the cell's nucleus must be unravelled and then duplicated (a process known as transcription). Anthracyclines bind to portions of the unwound strand of nuclear DNA, halting the transcription process, which in turn prevents cell replication. Among other details, scientists have found that anthracyclines inhibit the action of topoisomerase II ("Topo II"), an enzyme that unzips the DNA molecule for replication. It is anthracycline's interference with topoisomerase II that is credited with both its cardiotoxicity and mutagenic effects, since its Topo II inhibition leaves DNA breaks at even low concentrations, resulting in an accumulation of DNA damage following prolonged, repeated, or higher exposures (Pratt, 1999).

The biological activity of several well-known and widely used anthracycline antibiotics such as daunomycin and doxorubicin is thought to be associated to the hydroxyquinone structure (Young et al., 1981). Quinones are classified by the aromatic moieties present in their structure and naphthoquinone constitutes the naphthalenic ring (Silva et al., 2003).

The naphthoquinones are a class of compounds having cytotoxic properties that can be advantageous in treating cancer. Two essential mechanisms are linked to the effects of naphthoquinone, oxidative stress and nucleophilic alkylation (Bolton et al., 2000). These substances are able to accept electrons and generate reactive oxygen species (O_2^- , $HO\cdot$, H_2O_2), whose oxidative effects could explain the cytotoxicity produced by these compounds (Boveris et al., 1978; Silva et al., 2003; Witte et al., 2004).

Bolton et al. (2000) suggested that quinones are highly reactive molecules and can reduce the redox cycle using semi-quinone radicals, generating reactive oxygen species (ROS) that include superoxide radicals, peroxide radicals, hydrogen peroxide and hydroxyl radicals. ROS production can cause severe oxidative cell stress, forming oxidative macromolecule cells, affecting lipids, proteins and DNA.

Rajagopalan et al. (1988) demonstrated that Adriamycin, an anthracycline drug with a wide spectrum of clinical antineoplastic activity, stimulates the formation of OH in the isolated rat heart and suggests that this mechanism may be significant in Adriamycin-induced cardiotoxicity.

According to Minotti, Cairo and Monti (1999) the cardiotoxicity of anthracyclines is mediated by mechanisms that are distinct from those underlying the antitumor effects of these drugs. For these authors a major role in the development of cardiotoxicity has been assigned to iron, presumably because this metal can catalyze free radical reactions that overrule the antioxidant defenses of cardiomyocytes. For them some investigators have proposed mechanisms of cardiotoxicity that are independent altogether of both iron and free radicals. In an attempt to bridge the two extremes of this field, other studies have maintained a role for iron but not for free radicals, suggesting that anthracycline cardiotoxicity reflects disturbances in iron homeostasis within cardiomyocytes rather than the outcome of iron-catalyzed free radical injury.

2.3 Platinum compounds

The application of inorganic chemistry to medicine is a rapidly developing field, and novel therapeutic and diagnostic metal complexes are now having an impact on medical practice. Cisplatin, as one of the leading metal-based drugs, is widely used in the treatment of cancer. Significant side effects and drug resistance, however, have limited its clinical applications. Biological carriers conjugated to cisplatin analogs have improved specificity for tumor tissue, thereby reducing side effects and drug resistance (Kostova, 2006).

The history of platinum in cancer treatment began 150 years ago with the first synthesis of cisplatin, but it was not used in the clinic before 30 years ago. Then 3000 derivatives were synthesised and tested, with poor successes: three other derivatives only are available today. Clearly they are not more active, but they are less toxic than cisplatin, although two, carboplatin and nedaplatin, yield a cross-resistance, while one, oxaliplatin, does not. Their mechanisms of action are similar: these four pro-drugs form adducts with DNA, impairing DNA synthesis and repair then (Fig. 2). Their pharmacokinetics are complicated since we always measure two overlapping pharmacokinetics: those of the parent compound and of the bound platinum. Cisplatin is now recommended for few cancers, it is replaced by less-toxic carboplatin, and therefore more easily used in combination. Oxaliplatin give interesting results in a number of cancers (Desoize & Madoulet, 2002).

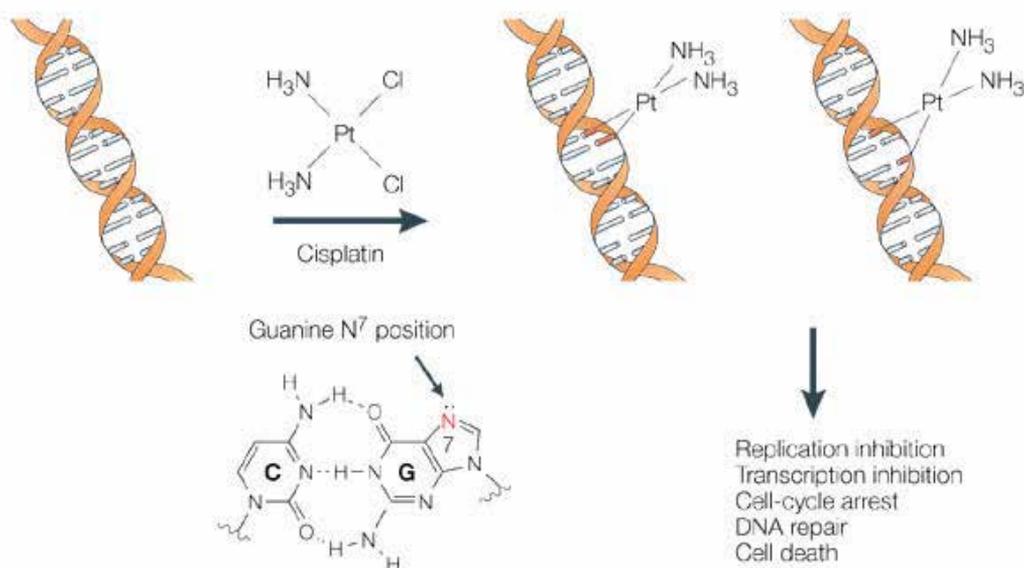


Fig. 2. The platinum atom of cisplatin binds covalently to the N7 position of purines to form 1,2- or 1,3-intrastrand crosslinks, and interstrand crosslinks. Cisplatin-DNA adducts cause various cellular responses, such as replication arrest, transcription inhibition, cell-cycle arrest, DNA repair and apoptosis (Wang & Lippard, 2005).

According to Boulikas and Vougiouka (2003) Cisplatin, carboplatin, oxaliplatin and most other platinum compounds induce damage to tumors via induction of apoptosis. Apoptosis is responsible for the characteristic nephrotoxicity, ototoxicity and most other toxicities of the drugs. The severity of cisplatin nephrotoxicity is related to platinum concentration in the kidneys. There is a growing amount of evidence that cisplatin-induced nephrotoxicity is

ascribed to oxidative damage resulting from free radical generation (Antunes & Bianchi, 2004). Reactive oxygen metabolites (superoxide, hydrogen peroxide, hydroxyl radical, and hypochlorous acid) are important mediators of renal damage in acute renal failure and glomerular and tubulointerstitial diseases (Klahr, 1997).

2.4 Radiation therapy

Radiation therapy has been used in cancer treatment for many decades. The primary focus in radiotherapy is to increase DNA damage in tumor cells, as double strand breaks are important in cell death. Another course of action is to alter cellular homeostasis, modifying signal transduction pathways, redox state, and disposition to apoptosis. The cellular changes ideally would enhance the killing of tumor cells while reducing the probability of normal cell death. Radiation damages cells by direct ionization of DNA and other cellular targets and by indirect effect through ROS. Indirect ionization happens as a result of the ionization of water, forming free radicals, notably hydroxyl radicals, which then damage the DNA (Fig. 3). Therefore, exposure to ionizing radiation produces oxygen-derived free radicals in the tissue environment; these include hydroxyl radicals (the most damaging), superoxide anion radicals and other oxidants such as hydrogen peroxide. Additional destructive radicals are formed through various chemical interactions (Borek, 2004a).

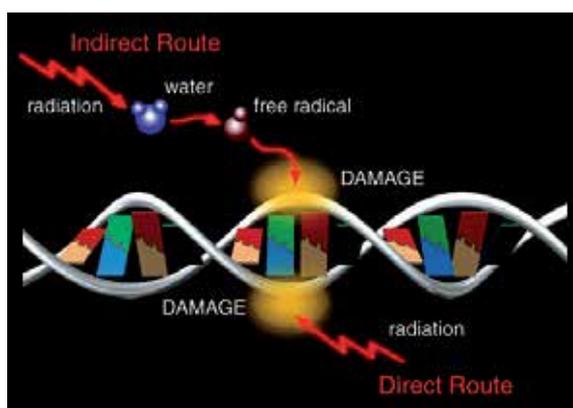


Fig. 3. There are two main ways radiation can damage DNA inside living cells. Radiation can strike the DNA molecule directly, ionizing and damaging it. Alternately, radiation can ionize water molecules, producing free radicals that react with and damage DNA molecules. Source unknown.

3. Antioxidants nutrients in cancer treatment

Cancer survivors receive a wide range of advice from many sources about foods they should eat, foods they should avoid, how they should exercise, and what types of supplements or herbal remedies they should take. Unfortunately, this advice is often conflicting (Doyle et al., 2006). Antioxidants vitamins show promise in cancer therapy by their palliative action, reducing painful side effects associated with treatment.

Examples of dietary antioxidants are vitamins A, C and E, selenium and flavonoids such as quercetin and genistein. In several *in vitro* and animal studies the hypothesis has been tested that antioxidants benefit patients receiving chemotherapy. In principle two opposing

mechanistic arguments could be advanced supporting or refuting this notion. On the one hand, antioxidants might protect cancer cells against the oxidative damage induced by chemotherapy, which would mitigate against their use. On the other hand they may enhance drug-induced cytotoxicity by blocking reactive oxidant species. (D'Incalci et al., 2007).

Antioxidant nutrients such as vitamin E, vitamin C, vitamin A, and Beta-carotene are involved in detoxification of the Reactive oxygen species (ROS). Vitamin E, A, and Beta-carotene are lipophilic antioxidants whereas vitamin C is hydrophilic antioxidant. Vitamin E function as a free radical chain breaker particularly it interferes with the propagation step of lipid peroxidation. The vitamin A and Beta-carotene have actions by quenching both singlet oxygen and other free radicals generated by photochemical reactions (Peerapatdit et al., 2006).

Simone II et al. (2007) wrote a review which showed that since the 1970s, 280 peer-reviewed *in vitro* and *in vivo* studies, including 50 human studies involving 8,521 patients, 5,081 of whom were given nutrients, have consistently shown that those non-prescription antioxidants and other nutrients do not interfere in therapeutic modalities of cancer. Furthermore, they enhance the killing of therapeutic modalities of cancer, decrease their side effects, and protect normal tissue. For them in 15 human studies, 3,738 patients who took non-prescription antioxidants and other nutrients actually had increased survival.

3.1 Vitamin A and carotenoids

Some studies show that vitamin A supplementation, during cancer treatment, shows no benefit in terms of survival (Meyskens et al., 1994; Culine et al. 1999; van Zandwijk et al., 2000). However, the term "vitamin A" commonly refers to either of two very different families of substances: retinol, or preformed vitamin A, and its synthetic analogues (retinoic acid and the carotenoids). It is important to understand that retinoids and carotenoids behave very differently in the body, each may act via a different mechanism even if they both have an anticancer effect (Hennekens et al., 1986).

A study involving a total of 18,314 smokers, former smokers, and workers exposed to asbestos evaluated the effects of a combination of 30 mg of beta carotene per day and 25,000 IU of retinol (vitamin A) in the form of retinyl palmitate per day on the primary end point, the incidence of lung cancer. After an average of four years of supplementation, the combination of beta carotene and vitamin A had no benefit and may have had an adverse effect on the incidence of lung cancer and on the risk of death from lung cancer, cardiovascular disease, and any cause in smokers and workers exposed to asbestos (Omenn et al., 1996). Other findings confirm of a lack of any benefit from administration of large doses of synthetic β -carotene in cancer prevention (Klerk et al., 1998). On the other hand the adjuvant effect of high-dose vitamin A was tested on 307 patients with stage I non-small-cell lung cancer. After curative surgery, patients were randomly assigned to either a group prescribed retinol palmitate administration (orally 300,000 IU daily for 12 months). The authors concluded that daily oral administration of high-dose vitamin A is effective in reducing the number of new primary tumors related to tobacco consumption and may improve the disease-free interval in patients curatively resected for stage I lung cancer (Pastorino et al., 1993). Antioxidants, when added adjunctively, to first-line chemotherapy, may improve the efficacy of chemotherapy and may prove to be safe (Drisko et al., 2003).

136 patients with advanced non-small cell lung cancer were randomized to receive chemotherapy (paclitaxel and carboplatin) alone or chemotherapy in combination with

ascorbic acid 6100 mg/day, dl-alpha-tocopherol (vitamin E) 1050 mg/day and beta-carotene 60 mg/day. The results do not support the concern that antioxidants might protect cancer cells from the free radical damage induced by chemotherapy (Pathak et al., 2005). The authors suggest that high-dose multiple antioxidants in conjunction with chemotherapy increase the response rates and/or survival time in advanced lung cancer.

A phase III randomized study, comparing treatment with fluorouracil, epidoxorubicin and methotrexate (FEMTX) with the best supportive care, was conducted in patients with unresectable or metastatic gastric cancer. During treatment, these patients received tablets containing vitamins A and E. This study concluded that treatment with fluorouracil, epidoxorubicin and methotrexate combined with vitamin A and E is a fairly well-tolerated treatment, giving a response rate of 29% in patients with advanced gastric cancer, and also prolonging patients' survival (Pyrhönen et al., 1995).

Alpha- and beta-carotene have been examined for *in vitro* tumor inhibitory activity against human neuroblastoma cell lines, and alpha-carotene was found to have 10 times the anti-tumor activity of beta-carotene (Murakoshi et al., 1989).

Twenty patients with advanced squamous cell carcinoma of the mouth, who received 60 Gy telecobalt therapy given in 30 daily fractions with synchronous chemotherapy comprising vincristine, methotrexate and bleomycin, were randomized to receive standard diet with supplemental beta carotene. The results reported suggest that a protective action of beta-carotene is exerted on the mucosal membrane within the radiation fields used (Mills, 1988).

Kucuk et al. (2002) conducted a clinical trial to investigate the biological and clinical effects of lycopene supplementation in patients with localized prostate cancer. Twenty-six men with newly diagnosed prostate cancer were randomly assigned to receive a tomato oleoresin extract containing 30 mg of lycopene or no supplementation for 3 weeks before radical prostatectomy. This study suggested that lycopene may have beneficial effects in prostate cancer. The authors state that preparation that was used in this study was a mixture of tomato carotenoids and other tomato phytochemicals. Although lycopene was the predominant carotenoid in the capsules, there were significant amounts of phytoene and phytofluene and other bioactive compounds. It is possible that the combination of the phytochemicals present in the tomato extract was responsible for the observed clinical effects rather than lycopene alone.

Because of the poor response of pancreatic cancer to conventional therapy Recchia et al. (1998) performed a phase II pilot study to evaluate whether beta-interferon and retinoids, added to active chemotherapeutic agents, could increase response rate and survival in a group of patients who had metastatic disease. Twenty-three chemotherapy-naïve patients were treated with epirubicin, mitomycin C, and 5-fluorouracil. Beta-Interferon, 1 x 10⁶ IU/m², subcutaneously three times a week, and retinol palmitate, 50,000 IU orally twice a day, were given between chemotherapy cycles. Eight patients responded (35%) and 8 (35%) had stable disease.

Prasad et al. (1999) has reviewed several studies showing the use of vitamin A and its analogs and its importance in cancer treatment. In this review the authors present a table (Table 1) on these studies. For them vitamins A derivatives at high doses, variable extents of tumor size reduction have been reported. Retinoids have been shown to have very little or no effect on several human tumors which included melanoma, non-small cell lung carcinoma, prostate cancer, breast cancer and neuroblastoma.

Tumor type	Design	Agents	Patient no.	Response
Actinic keratoses	Phase III (randomized)	Etretinate	54	84%
Advanced squamous cell carcinoma	Phase II	13 cRA	4	50%
Mycosis fungoides	Phase II	13 cRA	28	80%
Laryngeal papillomatosis	No record	13 cRA	123	60%
Oral leukoplakia	Phase II	b-carotene (synthetic)	6	60%
	Phase III randomized	13 cRA (high dose)	24	71%
Cervical cancer (CIN II or III)	Phase II	tRA (topical)	44	Marked regression
Cervical cancer (CIN II or III)	Phase II	tRA (topical)	20	50%
Cervical cancer (CIN II or III)	Phase III randomized	tRA (topical)	301	Increased regression of CIN II but not CIN III
Cervical cancer (CIN II or III)	Phase II	13 cRA + INFa	23	53%
Advanced cervical cancer	Phase II	13 cRA + INFa	26	50%
Advanced cervical cancer	Phase II	13 cRA + INFa	24	30%

Data from Prasad et al. (1999).

13 cRA 13-cis Retinoic acid.

INFa Interferon a.

Table 1. Efficacy of Retinoids in the Treatment of Human Tumors

13-cis-retinoic acid, even in short-term use, appears to be an effective treatment for oral leukoplakia and has an acceptable level of toxicity. 44 patients with this disease to receive 13-cis-retinoic acid (24 patients) or placebo (20), 1 to 2 mg per kilogram of body weight per day for three months, and followed them for six months. There were major decreases in the size of the lesions in 67 percent of those given the drug and in 10 percent of those given placebo. Dysplasia was reversed in 54 percent of the drug group (Hong et al., 1986). Some of the analogs of retinoids produce extreme toxicity. The use of single antioxidant vitamins which require very high doses for its effectiveness has no significant value in the

treatment of cancer, even though such doses may cause tumor regression of variable degrees (Prasad et al, 1999). The antitumor activity demonstrated for retinoids (especially retinoic acid) alone and in combination with other agents supports the need for targeted phase II trials to define the spectrum of responsive tumors and for laboratory studies to further delineate the biologic mechanisms associated with therapeutic responses. High priority should then be given to phase III trials to delineate optimal strategies for improving outcome by combining retinoid-based treatments with conventional chemotherapy and radiotherapy regimens (Smith et al., 1992).

Addition of nutrition supplements such as lycopene may have potential therapeutic benefit in the adjuvant management of high-grade gliomas. The results verified in fifty patients with high-grade gliomas were treated with surgery followed by adjuvant radiotherapy and concomitant paclitaxel. Patients were randomized to receive either oral lycopene 8 mg daily with radiotherapy (Puri et al., 2010).

Docetaxel is currently the most effective drug for the treatment of castration-resistant prostate cancer (CRPC), but it only extends life by an average of 2 months. Tang et al. (2011) proposed a study of the interaction between docetaxel and lycopene in CRPC models. Lycopene, an antioxidant phytochemical, has antitumor activity against prostate cancer in several models and is generally safe. In this study, the authors demonstrated that lycopene enhances the effect of docetaxel on the growth of CRPC cell lines both *in vitro* and *in vivo*. These data provide a rationale for the clinical investigation of the efficacy and safety profile of lycopene in combination with docetaxel in CRPC patients. In particular, combining lycopene with docetaxel may provide clinical benefit for men with metastatic CRPC, for whom morbidity and mortality remain high despite wide use of docetaxel chemotherapy.

3.2 Vitamin C

It has been claimed that high-dose vitamin C is beneficial in the treatment of patients with advanced cancer, especially patients who have had no prior chemotherapy. The possibility that this compound may be useful in the treatment of cancer was first raised by Cameron and Pauling (1976) that published research suggesting a survival benefit from vitamin C in cancer treatment. The results of this clinical trial were made in 100 terminal cancer patients that were given supplemental ascorbate, by intravenous infusion for 10 days and orally thereafter, as part of their routine management. This study was compared with 1000 similar patients treated identically, but who received no supplemental ascorbate. The mean survival time was more than 4.2 times as great for the ascorbate subjects (more than 210 days) as for the controls (50 days). In the same journal Cameron and Pauling (1978) published, 2 years later, additional cases, and in this paper the authors confirm the idea that patients who had ascorbate treatment benefited with enhanced quality and prolongation of life. However, in a double-blind study 100 patients with advanced colorectal cancer were randomly assigned to treatment with either high-dose vitamin C (10 g daily) (the same dose that Pauling and Cameron recommended), using oral doses only, or placebo. None had received any previous treatment with cytotoxic drugs. Vitamin C therapy showed no advantage over placebo therapy with regard to either the interval between the beginning of treatment and disease progression or patient survival (Moertel et al., 1985). Saul (2010) and González et al. (2010) contest the work of Moertel et al. (1985). For Saul (2010) it is important to note that the negative results in Moertel studies were not true replications of Cameron and Pauling's work, as A) they used oral doses only, and B) vitamin C was discontinued at the first sign of disease progression.

D'Andrea (2005) believes that the antioxidant perhaps most widely used for treating cancer is vitamin C. For the author neither study was able to show any objective improvement in disease progression or survival over placebo. However, Maciocia (2010) contests D'Andrea's conclusions. Maciocia (2010) reported that there is no evidence of significant decreases in efficacy from antioxidant supplementation during chemotherapy. For him many of the studies indicated that antioxidant supplementation resulted in either increased survival times, increased tumor responses, or both, as well as fewer toxicities than controls. He concluded that trials that assessed chemotherapy toxicities, including diarrhea, weight loss, nerve damage and low blood counts, showed that the antioxidant group suffered similar or lower rates of these side effects than the control group.

Cabanillas (2010) after trials which have included at least 1,609 patients over 33 years concluded that we still do not know whether Vitamin C has any clinically significant antitumor activity. Nor does he know which histological types of cancers, if any, are susceptible to this agent. He doesn't know with certainty which is the required plasma ascorbic acid level that will result in antitumor effects. Assuming that this level is 10 mM/L then the recommended dose of Vitamin C appears to be in the range of 1.5 g/kg three times weekly. According to Ohno et al. (2009) the administration of more than 10 g of ascorbate is proposed to achieve plasma concentrations of 1 to 5 mM. At this time, vitamin C at high plasma concentration may function as a pro-oxidant. This occurs in the presence of free transition metals, such as copper and iron, which are reduced by ascorbate and, in turn, react with hydrogen peroxide (H₂O₂), leading to the formation of highly reactive and damaging hydroxyl radicals. As normal tissue receives adequate blood flow and is rich in antioxidant enzymes (e.g. catalase, glutathione peroxidase) in the blood, any H₂O₂ formed will be immediately destroyed. Meanwhile, tumor tissue is often associated with reduced blood flow and antioxidant enzymes, and consequently formed H₂O₂ remains active leading to cell damage and death. González et al. (2010) says that doses of 50-100 g given intravenously may result in plasma concentrations of about 14,000 micromol/L. At concentrations above 1000 micromol/L, vitamin C is toxic to cancer cells but not to normal cells *in vitro*. However, it is important that when referring to the plasma concentrations necessary to achieve antineoplastic activity there are other numerous factors involved that will affect the specific response. Some of these include sensitivity of tumor, hypoxia inducible factor, intracellular Redox signal transduction and gene expression, apoptosis, autophagy, effect of collagen on tumor encapsulation and others.

Simone II et al. (2007) affirms that antioxidants and other nutrients do not interfere with cancer therapeutic modalities, enhance their killing capabilities, decrease their side effects, or protect normal tissues, and in 15 human studies, 3,738 patients actually had prolonged survival. Antioxidant and other nutrient food supplements are safe and can help to enhance cancer patient care.

Adriamycin (ADR) is effective against a wide range of human neoplasms. However, its clinical use is compromised by serious cardiac toxicity, possibly through induction of peroxidation in cardiac lipids. Ascorbic acid, a potent antioxidant, was examined for effect in reducing ADR toxicity in mice and guinea pigs. Ascorbic acid had no effect on the antitumor activity of ADR in mice inoculated with leukemia L1210 or Ehrlich ascites carcinoma, but it significantly prolonged the life of animals treated with ADR. The significant prevention of ADR-induced cardiomyopathy in guinea pigs by ascorbic acid was proved by electron microscopy. Ascorbic acid and the derivatives may delay general toxicity of ADR and also prevent the cardiac toxicity (Shimpo et al., 1991).

Borek (2004a) says in his studies that the antioxidants do protect against radiation-induced oncogenic transformation in experimental systems. However, she does not have comparable human studies that show the same association. Antioxidants do reduce the painful side effects of radiation therapy, thus supporting the beneficial effects of antioxidants in protecting normal cells in radiation therapy and in being used in conjunction with treatment for certain cancers. When considering antioxidant supplementation during treatment, it is doubtful whether high doses of radiation given in certain treatments would be rendered less effective if patients took a daily supplement of antioxidants.

Twenty consecutive symptomatic outpatients with endoscopically documented radiation proctitis seen in a single gastroenterology clinic were given a combination of vitamin E (400 IU tid) and vitamin C (500 mg tid). These patients presented with one or more of the following symptoms: rectal bleeding, rectal pain, diarrhea, or fecal urgency. There was a significant ($p < 0.05$; Wilcoxon rank) improvement in the symptom index (before treatment vs after treatment with vitamins E and C) for bleeding, diarrhea, and urgency. Patients with rectal pain did not improve significantly. Bleeding resolved in four of 11 patients, diarrhea resolved in eight of 16 patients, fecal urgency resolved in three of 16 patients, and rectal pain resolved in two of six patients. Lifestyle improved in 13 patients, including seven patients who reported a return to normal (Kennedy et al., 2001).

Cancer treatment by radiation and anticancer drugs reduces inherent antioxidants and induces oxidative stress, which increases with disease progression. Vitamins E and C have been shown to ameliorate adverse side effects associated with free radical damage to normal cells in cancer therapy, such as mucositis and fibrosis, and to reduce the recurrence of breast cancer. While clinical studies on the effect of anti-oxidants in modulating cancer treatment are limited in number and size, experimental studies show that antioxidant vitamins and some phytochemicals selectively induce apoptosis in cancer cells but not in normal cells and prevent angiogenesis and metastatic spread, suggesting a potential role for antioxidants as adjuvants in cancer therapy (Borek, 2004b).

3.3 Vitamin E

Vitamin E comprises a group of compounds possessing vitamin E activity. α -Tocopherol is the compound demonstrating the highest vitamin E activity, which is available both in its natural form as RRR- α -tocopherol isolated from plant sources, but more common as synthetically manufactured all-rac- α -tocopherol. Synthetic all-rac- α -tocopherol consists of a racemic mixture of all eight possible stereoisomers (Jensen & Lauridsen, 2007).

The ability of the vitamin E (RRR- α -tocopherol) derivatives α -tocopheryl succinate (α -TOS) and α -tocopheryloxyacetic acid (α -TEA) to suppress tumor growth in preclinical animal models has recently led to increased interest in their potential use for treating human cancer (Hahn et al., 2006).

With aim to evaluate the neuroprotective effect of antioxidant supplementation with vitamin E in patients treated with cisplatin chemotherapy Pace et al. (2003) evaluated, between April 1999 and October 2000, forty-seven patients assigned to either group one, which received vitamin E supplementation during cisplatin chemotherapy, or to group two, which received cisplatin chemotherapy alone. α -Tocopherol (vitamin E; 300 mg/d) was administered orally before cisplatin chemotherapy and continued for 3 months after the treatment suspension. The severity of neurotoxicity, measured with a comprehensive

neurotoxicity score based on clinical and neurophysiological parameters, was significantly lower in patients who were supplemented with vitamin E than in patients who were not supplemented with vitamin E. Thirty-one patients with cancer treated with six courses of cumulative cisplatin, paclitaxel, or their combination regimens were randomly assigned in two groups and followed by neurologic examination and electrophysiologic study. Patients assigned in Group I (n = 16) received oral vitamin E at a daily dose of 600 mg/day during chemotherapy and 3 months after its cessation were compared to patients of Group II (n = 15), who received no supplementation and served as controls. This study showed significantly that the vitamin E supplementation in cancer patients may have an important neuroprotective effect (Argyriou et al., 2005). Contradicting the idea of these authors, Kottschade et al. (2010) published new data where two-hundred seven patients were enrolled between December 1, 2006 and December 14, 2007, producing 189 evaluable cases for analysis. A phase III, randomized, double-blind, placebo-controlled study was conducted in patients undergoing therapy with neurotoxic chemotherapy (cytotoxic agents included: taxanes, cisplatin, carboplatin, oxaliplatin, or combination), utilizing twice daily dosing of vitamin E (400 mg)/placebo. The authors concluded that Vitamin E did not appear to reduce the incidence of sensory neuropathy in the studied group of patients receiving neurotoxic chemotherapy.

Prasad et al. (2003) showed in their review article that alpha-Tocopheryl Succinate is the most Effective Form of Vitamin E for Adjuvant Cancer Treatment. For them alpha-Tocopheryl Succinate inhibits the proliferation of rodent and human cancer cells without affecting the proliferation of most normal cells. In addition, they also show that alpha-Tocopheryl Succinate when used in combination with some standard and experimental cancer therapeutic agents may enhance their growth-inhibitory effect on cancer cells, while protecting normal cells against some of their toxicities.

Chemotherapy- and radiotherapy-induced oral mucositis represents a therapeutic challenge frequently encountered in cancer patients. This side effect causes significant morbidity and may delay the treatment plan, as well as increase therapeutic expenses (Köstler et al., 2001). A randomized, double-blind, placebo-controlled study was performed to evaluate the efficacy of topical vitamin E in the treatment of oral mucositis in patients receiving chemotherapy for various types of malignancy. A total of 18 patients, 17 of whom had solid tumors and one with acute leukemia, were included in this study. Lesions were observed daily prior to and 5 days after topical application of either vitamin E or placebo oil. Six of nine patients receiving vitamin E had complete resolution of their oral lesions. In eight of nine patients who received placebo, complete resolution of their oral lesions was not observed. This difference is statistically significant ($p = 0.025$ by Fisher's exact test). No toxicity was observed in this study. These results suggest that vitamin E may be an effective therapy in patients with chemotherapy-induced mucositis (Wadleigh et al., 1992).

Women with breast carcinoma were asked to complete a questionnaire that recorded their use of dietary supplements. Blood samples were obtained for the assessment of serum vitamin B12 and folate levels before and after the first cycle of chemotherapy and for weekly complete blood counts. Toxicity was evaluated by measuring absolute neutrophil counts and the frequency and severity of oral mucositis. Of the 49 women who submitted questionnaires, 35 (71%) took a combined total of 165 supplements. The decrease in neutrophil count caused by chemotherapy was ameliorated by dietary supplementation with a multivitamin or vitamin E. However, neither multivitamin use nor vitamin E use appeared to be associated with the severity of mucositis. (Branda et al., 2004).

Conditioning therapy preceding bone marrow transplantation usually consists of high-dose chemotherapy and total body irradiation. It has acute and delayed toxic effects on several tissues, possibly related to peroxidation processes and exhaustion of antioxidants (Clemens et al., 1997). Blood from 19 patients was examined for the essential antioxidants alpha-tocopherol and beta-carotene before, during, and after bone marrow transplantation (BMT). Marrow ablation and immunosuppression for BMT conditioning was achieved by treatment with high-dose chemotherapy, mostly combined with total body irradiation. All patients required total parenteral nutrition beginning 1 wk before BMT. After conditioning therapy the concentration of absolute and lipid-standardized alpha-tocopherol and beta-carotene in plasma decreased significantly, presumably as a result of an enhanced breakdown of these antioxidants. The loss of these lipid-soluble antioxidants has to be considered as a possible cause for early posttransplant organ toxicity (Clemens et al., 1990). Therefore, the antioxidant supplementation prior to conditioning therapy reduces peroxidation processes induced by conditioning therapy in bone marrow recipients.

In the Women's Health Study conducted between 1992 and 2004, 39 876 apparently healthy US women aged at least 45 years were randomly assigned to receive vitamin E or placebo and aspirin or placebo, using a 2×2 factorial design, and were followed up for an average of 10.1 years (Lee et al., 2005). The data from this large trial indicated that 600 IU of natural-source vitamin E taken every other day provided no overall benefit for major cardiovascular events or cancer, did not affect total mortality, and decreased cardiovascular mortality in healthy women. Therefore, these data do not support recommending vitamin E supplementation for cardiovascular disease or cancer prevention among healthy women.

On the other hand, Nechuta et al. (2011) conducted a population-based prospective cohort study of 4,877 women aged 20 to 75 years diagnosed with invasive breast cancer in Shanghai, China, between March 2002 and April 2006. Women were interviewed approximately 6 months after diagnosis and followed up by in-person interviews and record linkage with the vital statistics registry. Women who used antioxidants (vitamin E, vitamin C, multivitamins) had 18% reduced mortality risk and 22% reduced recurrence risk. Therefore, Vitamin supplement use in the first 6 months after breast cancer diagnosis may be associated with reduced risk of mortality and recurrence.

Bladder cancer is one of the most aggressive epithelial tumors characterized by a high rate of early systemic dissemination. Patients with metastatic bladder cancer are routinely treated with systemic chemotherapy such as methotrexate, vinblastine, doxorubicin, and cisplatin regimen, particularly in the setting of unresectable, diffusely metastatic, measurable disease. In a study was investigated the cytotoxic effect of vitamin E succinate (α -TOS) and the enhancement of chemosensitivity to paclitaxel by α -TOS in bladder cancer. KU-19-19 and 5637 bladder cancer cell lines were cultured in α -TOS and/or paclitaxel *in vitro*. For *in vivo* therapeutic experiments, pre-established KU-19-19 tumors were treated with α -TOS and/or paclitaxel. The results demonstrated the efficacy and therapeutic potential of α -TOS and its enhancement of chemosensitivity to paclitaxel in bladder cancer cells. α -TOS inhibits NF- κ B activity resulting in the promotion of apoptotic mechanisms in bladder cancer cell lines and also reduces activated NF- κ B induced by paclitaxel resulting in enhanced apoptosis *in vitro*. α -TOS displayed an antitumor effect and α -TOS in combination with paclitaxel demonstrated dramatic tumor inhibition in an *in vivo* s.c. KU-19-19 tumor model (Kanai et al., 2010). For these authors additional studies are needed to confirm its safety for use in clinical trials, the cytotoxic effect of α -TOS and its enhancement of paclitaxel treatment might provide a novel strategy for advanced or metastatic bladder cancer patients.

3.4 Selenium

Selenium was recognized as a nutritional essential only in the late 1950s. That it might also be anticarcinogenic was first suggested a decade later based on ecological relationships of cancer mortality rates and forage crop selenium contents in the United States. Since that time, a substantial body of scientific evidence indicated that selenium can, indeed, play a role in cancer prevention. This is supported by a remarkably consistent body of findings from studies with animal tumor and cell culture models, and by some, but not all epidemiologic observations (Combs Jr., 2005). Selenium is an essential element that is specifically incorporated as selenocystein into selenoproteins. It is a potent modulator of eukaryotic cell growth with strictly concentration-dependant effects. Lower concentrations are necessary for cell survival and growth, whereas higher concentrations inhibit growth and induce cell death (Selenius et al., 2010). The protective effect of this mineral is especially associated with its presence in glutathione peroxidase and thioredoxin reductase, enzymes that protect the DNA and other cellular components against oxidative damage caused by ROS. Several studies have demonstrated reduced expression of these enzymes in various types of cancer, especially when associated with a low intake of selenium, which may exacerbate the damage (Almond et al., 2010).

A total of 1312 patients (mean age, 63 years; range, 18-80 years) with a history of basal cell or squamous cell carcinomas of the skin were randomized from 1983 through 1991. Patients were treated for a mean (SD) of 4.5 (2.8) years and had a total follow-up of 6.4 (2.0) years. The patients were treated with oral administration of 200 µg of selenium per day or placebo. After a total follow-up of 8271 person-years, selenium treatment did not significantly affect the incidence of basal cell or squamous cell skin cancer. Therefore, Selenium treatment did not protect against development of basal or squamous cell carcinomas of the skin. However, results from secondary end-point analyses support the hypothesis that supplemental selenium may reduce the incidence of, and mortality from, carcinomas of several sites (Clark et al., 1996). Selenium treatment was associated with a significant (63%) reduction in the secondary endpoint of prostate cancer incidence during 1983-93 (Clark et al., 1998). A total of 974 men with a history of either a basal cell or squamous cell carcinoma were randomized to either a daily supplement of 200 microg of selenium or a placebo. Supplementation with a nutritional dose of the essential trace element selenium significantly reduced the incidence of prostate cancer in a population of patients with non-melanoma skin cancer. For the authors this was the first completed double-blind randomized controlled trial to specifically test if a dietary supplement can prevent prostate cancer. These results require confirmation in independent trials, but suggest that selenium supplementation may be important for both the primary and secondary prevention of prostate cancer.

A prospective study included 209 breast cancer patients treated by external beam radiotherapy from December 2007 until August 2008. Plasma selenium concentrations were determined before and at the end of the radiotherapeutic treatment. Sixty patients (28.7%) were in clinical stage I, 141 (67.5%) in clinical stage II and 8 (3.8%) in clinical stage III. At the beginning of radiotherapy, the mean selenium value for all patients was 86.4 µg/l and after radiation this value dropped to 47.8 µg/l. Multivariate analysis showed statistically significant difference in the plasma selenium concentration before and after radiotherapy. Therefore, significant reduction in plasma levels of selenium is recorded in patients undergoing radiotherapy, suggesting attention to the nutritional status of this micronutrient and other antioxidant agents (Franca et al., 2010).

However, a phase 2 randomized, double-blind, placebo-controlled clinical trial was conducted in men with localized nonmetastatic prostate cancer who had elected to forgo active treatment and be followed by active surveillance. A total of 140 men were randomized to placebo (n = 46), 200 µg/d (n = 47), or 800 µg/d (n = 47) selenium p.o. (as selenized yeast) and followed every 3 months for up to 5 years. Prostate-specific antigen (PSA) velocity was used as a marker of prostate cancer progression and was estimated using mixed-effects regression. Selenium supplementation did not show a protective effect on PSA velocity in subjects with localized prostate cancer. On the contrary, supplementation with high-dose selenium was observed to be a risk factor for increased PSA velocity in men with high baseline plasma selenium concentrations (Stratton et al., 2010).

3.5 Flavonoids

Flavonoids and their polymers constitute a large class of food constituents, synthesized by plants, many of which alter metabolic processes and have a positive impact on health. Flavonoids are a subclass of polyphenols (Beecher, 2003). Polyphenols are abundant micronutrients in our diet, and evidence for their role in the prevention of degenerative diseases such as cancer and cardiovascular diseases is emerging. The health effects of polyphenols depend on the amount consumed and on their bioavailability (Manach et al., 2004). The capacity of flavonoids to act as antioxidants *in vitro* has been the subject of several studies in the past years, and important structure–activity relationships of the antioxidant activity have been established. The antioxidant efficacy of flavonoids *in vivo* is less documented, presumably because of the limited knowledge on their uptake in humans (Pietta, 2000).

Sadzuka et al. (1998) investigated the effects of green tea and tea components on the antitumor activity of doxorubicin. We carried out the combined treatment of doxorubicin and green tea on Ehrlich ascites carcinoma tumor-bearing mice. The oral administration of green tea enhanced 2.5-fold the inhibitory effects of doxorubicin on tumor growth. The doxorubicin concentration in the tumor was increased by the combination of green tea with doxorubicin. In contrast, the increase in doxorubicin concentration was not observed in normal tissues after green tea combination. Furthermore, the enhancement of antitumor activity of doxorubicin induced by green tea was observed in M5076 ovarian sarcoma, which has low sensitivity to doxorubicin. These results suggest that drinking green tea can encourage cancer chemotherapy and may improve the quality of life of clinical patients.

A study demonstrates, for the first time, that cancer preventive effects of epigallocatechin gallate (EGCG) and quercetin can inhibit the self-renewal capacity of prostate cancer stem cells. In this study Tang et al. (2010) present data indicating that human prostate cancer cell lines contain a small population of CD44+CD133+ cancer stem cells and their self-renewal capacity is inhibited by EGCG. Furthermore, EGCG inhibits the self-renewal capacity of CD44+a2b1+CD133+ CSCs isolated from human primary prostate tumors, as measured by spheroid formation in suspension. EGCG induces apoptosis by activating capase-3/7 and inhibiting the expression of Bcl-2, survivin and XIAP in CSCs. Furthermore, EGCG inhibits epithelial-mesenchymal transition by inhibiting the expression of vimentin, slug, snail and nuclear b-catenin, and the activity of LEF-1/TCF responsive reporter, and also retards CSC's migration and invasion, suggesting the blockade of signaling involved in early metastasis. Interestingly, quercetin synergizes with EGCG in inhibiting the self-renewal properties of prostate CSCs, inducing apoptosis, and blocking CSC's migration and invasion. These data suggest that EGCG either alone or in combination with quercetin can eliminate cancer stem cell-characteristics.

Resistance of cancer cells to multiple chemotherapeutic drugs (a mechanism termed MDR) is a major obstacle to the success of cancer chemotherapy and has been closely associated with treatment failure. One of the most studied mechanisms of drug resistance is characterized by a decrease in drug accumulation resulting from over-expression of the 170 kDa plasma membrane, P-glycoprotein (Pgp). P-glycoprotein (Pgp), causes the efflux of chemotherapeutic drugs from cells and is believed to be an important mechanism in multidrug resistance (MDR) in human cancer (Khantamat et al., 2004). Khantamat et al. (2004) demonstrated that the flavonoid, i.e. kaempferol, could reverse the vinblastine resistant phenotype by inhibiting Pgp activity in KB-V1 cells, and the ability to affect the Pgp activity could be of relevance to the chemosensitization of this flavonoid towards anticancer drugs.

3.6 Melatonin

Melatonin, a derivative of an essential amino acid, tryptophan, was first identified in bovine pineal tissue and subsequently it has been portrayed exclusively as a hormone. Recently accumulated evidence has challenged this concept. Melatonin is present in the earliest life forms and is found in all organisms including bacteria, algae, fungi, plants, insects, and vertebrates including humans. Several characteristics of melatonin distinguish it from a classic hormone such as its direct, non-receptor-mediated free radical scavenging activity. As melatonin is also ingested in foodstuffs such as vegetables, fruits, rice, wheat and herbal medicines, from the nutritional point of view, melatonin can also be classified as a vitamin. It seems likely that melatonin initially evolved as an antioxidant, becoming a vitamin in the food chain, and in multicellular organisms (Tan et al., 2003).

Melatonin was found to be a potent free radical scavenger in 1993, since then over 800 publications have directly or indirectly confirmed this observation. Melatonin scavenges a variety of reactive oxygen and nitrogen species including hydroxyl radical, hydrogen peroxide, singlet oxygen, nitric oxide and peroxyxynitrite anion. The mechanisms of melatonin's interaction with reactive species probably involves donation of an electron to form the melatoninyl cation radical or through a radical addition at the site C3. Other possibilities include hydrogen donation from the nitrogen atom or substitution at position C2, C4 and C7 and nitrosation. Melatonin also has the ability to repair damaged biomolecules as shown by the fact that it converts the guanosine radical to guanosine by electron transfer. Unlike the classical antioxidants, melatonin is devoid of prooxidative activity and all known intermediates generated by the interaction of melatonin with reactive species are also free radical scavengers. This phenomenon is defined as the free radical scavenging cascade reaction of the melatonin family. Due to this cascade, one melatonin molecule has the potential to scavenge up to 4 or more reactive species. This makes melatonin very effective as an antioxidant. Under *in vivo* conditions, melatonin is often several times more potent than vitamin C and E in protecting tissues from oxidative injury when compared at an equivalent dosage (mmol / kg) (Tan et al., 2002).

Physiologic and pharmacologic concentrations of the pineal hormone melatonin have shown chemopreventive, oncostatic, and tumor inhibitory effects in a variety of *in vitro* and *in vivo* experimental models of neoplasia. Multiple mechanisms have been suggested for the biological effects of melatonin. Not only does melatonin seem to control development alone but also has the potential to increase the efficacy and decrease the side effects of chemotherapy when used in adjuvant settings (Jung & Ahmad, 2006).

Melatonin has a variety of functions in human physiology and is involved in a number of pathological events including neoplastic processes. The tissue protective actions of

melatonin are attributed to its antioxidant activity though, under certain conditions, melatonin might also exert oxidant effects, particularly in cancer cells. Büyükavcı et al. (2006) verified that these pro-oxidant actions of melatonin may assist in limiting leukemic cell growth. A similar study (Bejarano et al., 2011) evaluated the pro-oxidant effects of melatonin in tumour cell lines of human haematopoietic origin. Melatonin treatment was able to stimulate production of intracellular reactive oxygen species (ROS), as revealed by the increase in rhodamine-123 fluorescence, which was associated with significant cytotoxicity and activation of caspase activities. According to authors, this pro-oxidant action of melatonin may assist in limiting tumour cell growth. An increase in the activation of caspase-3, -8, -9 was also observed when melatonin was combined with vincristine in Ewing sarcoma cell line (Casado-Zapico et al., 2010).

A study was done with 14 women with metastatic breast cancer who did not respond to tamoxifen (TMX) therapy or progressed after initial disease stabilization. The study evaluated the biological and clinical effects of a concomitant melatonin therapy in women with metastatic breast cancer who had progressed in response to TMX alone. Melatonin was given orally at 20 mg/day in the evening, every day starting 7 days before TMX, which was given orally at 20 mg/day at noon. The authors concluded that this preliminary phase II study would suggest that the pineal hormone melatonin may amplify the therapeutic efficacy of TMX in women with metastatic breast cancer and induce objective tumour regressions in patients who have not responded to previous therapy with TMX alone (Lissoni et al., 1995).

A study included 70 consecutive advanced non-small cell lung cancer patients (NSCLC), with poor clinical status, were randomized to receive chemotherapy alone with cisplatin (20 mg/m²/day i.v. for 3 days) and etoposide (100 mg/m²/day i.v. for 3 days) or chemotherapy plus melatonin (20 mg/day orally in the evening). Cycles were repeated at 21-day intervals. Clinical response and toxicity were evaluated according to World Health Organization criteria. The percent of 1-year survival was significantly higher in patients treated with melatonin plus chemotherapy than in those who received chemotherapy alone. Finally, chemotherapy was well tolerated in patients receiving melatonin, and in particular the frequency of myelosuppression, neuropathy, and cachexia was significantly lower in the melatonin group. This study shows that the concomitant administration of melatonin may improve the efficacy of chemotherapy, mainly in terms of survival time, and reduce chemotherapeutic toxicity in advanced NSCLC, at least in patients in poor clinical condition (Lissoni et al., 1997).

Lissoni et al. (1999) evaluated effects of concomitant melatonin administration on toxicity and efficacy of several chemotherapeutic combinations in advanced cancer patients with poor clinical status. The study included 250 metastatic solid tumour patients (lung cancer, 104; breast cancer, 77; gastrointestinal tract neoplasms, 42; head and neck cancers, 27), who were randomized to receive melatonin (20mg/day orally every day) plus chemotherapy, or chemotherapy alone. Chemotherapy consisted of cisplatin (CDDP) plus etoposide or gemcitabine alone for lung cancer, doxorubicin alone, mitoxantrone alone or paclitaxel alone for breast cancer, 5-FU plus folinic acid for gastro-intestinal tumours and 5-FU plus CDDP for head and neck cancers. The 1-year survival rate and the objective tumour regression rate were significantly higher in patients concomitantly treated with melatonin than in those who received chemotherapy alone. The concomitant administration of melatonin significantly reduced the frequency of thrombocytopenia, neurotoxicity, cardiotoxicity, stomatitis and asthenia. This study indicates that the pineal hormone melatonin may

enhance the efficacy of chemotherapy and reduce its toxicity, at least in advanced cancer patients of poor clinical status.

A clinical trial was performed in locally advanced or metastatic patients with solid tumours other than renal cell cancer and melanoma. The study included 80 consecutive patients, who were randomized to be treated with interleukin 2 (IL-2) alone subcutaneously (3 million IU day⁻¹ at 8.00 p.m. 6 days a week for 4 weeks) or IL-2 plus melatonin (40 mg day⁻¹ orally at 8.00 p.m. every day starting 7 days before IL-2). Tumour objective regression rate was significantly higher in patients treated with IL-2 and melatonin than in those receiving IL-2 alone. The mean increase in lymphocyte and eosinophil number was significantly higher in the IL-2 plus melatonin group than in patients treated with IL-2 alone; on the contrary, the mean increase in the specific marker of macrophage activation neopterin was significantly higher in patients treated with IL-2 alone. The treatment was well tolerated in both groups of patients. This study shows that the concomitant administration of the pineal hormone melatonin may increase the efficacy of low-dose IL-2 subcutaneous therapy (Lissoni et al., 1994).

3.7 Dexrazoxane

Dexrazoxane received FDA approval in 1995 for reducing the incidence and severity of cardiomyopathy associated with doxorubicin administration in women with metastatic breast cancer who have received a cumulative doxorubicin dose of 300 mg/m² and who continue to receive doxorubicin therapy. For this use, dexrazoxane is given immediately before doxorubicin i.v. in a ratio (in milligrams) of 10:1 dexrazoxane: doxorubicin. In Europe, a 20:1 ratio is used. Thus, in Europe, a typical schedule for cardioprotection in a patient receiving 50 mg/m² of doxorubicin includes a single dose of 1,000 mg/m² of dexrazoxane given immediately prior to doxorubicin. From this experience, the sponsor selected the 3-day schedule used in the clinical studies. Renal excretion of dexrazoxane is substantial; based on a model of systemic exposure, the dose should be reduced by 50% in patients with creatinine clearance values <40 ml/minute. The possible benefit of alternative dose schedules has not been examined. The sponsor is conducting a population pharmacokinetic analysis to compare population parameter estimates and interindividual variability with literature values for dexrazoxane. There are no known drug interactions (Kane et al., 2008).

It has been proposed that dexrazoxane may act through its rings-opened hydrolysis product ADR-925, which can either remove iron from the iron-doxorubicin complex or bind to free iron, thus preventing iron-based oxygen radical formation. However, it is not known whether the antioxidant actions of dexrazoxane are totally dependent on its metabolism to its rings-opened hydrolysis product and whether dexrazoxane has any effect on the iron-independent oxygen free radical production. However, Junjing et al. (2010) demonstrated that dexrazoxane was an antioxidant that could effectively scavenge these free radicals and the scavenging effects of dexrazoxane did not require the enzymatic hydrolysis. In addition, dexrazoxane was capable to inhibit the generation superoxide and hydroxyl radicals in iron free reaction system, indicating that the antioxidant properties of dexrazoxane were not solely dependent on iron chelation. Thus the application of dexrazoxane should not be limited to doxorubicin-induced cardiotoxicity. Instead, as an effective antioxidant that has been clinically proven safe, dexrazoxane may be used in a broader spectrum of diseases that are known to be benefited by antioxidant treatments.

Between January, 1996, and September, 2000, children with high-risk acute lymphoblastic leukaemia (ALL) were enrolled from nine centres in the USA, Canada, and Puerto Rico. Patients were assigned by block randomisation to receive ten doses of 30 mg/m² doxorubicin alone or the same dose of doxorubicin preceded by 300 mg/m² dexrazoxane. In this study was established the long-term effect of dexrazoxane on the subclinical state of cardiac health in survivors of childhood high-risk ALL 5 years after completion of doxorubicin treatment. 100 children were assigned to doxorubicin (66 analysed) and 105 to doxorubicin plus dexrazoxane (68 analysed). 5 years after the completion of doxorubicin chemotherapy, mean left ventricular fractional shortening and end-systolic dimension Z scores were significantly worse than normal for children who received doxorubicin alone but not for those who also received dexrazoxane. The protective effect of dexrazoxane, relative to doxorubicin alone, on left ventricular wall thickness and thickness-to-dimension ratio were the only statistically significant characteristics at 5 years. Subgroup analysis showed dexrazoxane protection for left ventricular fractional shortening at 5 years in girls, but not in boys. Similarly, subgroup analysis showed dexrazoxane protection for the left ventricular thickness-to-dimension ratio at 5 years in girls, but not in boys. With a median follow-up for recurrence and death of 8.7 years, event-free survival was 77% for children in the doxorubicin-alone group, and 76% for children in the doxorubicin plus dexrazoxane group (Lipshultz et al., 2010). The authors concluded that dexrazoxane provides long-term cardioprotection without compromising oncological efficacy in doxorubicin-treated children with high-risk ALL. Dexrazoxane exerts greater long-term cardioprotective effects in girls than in boys.

Lopez et al. (1998) conducted a randomized trial to evaluate primarily the cardioprotective effect of dexrazoxane in patients with advanced breast cancer and soft tissue sarcomas treated with high-dose epirubicin. Patients with breast cancer (n = 95) or STS (n = 34) received epirubicin 160 mg/m² by intravenous (I.V.) bolus every 3 weeks with or without dexrazoxane 1,000 mg/m² I.V. In either disease, antitumor response rates, time to progression, and survival did not significantly differ between the two arms. There was little difference in noncardiac toxicity for the two treatment groups. All methods of cardiac evaluation clearly documented the cardioprotective effect of dexrazoxane. Dexrazoxane significantly protects against the development of cardiotoxicity when high single doses of epirubicin are used. Apparently, there was no evidence of an adverse impact of dexrazoxane on antitumor activity.

Choi et al. (2010) enrolled patients who were diagnosed as having solid tumors and treated them with the same chemotherapeutic regimen. Doxorubicin was administered at a dose of 30 mg/m² in combination with cisplatin 60 mg/m², cyclophosphamide 60 mg/kg, and etoposide 200 mg/m² at intervals of 4 weeks with or without chest radiation therapy. Doxorubicin was administered intravenously as a bolus infusion. Dexrazoxane was administered intravenously 30 min prior to each dose of doxorubicin in the 10:1 ratio dexrazoxane: doxorubicin. There was no clinically significant side effect associated with dexrazoxane administration. The authors concluded that dexrazoxane reduces the incidence and severity of early and late cardiotoxicity in children with solid tumors receiving doxorubicin chemotherapy. Administration of dexrazoxane was well tolerated and no second malignant neoplasm was observed during the follow-up period, which might be contributed by the limited follow-up period. For them, this study supports the benefit of dexrazoxane as a cardioprotective agent in children who are vulnerable to cardiac damage by anthracycline.

4. Conclusion

Until the present moment studies have shown that the association of antioxidant vitamins for the treatment of cancer is still a controversial issue. However, with few exceptions, we can say that most studies have reported positive findings from the interaction of antioxidants during cancer treatment. The majority of conventional chemotherapeutic agents cause cell death by directly inhibiting the synthesis of DNA or interfering with its function. Adverse effects such as cardiotoxicity of many drugs in cancer treatment are mediated by mechanisms that are distinct from those underlying the antitumor effects of these drugs. Most agents do not really depend that much on free radical damaging mechanisms of action. Thus, free radical generation would arise with an adverse effect and not as a primary mechanism of action. Although further studies are needed, the predominance of evidence supports a provisional conclusion that dietary antioxidants do not conflict with the use of chemotherapy in the treatment of a wide variety of cancers and may significantly mitigate the adverse effects of that treatment.

5. References

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Cytotoxic Plants: Potential Uses in Prevention and Treatment of Cancer

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1. Introduction

Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in 2008. Deaths from cancer worldwide are projected to continue to rise to over 11 million in 2030. [(2011) World health statistics]. Heredity and environmental changes affect the susceptibility to cancer affection. More than 30% of cancer could be prevented by modifying or avoiding key risk factors, including: tobacco use, being overweight or obese, low fruit and vegetable intake, physical inactivity, alcohol use, sexually transmitted HPV-infection, urban air pollution, indoor smoke from household use of solid fuels.

Plants have been used as a major source of remedies from the ancient time. The modern drug discovery and development is also dependent of medicinal plants (Saklani & Kutty, 2008). Using plants in the treatment of cancer has long history and dates back to ancient time. There are strong evidences about cancer preventing properties of various kinds of herbs used as food, fruit, spices, and vegetables (Dossus, 2008; Kruk, 2007; Moyad, 2004; Montesano, 2001; Lyman, 1992).

Dietary habits especially those involving fruits and vegetables have served the great interest in developing various preventive measures that influence cancer risk (Wu, 2009; Kurahashi, 2009). Phytochemicals of varied chemical structures from fruits and vegetables have already been studied extensively for their potential anticancer or chemopreventive efficacy (Ramos, 2008). Being the rich sources of vitamins, minerals, and fiber without posing "any side effects" made fruits and vegetables the best choice to lowering cancer risk and also in maintaining good general health.

The important role of plant derived compounds is undeniable. Paclitaxel (Wani et al., 1971), camptothecin (Wall, 1998), combrestatin (Cirla & Mann, 2003), epipodophyllotoxin (Canel et al., 2000) and Vinca alkaloids (vinblastine, vincristine) (Johnson et al., 1963) are some examples of herbal originated cancer treatments. These are also many other plant-derived compounds that are in clinical trials for cancers (Saklani & K. Kutty, 2008).

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Herbs and spices can defy the DNA damage which is the fundamental cause of cancer and can occur as a result of aging, genetic susceptibility, and exposure to an assortment of carcinogens.

Free radicals and different toxins have the important role in cancer development and progression through interaction with DNA. Numerous phytonutrients found in fruits, herbs and spices act as potent preventive agents against cancer by preventing the overproduction of toxic chemicals within the body, improving the body's detoxification processes.

Herbs and spices not only reduce the risks of developing cancer, but also act as efficient treatments for cancer. Herbs and spices are traditional cancer treatments of radiotherapy and chemotherapy enhancers, reducing the negative side effects of these therapies.

Edible vegetables, fruits, spices and whole grains contain significant amounts of bioactive phytochemicals, which are warranted health benefits beyond basic nutrition to reduce the risk of chronic disease and the process of carcinogenesis [Liu, 2004]

The National Cancer Institute (NCI) of the United States has introduced several plant-based foods that exert cancer-preventive properties, including garlic, soybeans, ginger, onion, turmeric, tomatoes and cruciferous vegetables (for example, broccoli, cabbage, cauliflower and Brussels sprouts). (Surh, 2003). Iridoids, phenols, phenolics, carotenoids, alkaloids, organosulfur compounds, and terpenoids are main class of phytochemicals.

Plants with cytotoxic effects respect to their sub family, species, phytochemical, and the stage of which they are in progress for cancer treatment are classified in table 1 to 38.

Each table is representative of phytochemical of a selected family. Each row illustrates the selected phytochemical of representative plant bioactives that have been shown to induce cytotoxic effects.

Anacardiaceae R.Brown

A family of 69 genera and 850 species mainly subtropical trees, shrubs, lianas or rarely perennial herbs with vertical resin - ducts in bark. The family contains dyeing and tanning materials and phenolic compounds (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Rhus verniciflua</i> Stokes	total extract	not stated	metastatic colorectal cancer (mCRC)	clinical trial	Lee et al, 2009

Table 1. Anacardiaceae Cytotoxic Phytochemicals

Apiaceae Lindley

(Umbelliferae A.L. de Jussieu)

The plant family consists of 434 genera and 3780 species most members are herbs with furrowed stems and hollow internodes, some are annuals, some biennials, and some perennials. The three subfamilies are as follows: 1) Hydrocotyloideae 2) Saniculoideae and 3) Apioioideae.

Constituents of the family include essential oils, coumarins, furocoumarins, chromonocoumarins, monoterpenes, sesquiterpenes, triterpenoid saponins, resins and acetylenic compounds. Alkaloids occur but are rare (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Angelica sinensis</i> (Oliv.) Diels	citronellol	terpenoids	improve their immune function, improving their ability to fight off the cancer	randomized, double-blind, placebo-controlled study	Zhuang et al., 2009
<i>Cuminum cyminum</i> L.	apigenin and luteolin	flavonoids	cancer chemopreventive activities	cell culture, animal study	Aggarwal et al., 2008; Patel et al., 2007; Manju & Nalini, 2007
<i>Foeniculum vulgare</i> Mill.	anethole, [1-methoxy-4-(1-propenyl)benzene], anethole dithiolethione	phenylpropanoid	chemopreventive activities as indicated by suppression of the incidence and multiplicity of both invasive and non-invasive adenocarcinomas	cancer cells cell culture	Aggarwal et al., 2008

Table 2. Apiaceae Cytotoxic Phytochemicals

Apocynaceae A.L. de Jussieu

This family contains 380 genera and 4700 species

mostly in tropical and subtropical but also in few temperate regions. The members are trees, shrubs, lianas, vines, sometimes succulent and cactuslike.

Constituents of the family are some types of alkaloids, cardioactive glycosides, cyanogenic glycosides leucoanthocyanins, saponins, tannins, coumarins, phenolic acids, cyclitols and triterpenoids (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Catharanthus roseus</i> (L.) G. Don	methyl jasmonate	cyclopentanone derivatives	induces apoptosis	A549 human lung adenocarcinoma cells, myeloid leukemia cells	Balbi & Devoto, 2007
<i>Rhazya stricta</i> Decne.	didemethoxycarbonyl-tetrahydrosecamine, sewarine, tetrahydrosecamine, tetrahydrosecaminediol diacetate vallesiachotamine DL-1-(oxo-3,4-thero-3,4,5-trihydroxy-1-pentyl)- β -carboline, 16-epi-Z-isositsirkine	alkaloids	cytotoxic	cell culture	Gilani et al., 2007

Table 3. Apocynaceae Cytotoxic Phytochemicals

Araliaceae A.L. de Jussieu

A family of 39 genera and 1425 species mainly tropical shrubs, lianas or trees to occasionally herbs, aromatic with secretory canals containing volatile oils and resins. other constituents include saponins, a few alkaloids, acetylenic compounds, coumarins, diterpenoids and triterpenoids (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Panax ginseng</i> C.A.Mey.	ginsenosides	saponins	anticancer effects: regulation of cell cycle, Induction of apoptosis, Inhibition of angiogenesis	<i>in vitro</i> and <i>in vivo</i>	Qi et al., 2010
<i>Panax ginseng</i> C.A.Mey.	ginsenosides	saponins	Re and Rg1 enhance angiogenesis, whereas Rb1, Rg3 and Rh2 inhibit it. Rh2, an antitumor agent,, <i>P. quinquefolium</i> has better anticancer effects	<i>in vitro</i> and <i>in vivo</i>	Chen et al., 2008
<i>Panax ginseng</i> C.A.Mey.	total extract	not stated	anticancer antitumor	cell culture	Xiang et al., 2008
<i>Panax ginseng</i> C.A.Mey.	ginsenosides	saponins	the risk of cancer was shown to be lower in those who used ginseng	prospective cohort study	Yun et al., 1998; Kiefer et al., 2003
<i>Panax ginseng</i> C. A. Mey.	ginsenosides	saponins	antitumor/cytotoxicity activities	<i>in vitro</i> and <i>in vivo</i> against a wide variety of cancer cell lines or <i>in vivo</i> neoplasms	Chang et al., 2003

Table 4. Araliaceae Cytotoxic Phytochemicals

Asteraceae Bercht. & J. Presl

(Compositae Giseke)

The family is the largest family of flowering plants and contains about 1590 genera and 23600 species that comprise of herbs, shrubs or trees. Asteraceae divided into 3 subfamilies: 1) Baradosioideae 2) Cichorioideae and 3) Asteroideae.

The Asteraceae contains a wide variety of chemical constituents. Some of the essential oils found in the family contain acetylenic compounds. sesquiterpenes known as azulenes. mono sesquiterpene lactones occur. Alkaloids of the pyridine, pyrrolizidine, quinoline and diterpenoid types also occur in the family, other constituents include triterpenoid saponins, cyclitols, coumarins and flavonols (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Artemisia annua</i> L.	different fractions	not stated	antiproliferative effect	cancer cell lines	Rabe et al., 2011

<i>Artemisia annua</i> L.	artemisinin	sesquiterpenoids	colon cancer	<i>in vitro</i>	McGovern et al., 2010
<i>Artemisia annua</i> L.	artemisinin	sesquiterpenoids	anticancer effects, cell cycle arrest, apoptosis, inhibition of angiogenesis, disruption of cell migration, and modulation of nuclear receptor responsiveness	in a variety of human cancer cell model systems	Firestone et al., 2009
<i>Artemisia annua</i> L.	different fractions	not stated	cytotoxic and pro-apoptotic	variety of cancer cell lines	Emami et al., 2009b
<i>Artemisia annua</i> L.	artemisinins	sesquiterpenoids	anticancer properties	in cell lines and animal models	Krishna et al., 2008
<i>Artemisia annua</i> L.	artemisinin	sesquiterpenoids	regulation of proliferation (BUB3, cyclins, CDC25A), angiogenesis (vascular endothelial growth factor and its receptor, matrix metalloproteinase-9, angiostatin, thrombospondin-1) or apoptosis (BCL-2, BAX, NF-kappaB). p53-dependent and -independent apoptosis	tumor cells	Efferth et al., 2007
<i>Artemisia annua</i> L.	total extract	not stated	cytotoxic activity	human Caucasian hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2)	Vahdati-Mashhadian et al., 2009
<i>Artemisia argyi</i> H.Lév. & Vaniot	isoscopoletin	coumarins	lung cancer	<i>in vitro</i>	McGovern et al., 2010

<i>Artemisia biennis</i> Willd.	different fractions	not stated	antiproliferative effect		Rabe et al., 2011
<i>Artemisia campestris</i> L.	total extract	not stated	cytotoxic activity	human Caucasian hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2)	Vahdati-Mashhadian et al., 2009
<i>Artemisia chamaemelifolia</i> Vill.	total extract	not stated	cytotoxic activity	human Caucasian hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2)	Vahdati-Mashhadian et al., 2009
<i>Artemisia ciniformis</i> Krasch. & Popov ex Poljak.	different fractions	not stated	antiproliferative effect	cancer cell lines	Rabe et al., 2011
<i>Artemisia diffusa</i> Krasch. ex Poljakov	different fractions	not stated	antiproliferative effect	cancer cell lines	Rabe et al., 2011
<i>Artemisia diffusa</i> Krasch. ex Poljakov	total extract	not stated	cytotoxic activity	human Caucasian hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2)	Emami et al., 2009a
<i>Artemisia fragrans</i> Willd.	total extract	not stated	cytotoxic activity	human Caucasian hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2)	Vahdati-Mashhadian et al., 2009
<i>Artemisia incana</i> Druce	total extract	not stated	cytotoxic activity	human Caucasian	Vahdati-Mashhadian

				hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2)	et al., 2009
<i>Artemisia khorassanica</i> Podlech	different fractions	not stated	cytotoxic activity	variety of cancer cell lines	Mahmoudi et al., 2009
<i>Artemisia kulbadica</i> Boiss. & Buhse	total extract	not stated	cytotoxic activity	human Caucasian hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2)	Emami et al., 2009a
<i>Artemisia persica</i> Boiss.	different fractions	not stated	antiproliferative effect	cancer cell lines	Rabe et al., 2011
<i>Artemisia persica</i> Boiss.	total extract	not stated	cytotoxic activity	human Caucasian hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2)	Vahdati-Mashhadian et al., 2009
<i>Artemisia santolina</i> Schrenk	different fractions	not stated		cancer cell lines	Rabe et al., 2011
<i>Artemisia santolina</i> Schrenk	total extract	not stated	cytotoxic activity	human Caucasian hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2)	Emami et al., 2009a
<i>Artemisia sieberi</i> Besser	total extract	not stated	cytotoxic activity	human Caucasian hepatocyte carcinoma (HepG-2) and human	Emami et al., 2009a

				Caucasian larynx carcinoma (Hep-2)	
<i>Artemisia turanica</i> Krasch.	total extract	not stated	cytotoxic activity	human Caucasian hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2)	Emami et al., 2009a
<i>Artemisia vulgaris</i> L.	different fractions	not stated	antiproliferative effect	cancer cell lines	Rabe et al., 2011
<i>Artemisia vulgaris</i> L.	total extract	not stated	cytotoxic activity	human Caucasian hepatocyte carcinoma (HepG-2) and human Caucasian larynx carcinoma (Hep-2)	Vahdati-Mashhadian et al., 2009
<i>Inula britannica</i> M.Bieb.	1-O-acetylbritannilactone	sesquiterpene lactones	cytotoxic, apoptotic	cell culture	Khan et al., 2010
<i>Inula britannica</i> M.Bieb.	1,6-O,O-diacetylbritannilactone	sesquiterpene lactones	cytotoxic, apoptotic	cell culture	Khan et al., 2010
<i>Inula britannica</i> M.Bieb.	6 α -O-(2-methylbutyryl)-britannilactone	sesquiterpene lactones	cytotoxic, apoptotic, inflammation	cell culture	Khan et al., 2010
<i>Inula britannica</i> M.Bieb.	neobritannilactone A	sesquiterpenes lactones	cytotoxic, apoptotic, inflammation	cell culture	Khan et al., 2010
<i>Inula britannica</i> M.Bieb.	neobritannilactone B	sesquiterpene lactones	cytotoxic, apoptotic, inflammation	cell culture	Khan et al., 2010
<i>Inula britannica</i> M.Bieb.	quercetin, spinacetin, diosmetin	flavonoids	antioxidant, cytotoxic	cell culture	Khan et al., 2010
<i>Saussurea costus</i> (Falc.) Lipsch.	C17 polyene alcohol	polyenes	anticancer, antitumor, moderate cytotoxicity	against the human tumor cell lines A549, SK-OV3, SK-MEL-2, XF 498, and	Wang et al., 2010

				HCT 15	
<i>Saussurea costus</i> (Falc.) Lipsch.	lappadilactone, dehydrocostuslactone, and costunolide	sesquiterpene lactones	most potent cytotoxicities	HepG2, OVCAR-3, and HeLa cell lines	Wang et al., 2010
<i>Saussurea costus</i> (Falc.) Lipsch.	total extract	not stated	cytostatic effects, inducer of apoptosis	AGS gastric cancer cell line	Wang et al., 2010
<i>Saussurea costus</i> (Falc.) Lipsch.= <i>Saussurea lappa</i> C.B.Clarke	costunolide	sesquiterpene lactones	Anticancer, induction of apoptosis	in HL-60 human leukemia cells	Pandey et al., 2007
<i>Saussurea costus</i> (Falc.) Lipsch.= <i>Saussurea lappa</i> C.B.Clarke	total extract	not stated	induced apoptotic cell death	AGS gastric cancer cell line	Pandey et al., 2007
<i>Saussurea costus</i> (Falc.) Lipsch.= <i>Saussurea lappa</i> C.B.Clarke	lappadilactone, dehydrocostuslactone and costunolide	sesquiterpene lactones	cytotoxic	HepG2, OVCAR-3 and HeLa cell lines	Pandey et al., 2007
<i>Saussurea costus</i> (Falc.) Lipsch.= <i>Saussurea lappa</i> C.B.Clarke	dehydrocostus lactone	sesquiterpene lactone	induced apoptosis	human leukemia HL-60 cells	Pandey et al., 2007
<i>Saussurea costus</i> (Falc.) Lipsch.= <i>Saussurea lappa</i> C.B.Clarke	cynaropicrin	sesquiterpene lactones	pro-apoptotic activity	leukocyte cancer cell lines, such as U937, Eol-1 and Jurkat T cells	Pandey et al., 2007
<i>Saussurea costus</i> (Falc.) Lipsch.= <i>Saussurea lappa</i> C.B.Clarke	costunolide	sesquiterpene lactones	anti-angiogenic effect	vascular endothelial growth factor (VEGF)	Pandey et al., 2007
<i>Saussurea costus</i> (Falc.) Lipsch.= <i>Saussurea lappa</i> C.B.Clarke	C-17 polyene alcohol	polyenes	moderate cytotoxicities	human tumor cell lines A549, SK-OV3, SK-MEL-2, XF 498 and HCT 15	Pandey et al., 2007
<i>Saussurea medusa</i> Maxim.	arctiin and arctigenin	lignans	remarkable antitumorpromoting effect on two-stage carcinogenesis test	mouse-skin tumors, mouse pulmonary tumors	Wang et al., 2010

<i>Saussurea</i> spp.	costunolide	sesquiterpene lactones	inducer of apoptosis	HL-60 human leukemia cells	Wang et al., 2010
<i>Saussurea</i> spp.	arctigenin		inhibition of TNF- α induction		Wang et al., 2010
<i>Saussurea</i> spp.	dehydrocostuslactone	sesquiterpene lactones	induced apoptosis	human leukemia HL-60 cells	Wang et al., 2010
<i>Saussurea</i> spp.	cynaropicrin	sesquiterpene lactones	inhibited the proliferation, potential anticancer agent against some leukocyte cancer cells	leukocyte cancer cell lines, such as U937, Eo1, and Jurkat T cells	Wang et al., 2010
<i>Saussurea</i> spp.	costunolide	sesquiterpene lactones	anti-angiogenic effect	human umbilical vein endothelial cells (HUVECs)	Wang et al., 2010
<i>Silybum marianum</i> (L.) Gaertn	total extract	not stated	prevention or treatment of liver dysfunction in patients undergoing anticancer therapy	patients with cancer	Ladas & Kelly, 2003
<i>Silybum marianum</i> (L.) Gaertn	silibinin, silymarin	flavonolignans	Prostate cancer, anticancer effects, inhibition of mitogenic and cell survival signaling	in different cancer cells, animal models	Singh et al., 2004
<i>Silybum marianum</i> (L.) Gaertn.	silibinin	flavonolignans	inhibition of multiple cancer cell signaling pathways, including growth inhibition, inhibition of angiogenesis, chemosensitization, and inhibition of invasion and metastasis	in animals and humans	Li et al., 2010
<i>Silybum marianum</i> (L.) Gaertn.	silybin, isosilybin, silychristin, silydianin and taxifoline	flavonolignans	suppression of the proliferation of a variety of tumor cells (e.g., prostate, breast,	cell culture, animal study and clinical trials	Agarwal et al., 2006

			ovary, colon, lung, bladder), angiogenesis (VEGF) and metastasis, as a chemopreventive agent, apoptosis induction, antitumor activity		
<i>Silybum marianum</i> (L.) Gaertn.	silybin and silymarin	flavonolignans	anticancer and canceroprotective	not stated	Kren et al., 2005
<i>Silybum marianum</i> (L.) Gaertn.	silymarin	flavonolignans	cancer prevention, adjuvant cancer treatment, and reduction of iatrogenic toxicity	clinical trials	Sagar et al., 2007
<i>Silybum marianum</i> (L.) Gaertn.	total extract	not stated	protective effect in certain types of cancer	clinical trials	Tamayo et al., 2007

Table 5. Asteraceae Cytotoxic Phytochemicals

Brassicaceae Burnett**(Cruciferae** A.L. de Jussieu)

A family of 321 genera and about 3400 species of herbs and a few undershrubs. Many members of the family contain glucosinolates. Cardiac glycosides occur in some genera and the seeds usually contain mucilage and fixed oil (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Arabidopsis thaliana</i> (L.) Heynh.	jasmonate	cyclopentanone derivatives	induces cell death and suppresses cell proliferation	in animals, several human cancer cell lines	Balbi & Devoto, 2007
<i>Brassica</i> spp.	indole-3-carbinol	glucosinolates	breast cancer, prostate cancer, endometrial cancer, colon cancer, and leukemic, induce G1/S arrest of the cell cycle, and induce apoptosis	<i>in vitro</i> and <i>in vivo</i> , clinical trials	Aggarwal & Ichikawa, 2005

Table 6. Brassicaceae Cytotoxic Phytochemicals

Campannlaceae A.L. de Juessieu

The family contains 79 genera and around 1900 species mostly herbs but some shrubs and pachycaul trees with a network of laticifers in phloem. The members of the family contain

phenolic compounds, tannins and triterpenoid glycosides (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Codonopsis pilosula</i> Nannf.	a mixture of citronellol and extracts of <i>Ganoderma lucidum</i> , <i>Codonopsis pilosula</i> and <i>Angelica sinensis</i>	not stated	Improvement of immune function, improving the ability to fight off the cancer, as well as any secondary infections that could compromise the treatment and the health	randomized, double-blind, placebo-controlled study	Zhuang et al., 2009

Table 7. Campannaceae Cytotoxic Phytochemicals

Cannabaceae Martynov

A family of 10 genera and 80 species usually trees or shrubs but also herbs or vines. Widely distributed in tropical to temperate regions (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Humulus lupulus</i> L.	hop acids	sesquiterpenoids	inhibiting cell proliferation and angiogenesis, by inducing apoptosis	<i>in vitro</i> and <i>in vivo</i>	Van Cleemput et al., 2009

Table 8. Cannabaceae Cytotoxic Phytochemicals

Clusiaceae Lindley

(**Guttiferae** A.L. de Jussieu)

The Clusiaceae contains about 30 genera and 1150 tropical species. They are trees shrubs or lianas with colored exudate in secretory canals or cavities. Constituents of the family include resins, volatile oils, alkaloids, xanthenes and seed oil (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Garcinia cantleyana</i> Whitmore	cantleyanones, 7-hydroxyforbesione, and deoxygaudichaudione A from	xanthenes	cytotoxic effect	variety of cancer cell lines	Han et al., 2009
<i>Garcinia gaudichaudii</i> Planch. & Triana	gaudichaudiones, gaudichaudic acids, gaudichaudione H xanthenes	xanthenes	potent antitumor activity	variety of cancer cell lines	Han et al., 2009
<i>Garcinia indica</i> Choisy	gambogic acid (gamboges or kokum)	pigments	cytotoxic, apoptotic, antiangiogenesis and anticancer	<i>in vitro</i> and <i>in vivo</i>	Aggarwal et al., 2008

<i>Garcinia</i> spp.	gaudichaudione A	xanthenes	induced the apoptosis	human leukemic cells	Han et al., 2009
<i>Garcinia</i> spp.	gambogic acid	pigments	induce apoptosis, can overcome the drug resistance, In vitro and in vivo studies	<i>in vitro</i> and <i>in vivo</i>	Han et al., 2009

Table 9. Clusiaceae Cytotoxic Phytochemicals

Combretaceae R. Brown

A family of 17 genera and 525 species, tropical and subtropical trees, shrubs or lianas, sometimes with erect monopodial trunk supporting a series of horizontal, sympodial branches. Members of the family usually rich in tannin (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Combretum caffrum</i> Kuntze	combretastatin A-4	phenolic compounds	potent antimitotic agent	cancer cells cell culture	Nam et al., 2003

Table 10. Combretaceae Cytotoxic Phytochemicals

Cornaceae Bercht. & Presl

A family of 2 genera and 80 species. Trees, shrubs or rarely rhizomatous herbs usually with iridoids, widespread; specially common in north temperate regions, rare in tropical and south temperate regions (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Camptotheca acuminata</i> Decne.	camptothecin and its derivatives	alkaloids	anticancer drugs	cancer treatment in patients	Sirikantaramas et al., 2007

Table 11. Cornaceae Cytotoxic Phytochemicals

Crassulaceae J. St. Hilaire

The Crassulaceae contains 92 genera and around 1380 species almost cosmopolitan especially south Africa, rare in Australia and west Pacific. succulent herbs to shrubs; often with cortical or medullary vascular bundles; with crassulacean acid metabolism (CAM), tannins present, often with alkaloids, sometimes cyanogenic (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Rhodiola rosea</i> L.	total extract	not stated	anticancer, decreased metastasis to the liver, and extended survival times	in animal experiments	Kelly et al., 2001

Table 12. Crassulaceae Cytotoxic Phytochemicals

Cupressaceae Gray

A family of 30 genera and 130 species. The family is subcosmopolitan of warm to cold temperate climate. Monoecious or dioecious resinous trees or shrubs. Leaves descussate or in whorls of 3 or 4, in young plants needle-like, usually small and scale-like in mature plants. Flowers, small, solitary, axillary or terminal on short shoots, cones terminal, woody, leathery or berry-like, cone-scales opposite or in whorls of 3, ovules usually several per scale. Seeds winged or not. Constituents of the family include essential oils, monoterpenes, sesquiterpenes, diterpenes, tannins and flavonoids (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Cupressus sempervirens</i> L. var. <i>horizontalis</i> (Mill.) Gordon	total extract	not stated	cytotoxic effect	cell culture	Emami et al., 2005
<i>Juniperus excelsa</i> M.Bieb. subsp. <i>excelsa</i>	total extract	not stated	cytotoxic effect	cell culture	Sadeghi-aliabadi et al., 2009a
<i>Juniperus excelsa</i> M.Bieb. subsp. <i>polycarpus</i> (K. Koch) Takhtajan	total extract	not stated	cytotoxic effect	cell culture	Sadeghi-aliabadi et al., 2009a
<i>Juniperus foetidissima</i> Willd.	total extract	not stated	cytotoxic effect	cell culture	Sadeghi-aliabadi et al., 2009b
<i>Juniperus sabina</i> L.	total extract	not stated	cytotoxic effect	cell culture	Sadeghi-aliabadi et al., 2009b
<i>Platycladus orientalis</i> (L.) Franco	total extract	not stated	cytotoxic effect	not stated	Emami et al., 2005

Table 13. Cupressaceae Cytotoxic Phytochemicals

Ericaceae A.L. de Jussieu

The Ericaceae contains 117 genera and 3850 species cosmopolitan except deserts usually montane in tropical regions. Trees, shrubs, lianas or subherbaceous, sometimes epiphytic, occasionally mycoparasitic herbs lacking chlorophyll, strongly associated with mycorrhizal fungi. The family produces phenolic acids, phenolic glycosides, aucubin glycosides, diterpenoids, triterpenoids, cyclitols and leucoanthocyanins. A few species are cyanogenic; saponins are absent (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Vaccinium macrocarpon</i> Aiton	not stated	not stated	induction of apoptosis in tumor cells, reduced ornithine decarboxylase activity, decreased expression of matrix	<i>in vitro</i> tumor models	Neto et al., 2008

<i>Vaccinium macrocarpon</i> Aiton	polyphenolic extracts flavonols, proanthocyanidin oligomers, and triterpenoids isolated from the fruit.	flavonoids	inhibit the growth and proliferation of breast, colon, prostate, lung, and other tumors, induction of apoptosis in tumor cells, reduced ornithine decarboxylase activity, decreased expression of matrix metalloproteinases associated with prostate tumor metastasis, and antiinflammatory activities including inhibition of cyclooxygenases	<i>in vitro</i> studies using a variety of tumor models	Neto et al., 2007
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Table 14. Ericaceae Cytotoxic Phytochemicals

Fabaceae Lindley**(Leguminosae** A.L. de Jussieu)

This is the second - largest family of flowering plants and contains 720 genera and 19500 cosmopolitan species. Herbs, shrubs, trees or vines / lianas climbing by twining or tendrils; with a high nitrogen metabolism and unusual aminoacids, often with root nodules containing nitrogen - fixing bacteria, sometimes with secretory canals or cavities, tannins usually present, often with alkaloids; sometimes cyanogenic, sieve cell plastids with protein crystals and usually also starch grains.

The family is divided into three subfamilies: 1) Papilionoideae; 2) Mimosoideae and 3) Caesalpinioideae (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Glycine max</i> (L.) Merr.	soy saponins	saponins	cancer protective effects	epidemiological studies	Kerwin et al., 2004
<i>Glycyrrhiza inflata</i> Batalin	licochalcone E,	flavonoids	potent cytotoxic effect	in tumor cells	Asl & Hosseinzadeh, 2008
<i>Glycyrrhiza</i> spp.	total extract	not stated	inhibits angiogenesis, induced apoptosis	<i>in vivo</i> and <i>in vitro</i>	Asl & Hosseinzadeh, 2008
<i>Glycyrrhiza</i> spp.	glycyrrhetic acid	triterpenoids	trigger the proapoptotic pathway	in tumor cells	Asl & Hosseinzadeh, 2008
<i>Glycyrrhiza</i> spp.	isoliquiritigenin (ILG)	triterpenoids	antiproliferative activity, trigger the proapoptotic pathway	in tumor cells	Asl & Hosseinzadeh, 2008
<i>Glycyrrhiza</i> spp.	glabridin dibenzoylmethane	triterpenoids	antiproliferative activity, trigger	in tumor cells	Asl & Hosseinzadeh,

	(DBM)		the proapoptotic pathway		2008
<i>Trigonella foenum graecum</i>	diosgenin	saponins	suppress proliferation, invasion inhibition, cytotoxic, apoptotic, anti-cancer activity	cell culture, animal study	Aggarwal et al., 2008

Table 15. Fabaceae Cytotoxic Phytochemicals

Ginkgoaceae Engler

A family contains only a monotypic genus, limited to remote mountain valleys of China; possibly extinct in the wild. From among the many groups of compounds isolated from *Ginkgo biloba* it is diterpenelactons and flavonoids which have been shown to possess therapeutic activity (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Ginkgo biloba</i> L.	total extract	not stated	chemopreventive action at various levels with antioxidant, antiangiogenic properties, reduce angiogenesis	cell culture, cancer model	Mahadevan et al., 2008
<i>Ginkgo biloba</i> L.	total extract	not stated	anticancer (chemopreventive) properties are related to its antioxidant, antiangiogenic and gene-regulatory actions	cell culture	Dubey et al., 2004
<i>Ginkgo biloba</i> L.	ginkgolide B	sesquiterpenoids	anticancer (chemopreventive) properties that are related to their antioxidant, anti-angiogenic and gene-regulatory actions	molecular, cellular and whole animal models	DeFeudis et al., 2003
<i>Ginkgo biloba</i> L.	not stated	flavonoids	anticancer (chemopreventive) properties that are related to their antioxidant, anti-angiogenic and gene-regulatory actions	molecular, cellular and whole animal models	DeFeudis et al., 2003

Table 16. Ginkgoaceae Cytotoxic Phytochemicals

Hypericaceae A. L. de Jussieu

The Hypericaceae consists of 9 genera and 540 species. Members of this family are trees, shrubs or herbs with clear or block resinous sap in secretory cavities (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Hypericum perforatum</i> L.	hypericin	naphthodianthrones	antineoplastic activity upon irradiation, photosensitizer can induce both apoptosis and necrosis	<i>in vivo</i> and <i>in vitro</i> , potential clinical anticancer agent	Agostinis et al., 2002

Table 17. Hypericaceae Cytotoxic Phytochemicals

Iridaceae A. L. de Jussieu

A family of 70 genera and 2000 species. The species are widely distributed usually geophytic herbs with rhizomes, corms or bulbs, less often evergreen or even shrubby, rarely annuals or achlorophyllous mycotroph. Constituents include quinones, aromatic ketones, carotenoid pigments, terpenoids, and flavonoids (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Crocus sativus</i> L.	total extract	not stated	antitumor and cancer preventive activities of saffron and its main ingredients	experimental <i>in vitro</i> and <i>in vivo</i> investigations	Abdullaev & Espinosa-Aguirre, 2004
<i>Crocus sativus</i> L.	total extract	not stated	apoptosis induction in MCF-7 and MDA-MB-231 breast cancer cells	<i>in vitro</i>	Mousavi et al., 2009; Chryssanthi et al., 2007

Table 18. Iridaceae Cytotoxic Phytochemicals

Lamiaceae Martynov**(Labiatae** A. L. de Jussieu)

A family of 238 genera and 6500 species. Trees, shrubs, annual or perennial herbs, rarely lianas. Young stems often 4 - angled. Leaves opposite and simple. Flower are bisexuals, usually bracteolate and in cymes, thyrse or verticillasters or single. The family divided into 7 subfamilies. The Lamiaceae contains many species that are economically important either for their volatile oils or for use as spices. Among the constituents found in the family are essential oils, saponins, tannins, quinones, irodoids: alkaloids appear to be rare (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Ocimum sanctum</i> L.	ursolic acid	triterpenoids	antitumor	not stated	Prakash & Gupta, 2005

<i>Phlomis armeniaca</i> Willd.	Phenyl propanoid caffeic acid, phenylethyl alcohol and phenylethylalcohol glycosides	phenolic compounds	anticancer activity	several kinds of cancer cells	Limem-Ben Amor et al., 2009
<i>Phlomis brunneogaleata</i> Hub.-Mor.	verbascoside, isoverbascoside, forsythoside B and 3-O-caffeoylquinic acid methyl ester	phenolic compounds	cytotoxic activity	L6 cell lines	Limem-Ben Amor et al., 2009
<i>Rosmarinus officinalis</i> L.	ursolic acid (3 β -hydroxy-urs-12-en-28-oic acid)	triterpenoids	suppress tumorigenesis, inhibit tumor promotion, and suppress angiogenesis, inhibited proliferation, induced apoptosis	<i>in vitro</i> and <i>in vivo</i>	Aggarwal et al., 2008
<i>Salvia chamelaeagnea</i> P.J.Bergius,	total extract	not stated	cyotoxic	animals and humans	Kamatou et al., 2008
<i>Salvia namaensis</i> Schinz	total extract	not stated	cyotoxic	animals and humans	Kamatou et al., 2008
<i>Salvia runcinata</i> L.f.	total extract	not stated	cyotoxic	animals and humans	Kamatou et al., 2008
<i>Salvia africana-caerulea</i> L.	total extract	not stated	cytotoxic effects on cancer cells	human cancer cell lines	Kamatou et al., 2008
<i>Salvia chamelaeagnea</i> P.J.Bergius	total extract	not stated	cytotoxic effects on cancer cells	human cancer cell lines	Kamatou et al., 2008
<i>Salvia dolomitica</i> Codd	total extract	not stated	cytotoxic effects on cancer cells	human cancer cell lines	Kamatou et al., 2008
<i>Salvia gariensis</i> E.Mey.	total extract	not stated	cytotoxic effects on cancer cells	human cancer cell lines	Kamatou et al., 2008
<i>Salvia hypargeia</i> Fisch. & Mey.	total extract	not stated	cytotoxic effects on cancer cells	human cancer cell lines	Kamatou et al., 2008
<i>Salvia lanceolata</i> Lam.	total extract	not stated	cytotoxic effects on cancer cells	human cancer cell lines	Kamatou et al., 2008
<i>Salvia multiorrhiza</i> Bunge	in mixture with <i>coriolus versicolor</i> in capsules	not stated	could be beneficial for promoting immunological function in post-treatment of breast cancer patients	clinical trial	Wong et al., 2005
<i>Salvia muiirii</i> L.Bolus	total extract	not stated	cytotoxic effects on cancer cells	human cancer cell lines	Kamatou et al., 2008
<i>Salvia namaensis</i> Schinz	total extract	not stated	cytotoxic effects on cancer cells	human cancer cell lines	Kamatou et al., 2008

<i>Salvia radula</i> Benth.	total extract	not stated	cytotoxic effects on cancer cells	human cancer cell lines	Kamatou et al., 2008
<i>Salvia repens</i> Burch. ex Benth.	total extract	not stated	cytotoxic effects on cancer cells	human cancer cell lines	Kamatou et al., 2008
<i>Salvia runcinata</i> L.f.	total extract	not stated	cytotoxic effects on cancer cells	human cancer cell lines	Kamatou et al., 2008
<i>Salvia verbenaca</i> L.	total extract	not stated	cytotoxic effects on cancer cells	human cancer cell lines	Kamatou et al., 2008
<i>Scutellaria barbata</i> D.Don	aqueous extract	not stated	anticancer activity in women with metastatic breast cancer (MBC)	the trial was an open-label, phase 1B, multicenter, dose escalation study	Perez et al., 2010
<i>Scutellaria</i> spp.	Wogonin, Baicalein and Baicalin	flavones	potent anticancer activities, scavenge oxidative radicals, to attenuate NF-kappaB activity, to inhibit several genes important for regulation of the cell cycle, to suppress COX-2 gene expression and to prevent viral infections	<i>in vitro</i> and <i>in vivo</i>	Li-Weber 2009
<i>Scutellaria lindbergii</i> Rech. f.	different fractions	not stated	cytotoxic effects in different cancer cell lines in which apoptosis plays an important role, potential chemotherapeutic agent	cancer cell lines	Tayarani-Najaran et al., 2009
<i>Scutellaria litwinowii</i> Bornm. & Sint. ex Bornm	different fractions	not stated	cytotoxic effects in different cancer cell lines in which apoptosis plays an important role, potential chemotherapeutic agent	cancer cell lines	Tayarani-Najaran et al., 2011

Table 19. Lamiaceae Cytotoxic Phytochemicals

Liliaceae A. L. de Jussieu

A widely distributed family of 16 genera and about 600 species of perennials with bulbs or rhizomes; aerial stem unbranched. Leaves oval to filiform, usually parallel - veined.

Inflorescences usually raceme sometimes umbel, thyrse or 1 – flowered. Many members of the family contain alkaloids, which are of the steroidal, isoquinoline or purine types, other steroidal substances include sterols, cardenolides bufadienolides and steroidal saponins. Other constituents include quinones, flavonoids, the gamma – pyrone chelidonic acid, cyanogenic substances and fructosan – type carbohydrates. Some volatile oils of the family have antimicrobial properties (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Allium sativum</i> L.	ajoene	thiosulfinates	anticancer activity, activation of the mitochondrial-dependent caspase cascade	<i>in vitro</i>	Kaschula et al., 2010
<i>Allium sativum</i> L.	not stated	not stated	garlic rich diet decreases risk of some cancers	epidemiological studies	Iciek et al., 2009
<i>Allium sativum</i> L.	not stated	not stated	Mortality in stomach cancer patients from the region where people have consumed high-garlic diet (about 20 g a day) was three times lower than in the second region in which consumption of plants of the family Allium was very low	trials carried out in China compared two big human populations living	Iciek et al., 2009
<i>Allium sativum</i> L.	not stated	not stated	negatively correlated with colon cancer, reduced risk of prostate cancer, reduced risk of breast cancer	epidemiological analysis,	Iciek et al., 2009
<i>Allium sativum</i> L.	DATS (diallyl trisulfide)	not stated	protection against gastric cancer	double-blind intervention study	Iciek et al., 2009
<i>Allium sativum</i> L.	alk(en)yl sulfides	thiosulfinates	anticancer effect	human colon cancer cells	Seki et al., 2008
<i>Allium sativum</i> L.	organosulfur compounds (OSCs)	thiosulfinates	inhibition of DNA adduct formation, upregulation of antioxidant defences and DNA repair systems, and suppression of cell proliferation by blocking cell cycle	epidemiological studies as well as laboratory data	Nagini et al., 2008

			progression and/or inducing apoptosis		
<i>Allium sativum</i> L.	diallylsulfides	thiosulfinates	not stated	not stated	Münchberg et al., 2007
<i>Allium sativum</i> L.	allicin and diallyltrisulfide	thiosulfinates	anticancer agents	cell culture	Münchberg et al., 2007
<i>Allium sativum</i> L.	allicin, methyl allyl trisulfide, and diallyl trisulfide	thiosulfinates	not stated	not stated	Ariga et al., 2006
<i>Allium sativum</i> L.	organosulfur compounds	thiosulfinates	anticarcinogenic and antitumorigenic	preclinical	Milner, 2006
<i>Allium sativum</i> L.	thiosulfinates such as allicin	thiosulfinates	anticancer and chemopreventive activities	not stated	Amagase, 2006
<i>Allium sativum</i> L.	organic allyl sulfur components	thiosulfinates	inhibitors of the cancer process, depression in nitrosamine formation and a reduction in carcinogen bioactivation	not stated	Milner et al., 2001
<i>Allium sativum</i> L.	thiosulfinates, allicin, S-allylcysteine, S-allylmercaptocysteine, N (alpha)-fructosyl arginine	thiosulfinates	not stated	not stated	Amagase et al., 2001
<i>Aloe arborescens</i> Mill.	in combination with pineal indole melatonin	not stated	stabilization of disease and survival, in patients with advanced solid tumors	a randomized study of chemotherapy versus biochemotherapy	Lissoni et al., 2009
<i>Aloe vera</i> L.	barbaloin, octapeptide, aloesin, aloe-emodin	anthraquinones	prolongation of the life span of tumor-transplanted animals, cytotoxicity, apoptosis	<i>in vitro</i> (acute myeloid leukemia (AML) and acute lymphocytes leukemia (ALL) cancerous cells) and <i>in vivo</i>	El-Shemy et al., 2010
<i>Aloe vera</i> L.	acemannan	polysaccharides	anti-tumour activity	<i>in vitro</i> models as well as in different animal species	Hamman et al., 2008
<i>Aloe vera</i> L.	gel	not stated	reduced tumour burden, tumour shrinkage, tumour necrosis and prolonged survival rates,	<i>in vitro</i> models as well as in different animal species	Hamman et al., 2008

			chemopreventative and anti-genotoxic effects on benzo[a]pyrene-DNA adducts		
<i>Aloe vera</i> L.	not stated	glycoproteins (lectins) and polysaccharides	anti-cancer effects, stimulation of the immune response	<i>in vitro</i> models as well as in different animal species	Hamman et al., 2008

Table 20. Liliaceae Cytotoxic Phytochemicals

Linaceae A. P. de Candolle ex Perleb.

A family of 10 genera and 280 species.

Lianas, shrubs and herbs sometimes cyanogenic. Leaves are spirals to opposites, simple, entire. Flowers bisexuals usually regulars and 5 - merous in cymose or racemose inflorescence. Constituents of the family in clued cyanogenaic glycosides, fixed oils, mucilage, diterpenes and triterpenes (Evans 2009; Judd et al., 2008; Mabberley 2008).

Family Linaceae

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Linum usitatissimum</i> L.	secoisolariciresinol	lignans	inhibited the invasion, inhibitory effect on breast and colon carcinoma, potent cytotoxic effect on human promyleocytic leukemia HL-60 cells, DNA damage and apoptosis, anticancer, antiapoptotic	cell culture	Sok et al., 2009
<i>Linum usitatissimum</i> L.	lariciresinol and pinoresinol	lignans	anticancer, antiapoptotic	cell culture	Sok et al., 2009
<i>Linum usitatissimum</i> L.	secoisolariciresinol diglucoside	lignans	anticancer, antiapoptotic	cell culture	Sok et al., 2009
<i>Linum usitatissimum</i> L.	hydroxymatairesinol, matairesinol	lignans	antitumor activity, anticarcinogenic effects	cell culture, animal experiment	Sok et al., 2009
<i>Linum usitatissimum</i> L.	enterolactone and enterodiol	Lignans	potential anticancer effects	randomized crossover study	Coulman et al., 2005

Table 21. Linaceae Cytotoxic Phytochemicals

Loranthaceae A. L. de Jussieu

A family of 84 genera and 950 species. Pantropical but no single genus spans both Old and New Worlds.

Typically brittle shrobllets on tree – branches; less often terrestrial shrubs, lianas or even trees. The family contains glycoproteins, polypeptides, lignans, flavonoids, etc. (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Viscum album</i> L.	total extracts	not stated	the evidence from RCTs to support the view that the application of mistletoe extracts has impact on survival or leads to an improved ability to fight cancer or to withstand anticancer treatments is weak.	randomized clinical trials (RCTs) and controlled clinical trials	Horneber et al., 2008
<i>Viscum album</i> L.	lectins	proteins	anti-metastatic effect, inhibition of tumour-induced angiogenesis, and in part due to an induction of apoptosis, suppress tumour growth	different tumour cell lines, in vivo	Pryme et al., 2006
<i>Viscum album</i> L.	aqueous extract (Isorel)	not stated	Isorel can improve immune competence and the overall health status of cancer patients undergoing surgery	clinical trial	Enesel et al., 2005
<i>Viscum album</i> L.	mistletoe lectins I, II, and III	proteins	improvement of quality of life in cancer patients	several preclinical studies, randomised phase III study	Stauder & Kreuser, 2002
<i>Viscum album</i> L.	total extract (Isorel)	not stated	benefit in terms of survival from combined postoperative chemotherapy and Isorel biotherapy, either adjuvant or palliative	Randomized and controlled study, patients with colorectal cancer stages Dukes C and D	Cazacu et al., 2003

Table 22. Loranthaceae Cytotoxic Phytochemicals

Lythraceae J. St. – Hilaire

A family contains 31 genera and 600 tropical with few temperate species. Herbs, less often sub shrubs or trees. Leaves are opposite and simple. Flowers are bisexuals, often heterostylons, solitary, fascicled in axils or terminal racemes, regular or not, with conspicuous hypanthium.

Constituents of the family include quinones, alkaloids and tannins (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Punica granatum</i> L.	total extract	not stated	potential chemopreventive and anticancer agent	<i>in vitro</i> and <i>in vivo</i> animal models	Syed et al., 2007
<i>Punica granatum</i> L.	total extract	not stated	cancer chemoprevention, anticancer activities, interference with tumor cell proliferation, cell cycle, invasion and angiogenesis	mouse mammary organ culture, <i>in vitro</i> and <i>in vivo</i> , open-label, singlearm, 2-year, phase-2, Simon two-stage clinical trial	Lansky et al., 2007

Table 23. Lythraceae Cytotoxic Phytochemicals

Malvaceae A. L. de Jussieu

The Malvaceae consists of 113 genera and 5000 cosmopolitan species. Trees, shrubs, lianas and herbs, rarely scandent, usually with tufted or stellate hairs and parenchyma typically with scattered mucilage cell, mucilage cavities or mucilage canals. The family divided into 9 subfamilies. Constituents of the family include alkaloids, cardiac glycosides, saponins, tannins, phenolic acids and mucilage (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Gossypium</i> spp.	gossypol	polyphenols	anticancer	not stated	Wang et al., 2009

Table 24. Malvaceae Cytotoxic Phytochemicals

Melanthiaceae Batsch ex Borkh.

A family of 16 genera and about 120 species distributed in temperate and/or montane habitats. Members of this family perennial herbs sometimes pachycaul, with rhizomes and spirals of or distichous leaves. Inflorescence are spikes or racemes of usually bisexual flowers, steroidal saponins and various tonic alkaloids often present (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Veratrum grandiflorum</i> O.Loos	resveratrol, trans-3,5,4'-trihydroxystilbene	stilbenoids	growth-inhibitory effects, suppression of angiogenesis, potentiate the	wide variety of tumor cells, including lymphoid and	Aggarwal et al., 2004

			apoptotic effects of cytokines (e.g., TRAIL), chemotherapeutic agents and gamma-radiation, chemopreventive effects, therapeutic effects against cancer	myeloid cancers; multiple myeloma; cancers of the breast, prostate, stomach, colon, pancreas, and thyroid; melanoma; head and neck squamous cell carcinoma; ovarian carcinoma; and cervical carcinoma	
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Table 24. Melanthiaceae Cytotoxic Phytochemicals

Meliaceae A. L. de Jussieu

The Meliaceae contains 50 genera and 650 tropical and subtropical species. Trees, often pachycaul, rarely subshrubs or suckering shrublets, dioecious polygamous, monoecious or with only bisexual flowers, bark bitter and astringent. Leaves pinnate to bipinnate, unifoliate or simple, in spirals with usually entire leaflets and basally swollen petiole sometimes spiny. Flowers if unisexual often with rudiments of opposite sex, in spikes to thyrses, axillary to supra - axillary. The family divided into two subfamilies. Significant constituents of the family are triterpenoids and limonoids (Evans 2009; Judd et al., 2008; Mabberley 2008).

Family Meliaceae

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Aglaiia elliptifolia</i> Merr.	rocaglamide	cyclopenta[b]benzofurans	antiproliferative activity, induce apoptosis in different human cancer cell lines	in a murine <i>in vivo</i> model, human cancer cell lines	Kim et al., 2006

Table 25. Meliaceae Cytotoxic Phytochemicals

Moraceae Gaudich.

A family of 38 genera and 1150 species of tropical and warm monoecious or dioecious trees, shrubs, lianas or rarely herbs, usually with laticifers with milky latex distributed in all parenchymatous tissues. The family divided into 5 tribes. Constituents of the family include cardenolides and pyridine alkaloids (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Ficus</i> spp.	6-O-acyl-d-glucosyl-sitosterol isoforms	latex	anti-proliferative activity, inhibited the growth of hepatic carcinoma xenografts by approximately 49%	in several tumor cell lines, <i>in vitro</i> in mice	Lansky et al., 2008

<i>Ficus</i> spp.	apigenin, carpachromene, and norartocarpetin	flavonoids	cytotoxic	In several cancer cell lines	Lansky et al., 2008
<i>Ficus</i> spp.	C-28 carboxylic acid functional groups	triterpenoids, phenanthroindolizidine alkaloids	cytotoxic	human cancer cell lines	Lansky et al., 2008
<i>Ficus septica</i> Burm. f.,	ficuseptine-A (6), (+)-tylophorine (19), and a mixture of (+)-antofine (1) and (+)-isotylocrebrine (12)	alkaloids	cytotoxic activity	in several cancer cell lines	Lansky et al., 2008
<i>Ficus hispida</i> L.f.	O-methyltylophorinidine	alkaloids	cytotoxic activity	in several cancer cell lines	Lansky EP 2008

Table 26. Moraceae Cytotoxic Phytochemicals

Oleaceae Hoffmannsegg & Link.

A family of 24 genera and 800 species of trees and shrubs, sometimes lianoid, usually with peltate secretory hairs. Sclereids often present, usually with phenolic glycosides. Other constituents of the family are saponins, tannins, coumarins and iridoid glycosides. Alkaloids are rare (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Olea europaea</i> L.	not stated	Phenolic compounds	inhibit colon cancer development	in large intestinal cancer cell models, animals, and humans	Corona et al., 2009
<i>Olea europaea</i> L.	oleic acid	fatty acids	induces apoptosis and cell differentiation, colorectal chemoprotection	<i>in vitro</i> and <i>in vivo</i> studies	Waterman et al., 2007
<i>Olea europaea</i> L.	squalene	triterpenoids	chemoprotective effect, lower incidence of skin cancer, inhibitory action on chemically-induced skin carcinomas.	epidemiological data, animal studies	Waterman et al., 2007
<i>Olea europaea</i> L.	monounsaturated fatty acids, squalene, tocopherols, and phenolic compounds	Phenolic compounds	associated with low incidence and prevalence of cancer, including colorectal cancer	epidemiologic, <i>in vitro</i> , cellular, and animal studies	Hashim et al., 2005

<i>Olea europaea</i> L.	squalene and terpenoids acteosides, hydroxytyrosol, ????tyrosol and phenyl propionic acids hydroxytyrosol and tyrosol secoiridoids and lignans	phenolic antioxidants and squalene???	scavenging singlet oxygen generated by UV light, chemopreventive effects against colorectal cancer, potential as chemopreventive agents	animal, cellular and metabolic studie	Owen et al., 2004
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Table 27. Oleaceae Cytotoxic Phytochemicals

Papaveraceae A. L. de Jussieu

The Papaveraceae contains 430 genera and 770 species widely distributed in mainly temperate regions; specially diverse in the Northern Hemisphere, but also in southern Africa and eastern Australia. Herbs to soft - wooded shrubs; stem with vascular bundles sometimes in several rings with laticifers present and plants with white, cream, yellow, orange, or red sap, or with specialized elongated secretory cells and sap then mucilaginous, clear, sap with various alkaloids. The family is rich in alkaloids (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Chelidonium majus</i> L.	total extract	not stated	has curative effects on a range of cancers	randomised clinical trials	Ernst & Schmidt, 2005

Table 28. Papaveraceae Cytotoxic Phytochemicals

Sapindaceae A. L. de Jussieu

The Sapindaceae is a family of 131 genera and 1450 species.

This family is divided into 3 subfamilies: 1) Hippocastanideae 2) Dodonaeoideae and 3) Sapindoideae. Mainly tropical and subtropical with a few genera most diverse in temperate regions. Trees, shrubs or lianas and herbaceous climbers. Constituents of the family included saponins, cyanogenic glycosides, cyclitols and coumarins. Alkaloids have been reported in a few species (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Litchi chinensis</i> Sonn.	epicatechin, procyanidin B2, procyanidin B4	flavonoids	inhibition of proliferation and induction of apoptosis in cancer cells through upregulation and down-regulation of multiple genes, anti-breast cancer	cell culture	Li et al., 2007

Table 29. Sapindaceae Cytotoxic Phytochemicals

Solanaceae A. L. de Jussieu

The family comprise of 91 genera and 2450 species. Subcosmopolitan especially in tropical America. Shrubs, trees, lianas and herbs with branched hairs and often prickles; internal phloem around pith; some dioecious. Leaves simple, or lobed to pinnate or 3 - foliate, usually in spirals. Flowers solitary or in appparent basically cymose inflorescence. The Solanaceae is divided into two subfamilies: 1) Solanoideae and 2) Browallioideae.

The family contains a wide range of alkaloids which are great taxonomic interest. Types of alkaloid recorded are tropane, alkaloidal amine, indole, isoquinoline, purine, pyrazole, pyridine, pyrrolidine, quinolizidine, stroid alkaloids and glycoalkaloids. Other constituents include stroidal saponins, withanolides, coumarins, cyclitols, pungent principles, flavones, caretenoids and anthra quinons (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Capsicum annuum</i> L.	capsanthin and capsorubin	carotenoids	potent P-gp inhibitors	cell culture	Molnár et al., 2010
<i>Capsicum chinense</i> Jacq.	capsaicin	capsaicinoids.	apoptosis inducer	cell culture	Meghvansi et al., 2010
<i>Capsicum</i> spp.	capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide)	alkaloids	blocks the translocation of nuclear factor kappa B (NF-kB), activator protein 1 (AP-1), and signal transducer and activator of transcription (STAT3) signaling pathway	cell culture	Oyagbemi et al., 2010
<i>Capsicum</i> spp.	capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide)	alkaloids	anticancer effects	cell culture, in animal models	Aggarwal et al., 2008
<i>Lycopersicon esculentum</i> Mill.	lycopene	carotenoids	prostate cancer	cell culture, animal, epidemiologic and case-control studies	Ansari & Ansari, 2005
<i>Lycopersicon esculentum</i> Mill.	lycopene	carotenoids	several types of cancer, including prostate cancer	<i>in vitro</i> and <i>in vivo</i> studies	Hwang et al., 2002
<i>Lycopersicon esculentum</i> Mill.	lycopene	carotenoids	cancer chemopreventive effects	animal models	Cohen et al., 2002
<i>Nicotiana tabacum</i> L.	alpha- and beta-2,7,11-cembratriene-4,6-diols	diterpenoids	anticancer activity	cell culture, mice, mouse, rats	El Sayed et al., 2007
<i>Solanum incanum</i> L.	diosgenin	saponins	suppress proliferation, invasion inhibition,	cell culture, animal study	Aggarwal et al., 2008

			Cytotoxic, apoptotic, anti-cancer activity		
<i>Solanum xanthocarpum</i> Schrad. & Wendl	diosgenin	saponins	suppress proliferation, invasion inhibition, Cytotoxic, apoptotic, anti-cancer activity	cell culture, animal study	Aggarwal et al., 2008

Table 30. Solanaceae Cytotoxic Phytochemicals

Ranunculaceae A. L. de Jussieu

A family of 56 genera and 2100 species. Widespread, but especially characteristic of temperate boreal regions of the Northern Hemisphere. Herbs, shrubs or occasionally vines. Leaves usually alternate and spiral, occasionally opposite, simples sometimes lobed or dissected, to compound, usually serrate, or crenate, with pinnate to occasionally palmate venation. Inflorescences determinate, sometimes appearing in de terminate or reduced to a single flower, terminal. Flowers usually bisexual. The family has diverse chemical constituents and is of considerable phytochemical and chemotaxonomic interest (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Nigella sativa</i> L.	Thymoquinone	not stated	suppress proliferation, invasion inhibition, Cytotoxic, apoptotic, anti-cancer activity, anti angiogenic	cell culture, animal study, <i>in vitro</i> and <i>in vivo</i>	Aggarwal et al., 2008

Table 31. Ranunculaceae Cytotoxic Phytochemicals

Rosaceae A. L. de Jussieu

A family of 85 genera and around 3000 species. The family is subcosmopolitan and most abundant in the northern Hemisphere. Herbs, shrubs, or trees, often rhizomatous, in frequently climbing; thorns sometimes present. This family is divided into 3 subfamilies and 17 tribes. Constituents of the Rosaceae include cyanogenic glycosides, saponins, tannins, sugar alcohols, cyclitols, terpenoids and mucilage; alkaloids and coumarins are rare (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Aronia arbutifolia</i> (L.) Pers.	not stated	anthocyanins and procyanidins	anticancer	<i>in vitro</i> and <i>in vivo</i> studies	Kokotkiewicz et al., 2010
<i>Aronia melanocarpa</i> (Michx.) Elliott	not stated	anthocyanins and procyanidins	anticancer	<i>in vitro</i> and <i>in vivo</i> studies	Kokotkiewicz et al., 2010

<i>Fragaria × ananassa</i> (Weston) Duchesne ex Rozier	ellagic acid, and certain flavonoids: anthocyanin, catechin, quercetin and kaempferol	flavonoids	anticancer activity, blocking initiation of carcinogenesis, and suppressing progression and proliferation of tumors	in several different experimental systems	Hannum et al., 2004
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Table 32. Rosaceae Cytotoxic Phytochemicals

Rubiaceae A. L. de Jussieu

The Rubiaceae consists of 563 genera and 10900 species. Members of the family are cosmopolitan, but most diverse in tropical and subtropical regions. The Rubiaceae divided into 4 subfamilies. In the family, alkaloids of indole, oxindole, quinoline and purine types are common; anthraquinones occur in some genera of the Rubiaceae. Other constituents of the family included anthocyanins, cyclitols, coumarins, diterpenoids, triterpenoids and iridoid glycosids.

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Morinda citrifolia</i> L.	total extract	not stated	potential anticancer activity, immunomodulatory activity	cell culture, phase I clinical study	McClatchey et al., 2002

Table 33. Rubiaceae Cytotoxic Phytochemicals

Rutaceae A. L. de Jussieu

A family of 158 genera and 1900 species. Nearly cosmopolitan, but mainly tropical and subtropical. Usually trees or shrubs, sometimes with thorns, spines, or prickles. The Rutaceae is divided into 5 subfamilies.

Constituents of this family include a wide variety of alkaloids, volatile oils, rhamno – glucosides, coumarins and terpenoids. Alkaloids include alkaloidal amines, imidazole, indole isoquinoline, pyridine, pyrrolidine, quinazoline types. Many of the fruits are rich in citric and other acids and in vitamin c (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Citrus</i> spp.	limonoids	triterpenoids	chemopreventive agents for cancer, Reduction of skin tumours	animal studies	Silalahi et al., 2002
<i>Citrus</i> spp.	nomilin	triterpenoids	inhibitor of carcinogenesis	animal studies for forestomach tumours	Silalahi, 2002
<i>Citrus</i> spp.	nobiletin and tangeretin dietary fibre	flavonoids	anticancer activity	<i>in vivo</i> and <i>in vitro</i>	Silalahi et al., 2002
<i>Citrus</i> spp.	β-carotene	not stated	pro-tumorigenic properties	not stated	Silalahi et al., 2002

<i>Citrus</i> spp.	ascorbic acid	not stated	prevents oxidation of specific chemicals to their active carcinogenic forms, protects against <i>in vivo</i> oxidation of lipids and DNA	<i>in vivo</i> , in humans	Silalahi et al., 2002
<i>Citrus</i> spp.	dietary fibre and pectin	polysaccharides	influence colon cancer by physical dilution of colon content, absorption of bile acids and carcinogens, decreased transit time, altered bile acid metabolism and the effects of fermentation, namely, the production of short-chain fatty acids, lowering of pH and stimulation of bacterial growth, absorbing carcinogens in the gastrointestinal tract, reducing the risk of bowel cancer,	in human	Silalahi et al., 2002
<i>Citrus</i> spp.	flavanone and flavone O- and C-glycosides and methoxylated flavones???	flavonoids	anti-inflammatory and anticancer actions	<i>in vitro</i> and <i>in vivo</i>	Manthey et al., 2001

Table 34. Rutaceae Cytotoxic Phytochemicals

Taxaceae Bercht. & J. Presl

A family of 6 genera and 28 species. Small or moderately sized dioecious trees or shrubs, usually not resinous or only slightly resinous, fragrant or not. Wood without resin canals, leaves simple, persistent for several years, shed singly, spirals, often twisted so as to appear 2 - ranked, linear, flattened, entire, acute at apex, with 0 - 1 resin canals. Some species of *Taxus* investigated for taxane alkaloids (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Taxus baccata</i> L.	total extract	not stated	cytotoxic effect	Cell culture	Emami et al., 2005

Table 35. Taxaceae Cytotoxic Phytochemicals

Theaceae Mirbel

A family of 7 genera and about 240 species. Usually evergreen trees and shrubs. Leaves simple, entire to toothed usually in spirals and coriaceous, often withering red. Flowers usually large and bisexual, hypogynous to epigynous, solitary and axillary. Among the constituents are purine alkaloids, saponins, tannins and fixed oil (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Camellia sinensis</i> (L.) Kuntze	(-)-epigallocatechin gallate (EGCG)	polyphenols	synergists with anticancer drugs	cell culture	Suganuma et al., 2011
<i>Camellia sinensis</i> (L.) Kuntze	catechins (specifically EGCG)	polyphenols	targeting of lipid rafts by EGCG	cell culture	Patra et al., 2008
<i>Camellia sinensis</i> (L.) Kuntze	catechins (specifically EGCG)	polyphenols	growth factor-mediated pathway, the mitogen-activated protein (MAP) kinase-dependent pathway, and ubiquitin/proteasome degradation pathways	phase I and II clinical trials	Chen et al., 2008
<i>Camellia sinensis</i> (L.) Kuntze	catechin, (-)-epigallocatechin	polyphenols	antioxidant, antiangiogenesis, and antiproliferative	review to summarize recent findings on the anticancer and medicinal properties of green tea	Cooper et al., 2005 b
<i>Camellia sinensis</i> (L.) Kuntze	epigallocatechin gallate (EGCG)	polyphenols	antioxidant, antiangiogenesis, and antiproliferative	review to summarize recent findings on the anticancer and medicinal properties of green tea	Cooper et al., 2005 a
<i>Camellia sinensis</i> (L.) Kuntze	catechin EGCG	polyphenols	antioxidant properties of the catechins with anticancer effects	preclinical research	Moyers et al., 2004
<i>Camellia sinensis</i> (L.) Kuntze	catechins and polyphenols	polyphenols	both cytostatic and cytotoxic activity towards cancer cells	<i>in vitro</i> and <i>in vivo</i> research	Colic & Pavelic, 2000

Table 36. Theaceae Cytotoxic Phytochemicals

Zingiberaceae Martynov

The Zingiberaceae family of 50 genera and 1500 species widespread in tropical regions; chiefly in shaded to semi - shaded forest understory habitats; occasionally in wetlands. Small to large, spicy - aromatic herbs, scattered secretory cells containing ethereal oils, various terpenes, and phenyl - propanoid compounds. The family is divided into two subfamilies: 1) Costoideae and 2) Zingiberoideae.

Volatile oils and pungent principles are a feature of the family. Other constituents include the colouring matters known as curcuminoids, tannins, phenolic acids, leucoantheyanins, flavonoids, ketones and terpenoids. Only a few isolated of alkaloids have been reported (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Curcuma longa</i> L.	curcumin, difluorocurcumin analogs	polyphenols	prevention of tumor progression and/or treatments of human malignancies	promising leads for conducting first in-depth animal studies and subsequently clinical trials	Padhye et al., 2010
<i>Curcuma longa</i> L.	curcumin	polyphenols	anticancer activities	not stated	Agarwal et al., 2007
<i>Curcuma longa</i> L.	curcumin	polyphenols	cancer chemoprevention, antitumor	cell epidemiological studies, Preclinical studies	Thangapazham et al., 2006
<i>Curcuma longa</i> L.	curcumin	polyphenols	cancer prevention	human clinical trials	Aggarwal et al., 2008
<i>Curcuma longa</i> L.	curcumin, demethoxycurcumin and bisdemethoxycurcumin	polyphenols	Anti-cancer activity	progress clinical trials	Jurenka et al., 2009
<i>Curcuma longa</i> L.	turmerone, atlantone, and zingiberone	sesquiterpenoids	anti-cancer activity	progress clinical trials	Jurenka et al., 2009
<i>Zingiber officinale</i> Roscoe	[6]-gingerol	phenolic compounds	cytotoxic, apoptotic, anti-cancer activity	cell culture, animal study	Aggarwal et al., 2008
<i>Zingiber officinale</i> Roscoe	pungent vallinoids, viz. [6]-gingerol and [6]-paradol, shogaols, zingerene	phenolic compounds	cancer preventive activity	in experimental carcinogenesis	Shukla et al., 2007
<i>Zingiber zerumbet</i> Smith.	zerumbone [2,6,9,9-tetramethyl-(2E,6E,10E)-cycloundeca-2,6,10-trien-1-one]	phenolic compounds	cytotoxic, apoptotic, anti-cancer activity	cell culture, animal study	Aggarwal et al., 2008

Table 37. Zingiberaceae Cytotoxic Phytochemicals

Zygophyllaceae R. Brown

A family of 26 genera and 280 tropical species. Small trees, shrubs to herbs with stems often sympodial and jointed at the nodes, xylem with vessels, tracheids, and fibers arranged in horizontally aligned tiers; usually producing steroidal or teriterpenoid saponins, sesquiterpenes and alkaloids (Evans 2009; Judd et al., 2008; Mabberley 2008).

Species	Compound	Class of Compound	Effect	Status	Ref.
<i>Larrea divaricata</i> Cav.	nordihydroguaiaretic acid	phenolic compounds	anticancer, antioxidant, antimicrobial, anti-inflammatory and immunosuppressive activities	phase I/II clinical trials as an anticancer agent	Chen et al., 2009

Table 38. Zygophyllaceae Cytotoxic Phytochemicals

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Combination Chemotherapy in Cancer: Principles, Evaluation and Drug Delivery Strategies

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1. Introduction

Cancer is a major public health problem since it is the second leading cause of illness-related death, only exceeded by heart disease (American Cancer Society [ACS], (2010)). Cancer results from structural and quantitative alterations in molecules that control different aspects of cell behavior. Genetic alterations probably represent the most common mechanisms for molecular changes that cause the development and progression of cancer (Dong, 2006). Great efforts have been made to identify common genetic modifications and the underlying target genes. Genetic alterations can be inherited, as in hereditary cancers, or induced by endogenous and exogenous carcinogenic factors as in most sporadic cancers (Dong, 2006). The six essential changes in cell physiology suggested to collectively dictate malignant growth are self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evading apoptosis (Hanahan & Weinberg, 2000).

Chemotherapeutic agents used in current clinical practice have played a significant role in reducing mortality/morbidity and in increasing patient's quality of life (Suggit & Bibby, 2005). Despite the recent advances in early diagnosis and in clinical protocols for cancer treatment, the development of antineoplastic agents that combine efficacy, safety and convenience for the patient remains a great challenge (Ismael et al., 2008).

Most anticancer drugs have narrow therapeutic index, develop multidrug resistance (MDR) and present unspecific biodistribution upon intravenous administration leading to unacceptable side effects to healthy tissues, mainly bone marrow and gastrointestinal tract. These limitations of conventional chemotherapeutic strategies frequently result in suboptimal dosing, treatment delay or discontinuance and reduced patient compliance to therapy (Ismael et al., 2008).

2. Combination chemotherapy

2.1 Principles and advantages

Combination therapy has been the standard of care, especially in cancer treatment, since it is a rationale strategy to increase response and tolerability and to decrease resistance.

Currently, there is a growing interest in combining anticancer drugs aiming at maximizing efficacy while minimizing systemic toxicity through the delivery of lower drug doses (Mayer & Janoff, 2007; Ramsey et al., 2005; Zoli et al., 2001).

The fundamentals of combination chemotherapy development have remained largely unchanged over the last decades. The general principles have been to: i) use drugs with non-overlapping toxicities so that each drug can be administered at near-maximal dose; ii) combine agents with different mechanisms of action and minimal cross-resistance in order to inhibit the emergence of broad spectrum drug resistance; iii) preferentially use drugs with proven activity as single drugs and iv) administer the combination at early stage disease and at a schedule with a minimal treatment-free period between cycles but still allowing the recovery of sensitive target tissues (Mayer & Janoff, 2007; Harasym et al, 2007; Ramsey et al., 2005; Zoli et al., 2001). The advantages attributed to combination chemotherapy include improved patient compliance due to the reduced number of administrations, emergence of additive or synergistic interaction effects, ability to overcome or delay MDR and reduction of drug dose with consequent diminishing of toxicity to healthy tissues (Chou, 2010, 2006; Ramsey et al., 2005).

As an example, multimodal combination treatments for hormone refractory prostate cancer (HRPC) have gained support in the clinical setting over the last decade (De la Taille et al., 2001). Given the complexity, heterogeneity, resistance and recurrence features of prostate cancer, rationally-designed drug combinations are necessary to achieve significant therapeutic progress (Armstrong & Carducci, 2006).

2.2 Preclinical vs. clinical drug combination studies

The majority of clinical protocols for cancer combination therapies are mainly obtained empirically, in the absence of supporting experimental data, or based on results derived from retrospective analysis of clinical trials (Zoli et al, 2001; Goldie, 2001). These studies investigate the sequencing and scheduling of drugs rather than determining the optimal drug interactions. Information obtained from clinical protocols is valuable, but is time-consuming, expensive and does not provide data on the biochemical and molecular mechanisms of drug interaction at cellular level resulted from combined treatments (Zoli et al., 2001). It is very difficult to determine whether drug combinations are acting in a synergistic, additive or antagonistic fashion in cancer patients. Ultimately, one can only determine whether a new combination provides a statistically significant increase in a specific end point such as response rate, time to progression or survival (Mayer, 2007).

Preclinical drug interaction studies allow a more rational design of clinical combination chemotherapy protocols, which are generally based on the empiric assumption that maximal efficacy will be achieved by co-administering each drug at their maximum tolerated doses (MTDs) (Mayer & Janoff, 2007; Harasym et al., 2007; Mayer et al., 2006). This “more-is-better” philosophy applied to anticancer combinations may result in higher toxicity with minimal therapeutic benefit due to concentration-dependent drug interactions (Mayer et al, 2006; Ramsey et al., 2005). Undoubtedly, there are several molecular and pharmacological factors that determine the effectiveness of drug combinations. A rationally-designed fixed drug combination is required since certain drug ratios can be synergistic, while others are additive or even antagonistic (Mayer & Janoff, 2007; Mayer et al., 2006).

The design of preclinical drug combination studies on established cell lines, primary cell cultures or animal models has to take into account several factors such as drug

concentration, exposure time, drug administration schedule and analytic method for evaluating the drug interaction (Zoli et al., 2001).

2.3 In vitro vs. in vivo drug combination studies

Evaluation of drug ratio-dependent effects in combination chemotherapy is frequently conducted in cell culture systems. During the course of the experiment, concentration and duration of administered drug(s) can be tightly controlled and the inhibition of tumor cell growth can be easily measured (Mayer & Janoff, 2007; Harasym et al., 2007; Chou et al., 2006). For the last two decades, in vitro experimentation with tumor-derived cell lines has been the most important resource for investigating molecular mechanisms of cancer pathogenesis (Mitchell et al., 2000). There are a number of advantages associated with the use of cell culture systems, e.g. availability of a wide range of human tumor cell lines, flexibility of culture conditions and easiness of protein/nucleic acid quantification (Harasym et al., 2007). Additionally, in vitro tests not only evaluate antiproliferative effects of tested drugs but also assess interference on cell cycle, induction of apoptosis and existence of molecular or biochemical interactions (Zoli et al., 2001).

Unfortunately, cell culture studies are of limited usefulness because the conditions are artificial, do not reflect the heterogeneity of clinical malignant disease and, hence, are unable to evaluate the therapeutic index (Budman et al., 2002). Unlike in vitro studies where drug concentration is relatively constant, in vivo models represents a dynamic system, where drug molecules undergo absorption, distribution, metabolism and elimination, thus leading to plasma drug concentration changes over time (Merlin, 1994). Nevertheless, when compared to in vitro studies, the determination of synergism or antagonism in vivo using animal models is more time consuming, more expensive and greater variability in measurements occurs. Therefore, in vivo drug combination studies are usually carried out, only for selected drugs, after in vitro evaluation and before clinical trials (Chou, 2010, 2006).

3. Drug interaction effects in combination chemotherapy

3.1 Definition and in vitro quantitative evaluation

A drug combination can result in synergistic, antagonistic or additive interaction effects at different concentration ratios. Synergy, additivity and antagonism are defined as the interaction between two or more components such that the combined effect is superior, equal or inferior, respectively, to the expected sum of individual effects. Additivity means that each constituent contributes to the effect in accordance with its own potency (Chou, 2006; Merlin, 1994).

Systematic screening analysis of drug combinations can identify additive or synergistic relationships previously unrecognized (Mayer & Janoff, 2007). In vitro synergistic activity is strongly dependent on drug:drug ratio and that dependence has profound implications on clinical application, since in vivo activity relies on the maintenance of those therapeutic ratios at the disease site (Mayer & Janoff, 2007; Harasym et al., 2007; Mayer et al., 2006). Therefore, in order to achieve maximal therapeutic efficacy in vivo, dosing schedule is essential to allow exposure of tumor cells to defined drug concentrations (Zhao et al., 2008). Several methods for the quantitative evaluation of drug-combined interaction effects have been used and were comparatively reviewed elsewhere (Zoli et al., 2001; Merlin, 1994). A brief description of the principles and limitations of the different methods is compiled in Table 1. However, in the present book chapter only the median effect analysis is extensively reviewed in the next section.

Method	Author	Principle	Limitations
Fractional product	Webb (1963)	Summation of the effects of two inhibitors is expressed by the product of the fractional activities	Method does not take into account the possible sigmoidicity of the dose response curves ($m > 1$ or $m < 1$) and is not applicable in the case of two mutually exclusive drugs or second-order mutually non-exclusive drugs
Classical isobologram	Loewe (1957)	Lines join doses or dose-combinations exerting the same effect (iso-effect)	Method requires a large number of data points, has poor computer software, the statistical approach is incomplete and only two-drug combinations can be evaluated. Method is not applicable in the case of two mutually non-exclusive drugs
Isobologram modified	Stell and Peckham (1979)	Envelope of additivity: a region delimited by confidence limits in which the cytotoxic agents are not significantly interacting	
Median effect analysis	Chou and Talalay (1984)	Enzyme kinetic system: mass action law, Michaelis–Menten and Hill equations	Method should not be applied when the dose–response curves are not sigmoidal because of the difficulty of applying linear regression analysis
Three-dimensional	Fraser (1972) Carter and Wampler (1986) Kanzawa (1997)	Michaelis–Menten equations Median effect principle	Model requires several mathematical functions and software for each different type of response surface. The complex execution prevents it from being widely used in preclinical studies

Table 1. Methods for the quantitative evaluation of drug combination effects. Data was adapted and compiled from several literature references (Chou, 2006, 1994; Chou & Talalay, 1984; Zoli et al., 2001; Merlin, 1994).

3.2 The median effect analysis

By far the most prevalent method used for quantitative evaluation of drug combinations is the median effect analysis proposed by Chou and Talalay (Chou, 2010, 2006, 1994; Chou & Talalay, 1984). The fundamental equations of this method were derived from mass action enzyme kinetic models, previously established for enzyme-substrate interactions and then extended to multiple drug combinations (Chou, 1976). The equations underlying the median effect principle can be considered as a generalized form including the concepts of fractional product and isobologram analysis (Table 1) (Merlin, 1994; Chou & Talalay, 1984).

Regardless of the shape of the dose-effect curve or the drug mechanism of action, the median effect equation correlates drug dose and corresponding effect (cell growth inhibition) and is given by:

$$f_a / f_u = (D/D_m)^m \quad (1)$$

Where f_a and f_u are the fractions of cells affected and unaffected, respectively, by a dose (D); D_m is the dose causing the median effect and m the coefficient traducing the shape of the dose effect curve ($m = 1$, >1 and < 1 , indicate hyperbolic, sigmoidal and negative sigmoidal,

respectively). The m and D_m parameters are easily determined from the median effect plot since they correspond to the slope and to the antilog of the x-intercept, respectively (Chou 2006, 1994; Chou et al. 1994). When m and D_m are determined, the entire dose-effect relationship is described since for a given dose (D) it is possible to calculate the effect (f_a) and vice-versa (Chou, 2010, 1994). Application of equation 1 allows the linearization of hyperbolic ($m = 1$) as well as sigmoidal curves ($m \neq 1$) which are often encountered in chemotherapy treatment data (Merlin, 1994; Chou & Talalay, 1984). Plotting $x = \log(D)$ vs. $y = \log(f_a/f_u)$ based on the logarithm form of equation 1 is called the median effect plot (Chou, 2010, 2006) (Fig. 1).

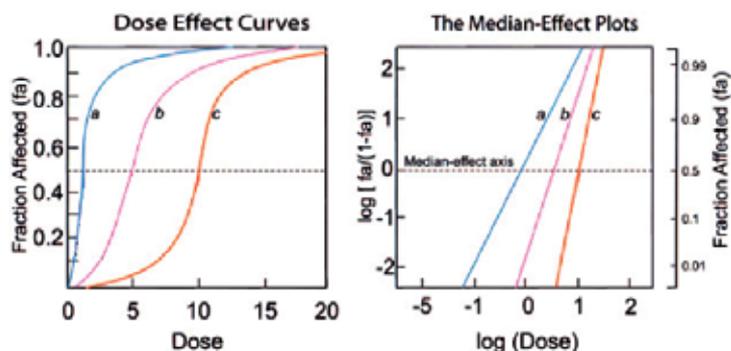


Fig. 1. Graphical representations executed in the median effect analysis method. Three sigmoidal dose-effect curves (a, b, c) (left graph) and respective transformation into the corresponding linear forms by the median effect plot (right graph), where $y = \log(f_a/f_u)$ vs. $x = \log(D)$. Adapted from reference (Chou, 2006).

The conformity of the data to the median effect principle can be readily manifested by the linear correlation coefficient (r) of the plot, in which $r = 1$ indicates perfect conformity (Chou, 2010, 2006, 1994). The fractional effect associated with a range of concentrations is determined for each individual drug and for their combination. The median effect plot gives parallel lines if the drugs have the same or similar modes of action and the effects are then considered mutually exclusive; if the plots for single drugs are parallel but the mixture plot is concave upward with the tendency to intersect the plot of the more potent drug, the drugs act independently and their effects are considered mutually non-exclusive (Chou & Talalay, 1984).

The combination index (CI) quantitatively evaluates the nature of drug interaction and is defined by the following equation:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \alpha \frac{(D)_1(D)_2}{(D_x)_1(D_x)_2} \quad (2)$$

Where $\alpha = 0$ and $\alpha = 1$, for drugs with mutually exclusive or non-exclusive mechanisms of action, respectively (Chou, 2010, 2006, 1994; Chou & Talalay, 1984). Denominators $(D_x)_1$ and $(D_x)_2$ are drug doses required to achieve a given effect level (f_a). Numerators $(D)_1$ and $(D)_2$ are doses of each drug in a given mixture which originates the same f_a . For three-drug combinations, a third term $(D)_3/(D_x)_3$ is added to equation 2. A plot of CI as a function of

effect level (f_a) is represented in Fig. 2. CI values reflect synergism, additivity or antagonism when inferior, equal or superior to 1, respectively.

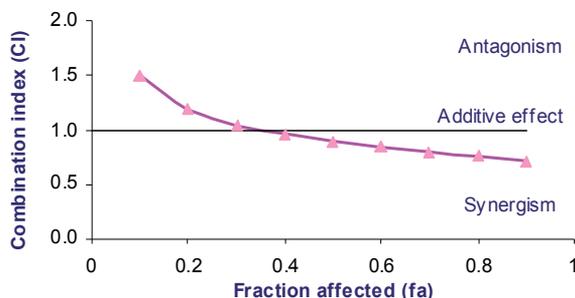


Fig. 2. Graphical representation of an exemplificative CI - f_a plot. Combination index (CI) < 1, = 1, > 1 indicate synergism, additivity and antagonism, respectively.

If the nature of drug mechanisms is not clear, the authors of the method suggest that CI value must be determined by both mutually exclusive ($\alpha = 0$) and mutually non-exclusive ($\alpha = 1$) assumptions. The later approach is more conservative as the addition of a third term in equation 2 results in higher CI values than the former one (Chou, 2006, 1994, Chou & Talalay, 1984). Even though the CI value can be expressed for any effect level, the most accurate determination is for $f_a = 0.5$ since the median effect plot may be unreliable at the extremes as it represents a linear approximation of a non-linear function (Kreis et al., 2001). The median effect analysis is a simple quantitative method that takes into account not only the potency (D_m) of each drug and of their combination but also the shape (hyperbolic or sigmoidal) of their dose-effect curves (Chou, 1994; Chou et al., 1994). Furthermore, this method evaluates interaction effects at different drug ratios, at different effect levels and up to three agents can be evaluated simultaneously (Chou et al., 1994; Chou & Talalay, 1984). It is recommended that an experiment should be carried out using a constant equipotency ratio (e.g. $(IC_{50})_1/(IC_{50})_2$) so that the effect contribution of each drug to the combination can be roughly equal (Chou, 2010, 2006; Chou & Talalay, 1984). When evaluating anticancer drug combinations, the dose range administered must be wide enough to allow extrapolation of the results up to high levels of activity, i.e. $f_a \geq 0.5$, owing to the fact that tumor growth inhibition below that level is not clinically meaningful (Chou, 2010; Harasym et al., 2007; Merlin, 1994).

Dose reduction index (DRI) is a measure of how many folds the dose of a combined drug may be reduced at a given effect level as compared to the dose of the drug alone (Chou, 2006; Mayer et al., 2006). DRI is an important parameter in clinical practice because a favorable value (> 1) may lead to reduced systemic toxicity toward healthy tissues while maintaining therapeutic efficacy (Chou, 2010, 2006, 1998, 1994; Chou & Talalay, 1984).

4. Nanoparticles as drug delivery systems

In face of the difficulties and high costs inherent to the development of new therapeutic molecules, the strategy of most pharmaceutical companies seems to rely on the optimization of the existing drugs, namely those characterized by a low therapeutic index. In particular,

the application of nanotechnology-based drug delivery systems, such as liposomes, to cancer chemotherapy has been an exciting and promising area of research and constitutes an important ongoing effort to improve specificity and efficacy of anticancer drugs.

4.1 Different types of nanoparticles

Several drugs have physical and biological properties which hinder their clinical applicability, namely poor water solubility, rapid metabolism, instability under physiological conditions, unfavorable pharmacokinetics and unspecific biodistribution to healthy tissues (Allen, 1998). Particularly, in the case of anticancer drugs, such features ultimately lead to inadequate delivery of effective therapeutic drug concentrations to tumor tissue and/or unacceptable toxic effects (Andresen et al., 2005; Cattel et al., 2003). Therefore, it is crucial to develop nanotechnology-based platforms (lipid or polymer-based nanocarriers such as liposomes, micelles, polymeric nanoparticles or dendrimers) to promote and control delivery of some anticancer drugs to tumors (Devalapally et al., 2007; Peer et al., 2007; Dutta, 2007) (Fig. 3).

Nanoparticles with medical applications differ in terms of structure, size and composition, thus resulting in different characteristics, namely drug loading capacity, physical stability and targeted delivery ability (Haley & Frenkel, 2008). It is beyond the scope of this chapter to review the current drug delivery nanocarriers since they have been widely reviewed in recent publications (Devalapally et al., 2007; Peer et al., 2007; Dutta, 2007; Haley & Frenkel, 2008; Lammers et al., 2008; Cho et al., 2008; Alexis et al., 2008). Therefore, in this chapter an overview will be restricted to liposomes, since these are probably the most used drug delivery system for small drug molecules.

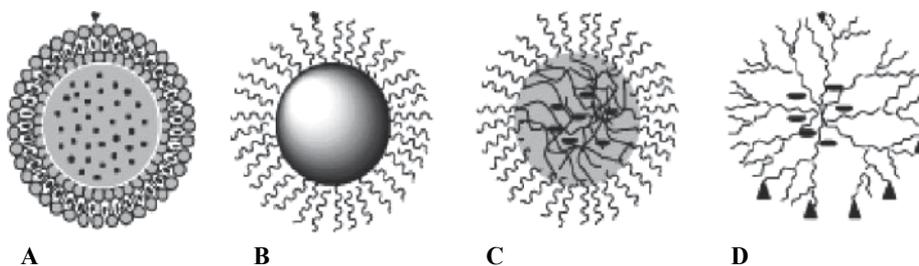


Fig. 3. Schematic representation of exemplificative nanocarriers for drug delivery. A Liposome; B Polymeric nanoparticle; C Micelle; D Dendrimer. Adapted from reference (Devalapally et al., 2007).

4.2 Liposomes

4.2.1 General definition and main features

Liposomes were firstly described by Bangham *et al.* (Bangham et al., 1965) and were originally called phospholipid spherules. Liposomes are self-assembling closed colloidal lipid vesicles (Fig. 4) with considerable potential for delivery of therapeutic agents due to several features: biodegradability, biocompatibility, simplicity, scaled-up production, low inherent toxicity, weak immunogenicity, versatility in structure and in physicochemical properties (lipid composition, size and surface charge) and ability to undergo surface engineering towards conjugation of polymers and targeting ligands (Immordino et al., 2006; Hofheinz et al., 2005; Cattel et al., 2003). Due to those specific attributes, liposomes have the

ability to modulate in vivo behavior (pharmacokinetics and biodistribution profile) and/or solubility properties of drugs and to protect them from premature degradation or inactivation after intravenous administration (Fenske et al., 2008; Immordino et al., 2006; Drummond et al., 1999; Allen, 1998). In general, when a drug is encapsulated within a carrier, such as liposome (Fig. 4) the plasma clearance and volume of distribution decrease while the plasma circulation half-life ($t_{1/2}$) and area under the plasma concentration *vs.* time curve (AUC) increase (Gabizon et al., 2003).

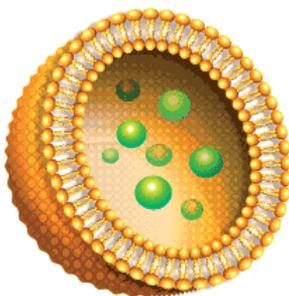


Fig. 4. Schematic representation of a liposome vehicle with drug molecules encapsulated in the aqueous internal compartment.

The pharmacokinetics, biodistribution and biological activity of a liposomal drug formulation are influenced by size, surface charge, lipid and drug doses, lipid composition, steric stabilization and route of administration (Charrois & Allen, 2004, 2003; Nagayasu et al., 1999; Mayer et al., 1989).

The development of a successful therapeutic liposomal drug formulation must comply with three fundamental requisites: i) clear knowledge of the biology and physiology of the disease to be treated; ii) good understanding of the physicochemical properties of both the carrier and the drug and iii) determination of the pharmacokinetic and biodistribution changes induced in the drug by the liposomal vehicle (Allen, 1998).

Liposome structural types, physicochemical composition and preparation methods will not be overviewed in the present chapter since they have been thoroughly reviewed over the last two decades, in particular by Dr. Allen (Allen, 1998, 1997, 1994) and Dr. Lasic (Lasic et al., 1999) research groups.

4.2.2 Medical applications

In the past decades there have been major advances in the development of liposomal drug formulations suitable for several medical applications. In addition to their feature as drug delivery systems in the treatment of cancer, bacterial infections or ophthalmic disorders, current clinical applications of liposomes also include gene delivery, diagnostic imaging, vaccine adjuvant, photodynamic therapy, dermatology, hemoglobin or chelating agent transporter and enzyme replacement therapy (Fenske et al., 2008; Torchillin, 2007; Immordino et al., 2006; Torchillin, 2005; Gregoriadis & Florence, 1993). The ultimate goal of an anticancer liposomal formulation is to improve overall therapeutic index of encapsulated drugs by increasing the antitumor activity and/or by reducing the toxicity profile, due to preferential delivery and accumulation at tumor tissue as compared to free drugs (Drummond et al., 1999; Gabizon, 1992).

4.2.3 In vivo behavior: From conventional to sterically stabilized liposomes

Ideally, liposomal drug formulations should have a mean diameter centered on 100 nm, a high drug-to-lipid ratio, an excellent retention of encapsulated drug(s) (while circulating in the blood) and a long circulation lifetime (from hours to days) (Fenske & Cullis, 2005). In general, the short blood residence time and in vivo drug leakage profile of conventional liposomes hinder their clinical applicability. Liposomes are recognized and bounded by serum proteins (opsonins) (Fig. 4) and by complement system after which they are cleared from systemic circulation by reticulo-endothelial system (RES) cells of the liver, spleen and bone marrow (Immordino et al., 2006; Chonn et al., 1992; Papadadjopolous et al., 1991). The physicochemical properties of liposomes, such as net surface charge, hydrophobicity, size, fluidity and packing of the lipid bilayer, influence their stability and the type of proteins that bind to them (Chonn et al., 1992). Moreover, lipid exchange with plasma lipoproteins can destabilize liposomes and lead to their rupture with release of entrapped content (Immordino et al., 2006). The use of saturated phospholipids with high phase transition temperature associated with cholesterol, but mostly surface coating with a synthetic hydrophilic polymer such as poly(ethylene glycol) - PEG (Fig. 4) or with ganglioside G_{M1} , significantly extend bloodstream circulation time to several days and reduces RES clearance (Immordino et al., 2006; Gabizon et al., 2003; Allen, 1994, Papahadjopoulos et al., 1991; Gabizon & Papahadjopoulos, 1988). Nevertheless, there are some disadvantages associated with PEG coating. There is some evidence that pegylated liposomes are not completely inert and can still induce activation of complement system (Immordino et al., 2006). Furthermore, the presence of PEG may hinder drug release to the target cell population (Harrington et al., 2002). Attempts have been made to solve these limitations by generating liposomes that are reversibly pegylated as described in detail elsewhere (Immordino et al., 2006; Harrington et al., 2002).

Pegylated liposomes are named sterically stabilized liposomes (SSL) or Stealth[®] due to a highly hydrated surface, constituted by the hydrophilic PEG and water molecules, that acts as a steric barrier and prevents protein adsorption and opsonization (Fig. 5) (Immordino et al., 2006; Andresen et al., 2005; Gabizon et al., 2003).

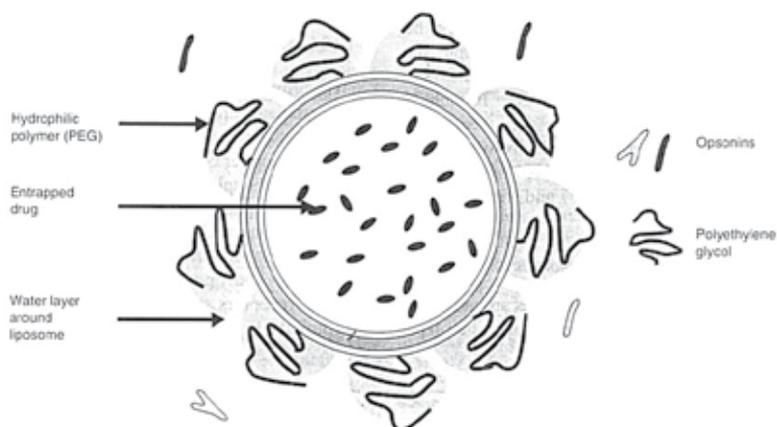


Fig. 5. Graphical representation of PEG-coated liposome (Stealth[®]). Extent of opsonization of pegylated liposomes is significantly diminished due to their highly hydrated surface.

Adapted from reference (Allen, 1997).

4.2.4 The enhanced permeability and retention effect in tumor tissues

Most solid tumors possess unique pathophysiological characteristics that are absent in normal tissues, such as extensive and unregulated angiogenesis, defective vascular architecture, enhanced vascular permeability, dysfunctional lymphatic drainage and increased production of a number of permeability mediators (Gabizon et al., 2006; Maeda et al., 2000). This enhanced permeability and retention effect (EPR) inherent to solid tumors (Fig. 6) (Maeda et al., 2000; Drummond et al., 1999) has been described in several experimental tumors and depends mainly on tumor volume, vascularization and leakage from blood vessels (Gabizon et al., 2006; Yuan et al., 1995). Long-circulating liposomal drug formulations with size diameter within the range of 100-150 nm demonstrate preferential extravasation through leaky tumor vasculature and passively accumulate in the interstitial space due to the EPR effect. The release of drug molecules from liposomes into the tumor interstitium provides locally drug delivery at therapeutic dose levels (Abraham et al., 2005; Drummond et al., 1999; Gabizon & Papahadjopoulos, 1988). Interestingly, a particular study on tumor xenograft animal models reported that liposomes up to 400 nm can extravasate across tumor vessels and penetrate into tumor interstitium, suggesting that the threshold vesicle size of the pores is generally between 400 and 600 nm in diameter (Yuan et al., 1995). Nevertheless it is important to emphasize that the cut-off range mostly depends on tumor type. The extent of accumulation within the tumor is largely determined by the circulation lifetime of the liposomes (Song et al., 2006). Moreover, the impaired lymphatic drainage in the tumor interstitium favors the retention of liposomal formulations at the extravasation site, accentuating the passive targeting to solid tumors (Fig. 5) (Gabizon et al., 2006; Maeda et al., 2000; Drummond et al., 1999).

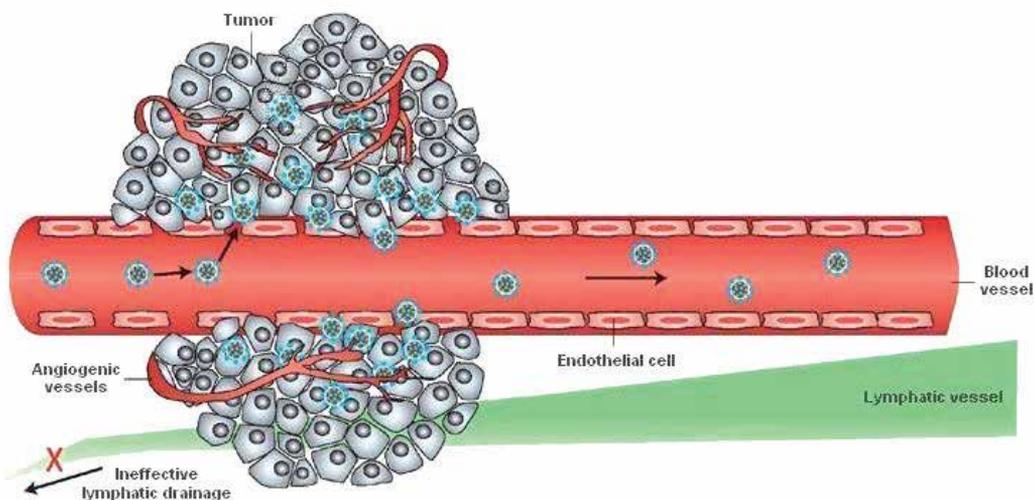


Fig. 6. Enhanced permeability and retention effect (EPR). Liposomes are shown as representative nanoparticles. Tumor targeting is achieved by passive extravasation of liposomes, from blood to tumor interstitium through highly permeable vasculature, and by accumulation in interstitial space due to non-functional lymphatic system in solid tumors. Adapted from reference (Peer et al., 2007).

4.2.5 Liposomal single drug formulations approved for clinical use or under clinical evaluation

The success of liposomes as drug delivery systems has been reflected by the significant number of formulations which are FDA or EMA-approved for clinical use (Table 2) or undergoing clinical evaluation (Table 3).

Representative examples of liposomal single drug formulations that have gained regulatory approval for clinical use are Doxil[®] and Myocet[®]. These LUV formulations encapsulating doxorubicin are being marketed for the treatment of several cancers, either as an individual formulation or in combination treatments (Table 2). Liposomal anthracyclines, namely doxorubicin, have raised significant interest due to their ability to decrease drug-related toxicity (cardiomyopathy, which can lead to congestive heart failure and death, bone marrow suppression, alopecia or nausea) with no associated loss of therapeutic activity (Abraham et al., 2005; Gabizon et al., 2003, 1998).

Formulation brand name	Drug name	Dosage form / route	Therapeutic indication	Date	Company name
Abelcet [®]	Amphotericin B	Lipid complex /injection	Systemic fungal infection	1995	Enzon
AmBisome [®]		Liposomal/ injection	Systemic fungal infection	1997	Astellas Pharma
Amphotec [®]		Lipid complex /injection	Systemic fungal infection	1996	Three Rivers Pharms
DaunoXome [®]	Daunorubicin citrate	Liposomal/ injection	AIDS-related Kaposi's sarcoma Breast cancer and other solid tumors	1996	Diatos S.A.
DepoCyt [®]	Cytarabine	Liposomal/ injection	Lymphomatous meningitis (intrathecal application)	1999	Pacira Pharmaceuticals Inc.
DepoDur [®]	Morphine sulfate	Liposomal/ epidural	Post-surgical pain reliever	2004	Pacira Pharmaceuticals Inc.
Doxil [®] (USA)	Doxorubicin.HCl	Liposomal/ injection	AIDS-related Kaposi's sarcoma	1995	Centocor Ortho Biotech Inc.
Caelyx [®] (Europe)			Advanced ovarian cancer Metastatic breast cancer Multiple myeloma (combination with bortezomib)	1996	Schering Plough Europe
Myocet [®]	Doxorubicin.HCl	Liposomal/ injection	Metastatic breast cancer (in combination with cyclophosphamide)	2000	Cephalon Europe
Visudyne [®]	Verteporfin	Liposomal/ injection	Age-related macular degeneration	2000	QLT Inc. /Novartis

Table 2. Current FDA or EMA-approved liposomal single drug formulations for different clinical applications. Source: official website of USA Food and Drug Administration (FDA) - <http://www.fda.gov> (2010) and European Medicines Agency (EMA) - <http://www.ema.europa.eu> (2010).

Formulation brand name	Therapeutic agent	Therapeutic indication	Company name	Status
Annamycin		Acute lymphocytic leukemia	Callisto Pharmaceuticals	Phase I/II
		Acute myelogenous leukemia Breast cancer	New York University School of Medicine	Phase I/II
Aroplatin	Platinum agent NDDP	Colorectal cancer	Aronex Pharmaceuticals	Phase II
Atragen	Tretinoin	Hodgkin's lymphoma	M.D. Anderson Cancer Center	Phase II
		Metastatic kidney cancer	Weill Medical College of Cornell University	Phase II
	Cisplatin	Lung cancer	Transave	Phase II
LE-SN28	Irinotecan metabolite SN38	Advanced cancer	NeoPharm	Phase I
LEP-ETU	Paclitaxel	Advanced cancer	NeoPharm	Phase I
Marqibo®	Vincristine	Acute lymphoblastic leukemia	Hana Biosciences	Phase II
		Malignant melanoma		Phase I/II
	Mitoxantrone	Advanced cancer	NeoPharm	Phase I
	Nystatin	Systemic fungal infection in patients with hematologic cancer	Aronex Pharmaceuticals	Phase III
OSI-211	Lurtotecan	Ovarian cancer Small cell lung carcinoma	OSI Pharmaceuticals	Phase II
SPI-77	Cisplatin	Ovarian cancer	New York University School of Medicine	Phase II
Stimuvax®	BLP25 vaccine	Non-small cell lung cancer	EMD Serono and Oncothyreon	Phase III
	Topotecan	Small cell lung cancer Ovarian cancer Other solid tumors	Hana Biosciences, Inc	Phase I
	Vinorelbine	Advanced solid tumors Non-Hodgkin's lymphoma Hodgkin's lymphoma	Hana Biosciences, Inc	Phase I

Table 3. Examples of emerging liposomal single drug formulations currently undergoing clinical evaluation for cancer treatment. Currently, some liposomal drugs have no brand name and, therefore, are identified by the drug name. Data was compiled from <http://www.phrma.org> (2009) and from literature references (Lammers et al., 2008; Dutta, 2007; Torchillin, 2007, 2005; Immordino et al., 2006; Hofheinz et al., 2005) with actualization of current clinical status after consult of <http://clinicaltrials.gov/> (2010).

Currently, there are no approved liposomal drugs for treatment of urologic cancers. Nevertheless, some liposomal drug formulations listed in Table 4 are under clinical evaluation for prostate cancer treatment.

Formulation name	Treatment	Therapeutic indication	Company name	Status
Pegylated liposomal doxorubicin hydrochloride	Monotherapy	Prostate cancer (associated with hyperthermia treatment)	Celsion	Phase I
	Monotherapy		Ireland Cancer Center	Phase II
Doxil [®]	In combination with estramustine	Hormone-refractory prostate cancer (HRPC)	Ortho Biotech, Inc.	Phase I/II
Doxil [®]	In combination with thalidomide		Ortho Biotech, Inc.	Phase II
Doxil [®]	In combination with Taxotere [®]		James Graham Brown Cancer Center	Phase I/II

Table 4. Liposomal single drug formulations currently under clinical evaluation for prostate cancer treatment. Data was compiled after consult of <http://clinicaltrials.gov/> (2010).

4.2.6 Liposomal formulations of anticancer drug combinations

4.2.6.1 General considerations

The use of drug combinations has been standard of care for the treatment of cancer over the last decades. Nevertheless, the application of liposomes as carriers for anticancer drug combinations has been described in literature only in the last few years (Tardi et al., 2009; Harasym et al., 2007; Tardi et al., 2007; Mayer et al., 2006). To our knowledge, there are no liposomal drug combinations approved for clinical application. As previously mentioned in section 3.1 drug combinations can act synergistically, additively or antagonistically depending on the ratio of the agents being combined (Chou, 2006). While this relationship can be readily evaluated *in vitro*, where drug ratios can be controlled, the translation of those ratios to the clinical setting is complex due to the independent pharmacokinetics, biodistribution and/or metabolism of the individual drugs intravenously administered as aqueous-based free drug cocktail (Mayer et al., 2006; Lee, 2006). Therefore, the referred uncoordinated pharmacokinetics results in exposure of tumor cells to drug concentrations below therapeutic threshold level or to antagonistic drug ratios with concomitant loss of therapeutic activity (Mayer & Janoff, 2007; Harasym et al., 2007). The inability to control drug ratios in systemic circulation, and mainly in tumor tissue, may partly explain the short outcome in clinical efficacy seen for conventional free drug combinations (Mayer & Janoff, 2007).

Drug delivery systems, such as liposomes, can control the release of drug combinations such that fixed drug ratios are maintained after systemic administration. This tight control provides significant improvements in efficacy as compared to free drug cocktail and to individual liposomal drugs (Tardi et al., 2009, Mayer & Janoff, 2007; Harasym et al., 2007; Mayer et al., 2006; Lee, 2006). In 2006, Mayer and colleagues were the first to investigate the importance of maintaining an optimal drug combination ratio *in vivo* through drug encapsulation in liposomes (Mayer et al., 2006). Further studies (Tardi et al., 2009; Harasym et al., 2007) have demonstrated that *in vitro* drug interaction effects can be translated *in vivo* since liposomes can synchronize pharmacokinetics and biodistribution of drug combinations and deliver them to tumor tissue at a specific drug ratio (Fig.7, lower panel).

This “ratiometric” dosing approach has the potential to be applied to other diseases besides cancer in which multiple interacting mechanisms are responsible for disease progression or response to therapeutic interventions (Mayer & Janoff, 2007). In contrast, the combination injected as a free drug cocktail rapidly distributes into healthy and tumor tissues at drug ratios that differ from the administered one (Fig.7, upper panel).

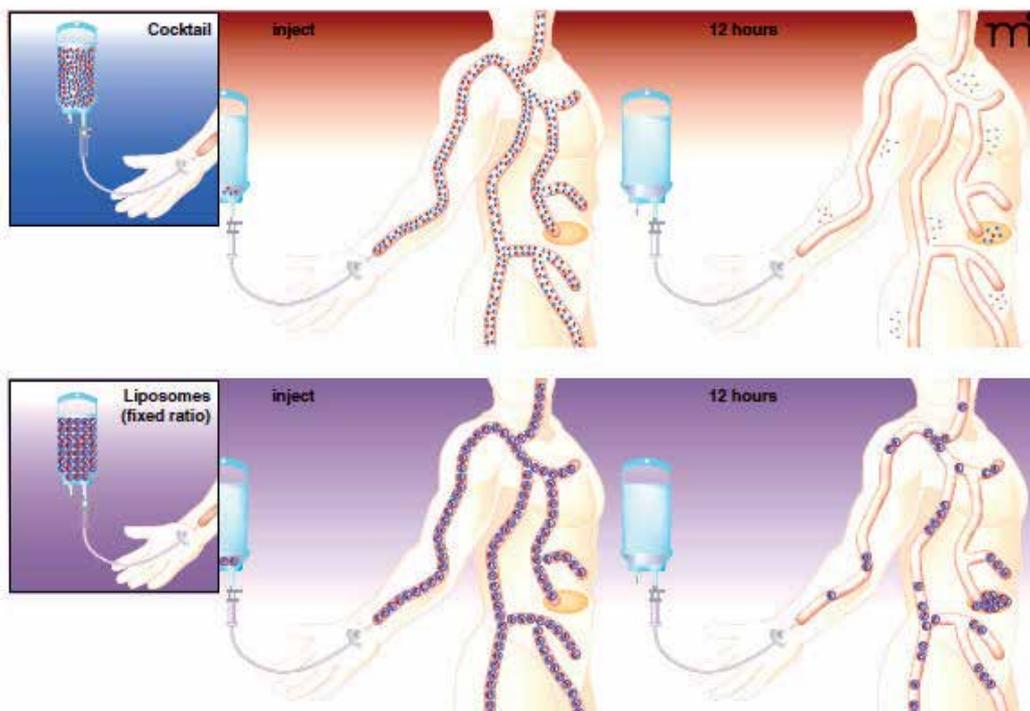


Fig. 7. Description of how clinical application of drug–drug synergy depends on controlled delivery of the desired drug ratio to the in vivo target. Upper panel - a drug cocktail is prepared at the desired ratio but biodistribution, metabolism and excretion processes will act differentially on the two drugs and cause the ratio to vary after intravenous injection. The two drugs distribute extensively into tissues shortly after injection and the ratio that reaches the tumor has been displaced 5-fold from the initially injected 1:1 ratio. Lower panel - liposomes that contain the synergistic 1:1 ratio maintain and selectively deliver this drug ratio to the tumor. The appropriately designed drug delivery vehicle maintains the drugs in the blood at higher concentrations for extended periods of time and, most importantly, at the effective synergistic ratio. Reproduced from reference (Mayer & Janoff, 2007).

Therefore, it can be concluded that liposome nanotechnology constitutes a valuable tool for preclinical assessment of drug combinations for clinical development (Lee, 2006). Advantages of liposomal drug combination delivery are summarized in Table 5.

Celator Pharmaceuticals were pioneers in liposomal drug combination design and have some products under clinical development and preclinical programs, which are summarized in Table 6. Currently, CPX-351 and CPX-1 are the only liposomal drug combinations under testing in clinical trials (<http://clinicaltrials.gov> (2010)) but none of them is intended for prostate cancer therapy.

1. Injection of multiple drugs simultaneously
2. Identical pharmacokinetic profile for multiple drugs reflecting the profile of the lipid carrier
3. Tight control of drug concentration at the target sites by changing the drug combined ratio in the liposome
4. Maximal combination effects can be achieved by synergistic action of multiple drugs after cellular uptake
5. Versatile design of the loading methods and membrane lipid composition to control drug release
6. Improvement of patient compliance and quality of life due to reduced number of injections and of side effects while increasing efficacy

Table 5. Advantages of liposomal drug combination delivery. Adapted from reference (Bae et al., 2007)

Product name	Liposomal drug combination	Therapeutic indication	Status
CPX-351	Cytarabine : daunorubicin	Acute myeloid leukemia	Phase II
CPX-1	Irinotecan HCl : floxuridine	Colorectal neoplasms	Phase II
CPX-571	Irinotecan HCl : cisplatin	Small cell lung cancer	Preclinical
CPX-8XY	Unknown	Unknown	Research

Table 6. Liposomal drug combinations developed by Celator Pharmaceuticals. Source: Celator Pharmaceuticals (<http://www.celatorpharma.com> (2010)).

4.2.6.2 Design of liposomal formulations for drug combination delivery

The concept of combining drugs, with dissimilar physicochemical properties, into a single vehicle, that efficiently encapsulates both drugs and releases them at the same rate after administration *in vivo*, represents a major scientific and technical challenge. Presently, liposomal encapsulation represents a new paradigm for formulating anticancer drug combinations. Although this approach has emerged as a promising strategy for cancer treatment, there are a limited number of research studies reporting successful drug co-loading in the same carrier (Tardi et al., 2009; Zhao et al., 2008; Harasym et al., 2007; Mayer et al., 2006). This is likely the result of technical difficulties associated with the efficient and stable encapsulation of two drugs inside a single carrier as well as challenges in controlling the drug leakage rate and still maintaining the entrapped drug:drug ratio after systemic administration (Harasym et al., 2007; Tardi et al., 2007).

There are three different approaches to formulate a drug combination involving liposomal design: i) combination of a liposomal drug with a free drug; ii) encapsulation of two drugs in individual liposomal carriers that are subsequently combined at the desired ratio and iii) co-encapsulation of two drugs in the same carrier by means of a simultaneous or a sequential drug loading. The advantages and limitations of each strategy are discussed below in more detail and in the mentioned order:

- i. A liposomal drug formulation can be administered together with a free drug but unfavorable liposome-free drug interactions may occur, such as hydrophobic interactions or loading of the free drug into liposomes exhibiting a pH gradient

- (Waterhouse et al., 2001; Mayer et al., 1999). Therefore, these interactions may induce changes in the pharmacokinetic parameters of the free and encapsulated drugs as well as of the lipid carrier, leading to decreased efficacy and/or increased toxicity (Waterhouse et al., 2001).
- ii. Perhaps the most straightforward approach to coordinate the pharmacokinetics of a drug combination would be to encapsulate each drug independently into different liposomes that provide the required drug retention properties and, subsequently, to mix the liposomes in a single suspension at the desired drug:drug ratio. Nevertheless, this protocol of formulating drugs in individual liposomes and ultimately administrate to patients would be extremely expensive due to the high costs inherent to lipid constituents and to the manufacturing process of two separate formulations (Harasym et al., 2007).
 - iii. Co-encapsulation of two drugs in the same liposomal carrier seems to be a preferable solution as compared to administration of individual liposomal drugs since it reduces cost production, minimizes lipid load to the patient, which has been associated to infusion-related side effects, and eliminates the potential interference that each liposome population may exert in the pharmacokinetic profile of the other (Harasym et al., 2007; Tardi et al., 2007). Furthermore, co-encapsulation overcomes potential uncertainties about drug biodistribution provided by the different liposome compositions. By encapsulating a drug combination into a single liposome, the two agents are no longer metabolized and eliminated independently but rather distributed as a unit, dictated by the characteristics of the carrier. However, this approach represents a technical challenge in order to develop a liposomal formulation that matches drug release kinetics for both drugs. To accomplish such purpose, experimental parameters and liposome features, such as drug loading methods and lipid composition, must be systematically optimized during formulation development (Harasym et al., 2007).

4.2.6.3 Development of a liposomal drug combination for prostate cancer: an example

In the last few years our group has performed an extensive and systematic preclinical study to evaluate the *in vitro* biologic activity of traditional and novel anticancer drugs and, ultimately, identify new drug combinations with therapeutic potential for the treatment of prostate cancer. Combinations were selected among different drugs (ciprofloxacin, docetaxel, doxorubicin, etoposide, imatinib, mitoxantrone and vinblastine) representative of distinct mechanisms of action. Several treatment schemes were evaluated by varying schedule, type of administration (simultaneous or sequential) and drug:drug molar ratio. The nature of antiproliferative combined effects (synergism, additivity or antagonism) against metastatic prostate cancer cell lines was quantitatively assessed by the median effect analysis method, whose main parameters are combination index (CI) and dose reduction index (DRI) (Pinto et al. 2011a, 2009).

Combination of two drugs tested simultaneously, comprising at least one topoisomerase II inhibitor, result in mild antagonistic effects against PC-3 and LNCaP cells. This antagonism of growth inhibition effects is translated by a CI superior to 1 and by a DRI inferior to twofold. In contrast, imatinib-mitoxantrone and ciprofloxacin-etoposide simultaneous combinations interact additively in inhibiting PC-3 cell growth, yielding CI values close to 1 and DRI values of 2.6 and 3.5-fold for mitoxantrone and etoposide, respectively (Pinto et al. 2011a, 2009).

The use of liposomes as drug delivery systems has been exploited in order to improve overall therapeutic index of anticancer drugs by increasing their antitumor activity and/or by reducing their toxicity profile. The main goal of our studies was to develop and characterize a novel liposomal formulation, for simultaneous co-loading and delivery of a drug combination previously identified in cytotoxicity screening studies performed in our laboratory, and to evaluate its *in vitro* and *in vivo* antitumor activity against HRPC preclinical models. The rationale for selecting imatinib-mitoxantrone combination was to investigate if co-loading of those two drugs into a liposome could translate the additive growth inhibition effects exerted on PC-3 cells when these drugs were combined simultaneously in the free form. Another selection criterion was that these drugs exhibit non-overlapping toxicity profiles and different mechanisms of action, so potential side effects and resistance phenomena could be minimized. Moreover, this combination is innovative for prostate cancer therapy since it conciliates a conventional antineoplastic drug (mitoxantrone), which is standard of care for palliative treatment of HRPC, with a molecular-targeted agent (imatinib), which has exhibited antitumor activity against *in vitro* and *in vivo* HRPC models.

Systematic development studies, by varying drug loading methods and incubation conditions, were carried out in order to design a liposomal imatinib-mitoxantrone (LIM) formulation that while being stable would exhibit adequate features for intravenous administration. Obtained results provide clear evidence that the two drugs can be simultaneously loaded, with high encapsulation efficiency (> 95%), in a single liposomal carrier using a transmembrane (NH₄)₂SO₄ gradient-based procedure. According to literature, our study was the first to report an active loading method for imatinib (Pinto et al. 2011b).

In vitro studies performed on PC-3 cells showed that LIM formulation, at an optimized drug:drug molar ratio, exhibits enhanced tumor cell growth inhibition and promotes a 2.6-fold reduction of IC₅₀ as compared to single liposomal mitoxantrone. This dose reduction is equivalent to the one found for mitoxantrone in free drug combination against the same cell line (Pinto et al. 2011a). Therefore, the therapeutic gain in mitoxantrone efficacy, mediated by imatinib and that result from free drug combination, is also attainable after liposomal encapsulation of the drugs at an optimized drug:drug ratio (Pinto et al. 2011b).

In vivo therapeutic activity of developed liposomal formulations, comprising different doses of single or imatinib-combined mitoxantrone, was evaluated in a nude mice bearing subcutaneous PC-3 xenograft model. Obtained results clearly demonstrate that intravenous administration of the liposomal formulation co-loading a low mitoxantrone dose (0.5 mg/kg) with imatinib (10 mg/kg) enables a tumor growth inhibition similar to the one yielded by single liposomal mitoxantrone (2.0 mg/kg), i.e. with a 4-fold inferior dose. This dose reduction could minimize the occurrence of side effects and hence increase therapeutic index of mitoxantrone (Pinto et al. 2011b).

Our results clearly emphasize the potential of incorporating clinically relevant drug combinations, at specific therapeutic ratios, within a lipid-based delivery system. Our research study is the first to provide a proof-of-principle for imatinib use in improving *in vitro* and *in vivo* antitumor efficacy of liposomal mitoxantrone. Overall, the developed LIM formulation constitutes a novel nanotechnology-based drug combined platform with improved therapeutic outcome against HRPC.

5. Conclusion

The up-to-date approach intended to develop novel chemotherapeutic drug combinations should be based on a rational selection of the drugs to be combined and on a systematic and quantitative screening of the ratio-dependent antiproliferative effects against human tumor cell lines. Drug combination studies on tumor cell lines, using a quantitative method to evaluate the nature of drug interactions, allow a more rational design of future chemotherapy protocols.

The translation of specific drug ratios, previously selected in vitro, to the clinical setting is complex due to the independent pharmacokinetics and biodistribution of individual drugs intravenously administered as aqueous-based free drug cocktail. The referred uncoordinated pharmacokinetics results in exposure of tumor cells to drug concentrations below therapeutic threshold level or to antagonistic drug ratios with concomitant loss of therapeutic activity. The inability to control drug ratios in systemic circulation, and mainly in tumor tissue, may partly explain the short outcome in clinical efficacy seen for conventional free drug combinations.

The extensive in vitro information on drug ratios can be used to formulate drug combinations in drug delivery systems. The use of liposomes as drug delivery systems has been successfully exploited in order to improve overall therapeutic index of anticancer drugs by increasing their antitumor activity and/or by reducing their toxicity profile. Successful clinical application of this rationally-designed approach to cancer therapy depends on the development of a liposomal formulation, with specific features, that delivers the drug combination in vivo so that the effective drug ratio is maintained after systemic administration and is ultimately exposed to tumors.

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Multimodal Therapies for Upper Gastrointestinal Cancers – Past, Now, and Future

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1. Introduction

Gastric and esophageal cancers are one of the most aggressive malignancies worldwide. Complete surgical resection offers the only chance of cure but many cases are presented at advanced stages or recur even after R0 resections. In gastric cancer (GC), 5-year survival rates (SRs) of stages II and III after curative resection range respectively from 30%-49% and 10-20% in Western countries, and respectively 70-80% and 40-50% in Japan and Korea (Fujii et al., 1999; Hundahl et al., 2000; J.P.Kim et al., 1998; Shimada et al., 1999). With regard to esophageal cancer (EC), the 5-year SRs in older series were 14-20% in the United States (Daly et al., 1996; Ries et al., 2007) and ranging from 3 to 11% in European countries, with an average of 10% (Faivre et al., 1998) while they improved to 42% in a recent series (Rice et al., 2009; Ruol A et al., 2009), which is comparable to those (36-46%) after an esophagectomy in Japan (The Japan Esophageal Society). Even in Japan, however, T3 or stage III EC, the most frequently encountered tumor depth or tumor stage, has exhibited respectively lower 5-year SRs of 27-29% and 17-20% after esophagectomy (Ando et al., 2000; The Japan Esophageal Society). Although stage-specific survival rates differ between reports from Western and Asian institutions, the survival decline according to the stage progression suggests that recurrence does occur even for those who undergo curative resection. Furthermore, a more extended (>D2) lymph node dissection (LND) in GC unfortunately fails to improve survival outcomes (Sasako et al., 2008).

Such disappointing treatment results in GC and EC even in Japan -where aggressive LND has been performed- have reminded researchers a plateau effectiveness of, and little likelihood of further improvement by, surgical therapy only. These facts have encouraged physicians to establish more effective multimodal strategies to improve survival, including newly developed regimens incorporating molecular targeting agents as well as to determine candidate molecules or genes that could help establish individualized therapies. These multimodal therapies have mainly consisted of chemotherapy (CTx), radiotherapy (RTx), and chemoradiotherapy (CRTx) administered after curative resection (adjuvant settings), or for recurrent or inoperable diseases (advanced settings), or before surgical resection (neoadjuvant settings). Recently, CRTx has become a focus of research, because of the radiosensitizing properties of some chemotherapeutic agents that could potentiate the anticancer activities of both CTx and RTx (Kleinberg et al., 2007).

Table lists the theoretical advantages and disadvantages of adjuvant and neoadjuvant treatments (Rice et al., 2003). Adjuvant and neoadjuvant treatments require balancing advantages and disadvantages to maximize treatment effects. This chapter focuses on past, current, and potential future directions in the field of multimodal therapies in GC and EC.

	Advantages	Disadvantages
Adjuvant treatment	<p>Prevention of potential recurrence from occult micrometastasis which would become overt postoperatively.</p> <p>Less consideration for increase in morbidity and mortality that would be sometimes harm in case of neoadjuvant treatment.</p> <p>Treatment decision based on the full staging information, which can avoid unnecessary treatment in patients who may not otherwise require it, i.e., in patients with earlier stage of the disease than expected.</p> <p>Especially in EC, relief of tumor-associated complaints such as dysphagia or alimentary support by surgically-placed feeding tube, allowing for better tolerance of postoperative therapy.</p>	<p>Inability to assess final treatment effects until diseases recur.</p> <p>Destruction of vasculature that decreases delivery of drugs or oxygen compromising CTx and/or RTx effects.</p> <p>Preclusion of early commencement of therapy if patient recovery delays due to postoperative complications or reduced functional status. Requirement of longer period to recover from surgery allows tumor cells for further growth.</p> <p>In RTx, removal of target that make definition of radiation field more difficult.</p>
Neoadjuvant treatment	<p>Increased surgical R0 resection rate by downstaging which would otherwise have been regarded as incurable (R1/R2 resection).</p> <p>Early elimination of undetectable micrometastasis.</p> <p>Intact vasculature that maintains accessibility of drugs to the tumor bed and oxygenation, realizing more effective CTx and/or RTx.</p> <p>Determination of tumor and patient specific chemo(radio)sensitivity that would be applicable to postoperative treatment.</p> <p>Possibility to administer a more intensive regimen because of the maintained physical and nutritional state. The same regimen would become more toxic if given postoperatively due to surgical-related complication.</p>	<p>Surgical delay due to toxicities that would cancel the potential survival benefit of responders.</p> <p>Loss of opportunity to undergo surgery by tumor growth during treatment period in nonresponders. No reliable methods available to predict tumor response that could discriminate responders and non-responders before treatment.</p> <p>Increased surgical complaints due to preoperative treatment-related toxicities.</p> <p>Preclusion of effective regimen due to dysphagia or tumor related worse nutritional state, especially in EC.</p> <p>Possibility of overtreatment for early stage tumor because treatment is based on clinical stage that is not necessarily accurate.</p>

Table 1. Advantages and disadvantages of adjuvant and neoadjuvant treatment [Rice TW, et al. 2003].

2. General considerations

In consideration of the establishment of multimodal therapies against GC and EC, one should bear in mind the difference in surgical treatment and pathology between Japan and the West. First, the scope of LND influences local tumor control rate and postoperative survival. A wider scope of LND has the theoretical potential to reduce local recurrence rates, which may improve postoperative survival. Second, it is also a fact that a wider scope of LND increases postoperative morbidity and mortality, which may cancel the anticipated survival benefit of LND. This phenomenon is especially observed in the West, providing one reason for the difference in the standard scope of LND between here and Japan. As a result, for GC, the standard scope of LND is a D2 (second-tier node dissection) in Japan but a D1 (first-tier node dissection) in the West, and for EC, the routinely performed node clearance encompasses three fields (cervical, thoracic, and abdominal) or at least any two of the three fields in Japan, while it encompasses a limited area in the West. Since the effect of hospital volume on a perioperative mortality hazard is larger than those on hazards for death at 5 years postoperatively (Bilimoria et al., 2008; Birkmeyer et al., 2002), the perioperative patient control reflects the subsequent overall survival; thus, LND by specialists is now an important issue. Third, a wider scope of LND results in a more accurate nodal staging than a narrower one. Therefore, under conditions of a limited extent of LND, judgement regarding R0 resection is not always accurate, and resections deemed R0 might sometimes be actually equivalent to R1 resection. For example, in a Dutch trial, 38% of GC patients classified as stage II on D1 dissection were reclassified as stage IIIA on D2 dissection (Bunt, 1995). This discordance is reflected by the stage migration phenomenon which implies that a narrower scope of LND results in a significant risk of understaging. Therefore, results of clinical trials of multimodal therapies are undoubtedly influenced if they recruit such understaged patients. Fourth, the scope of LND depends on the pathological characteristics of the tumor. With regard to EC, adenocarcinoma is observed at a substantial ratio (43-56%) in the United States (Daly et al., 1996; Trivers et al., 2008), while squamous cell cancer comprises a majority in Japan (The Japan Esophageal Society), the incidences twice those of the United States (90% vs. 38%) (Trivers et al., 2008). Such histological differences reflect tumor location and surgical approach. In Japan, the incidences of middle and lower third thoracic EC were respectively 50% and 25% (The Japan Esophageal Society), whereas those in the United States were respectively 25% and 50% (Daly et al., 1996). Considering that upper thoracic or cervical nodal involvement occurs more frequently in the middle third than lower third thoracic EC, such histological and topographical differences also influence surgical approach and ultimately the scope of LND. In Japan, a right thoracotomy is a main approach while a transhiatal approach is considered in the West (Hulscher et al., 2001). Finally, the different stances in surgical therapy between Japan and the West reflect the different stances of multimodal therapies. Since a wider scope of LND is more effective in local tumor control than a limited one, CTx rather than CRTx has been developed in Japan for the purpose of preventing systemic relapse rather than local recurrence, whereas CRTx rather than CTx has been developed in the West for the purpose of preventing both local and systemic relapse.

3. Gastric cancer

3.1 Adjuvant setting

Many randomized controlled trials (RCTs) have been conducted to assess the advantage of adjuvant CTx on survival compared with surgery alone. However, they have shown mixed

results and have been mostly disappointing. The difficulties in making definitive conclusions concerning the significance of adjuvant CTx are accounted for -at least in part- by the small size of the studies and suboptimal CTx regimens. Many trials recruited relatively small numbers of patients -usually less than 200- and were therefore inadequate to detect clinically significant survival differences between the CTx arm and surgery alone arm. In addition, many CTx regimens employed old types of drug with low response rates (RRs) and short durations of response. Therefore, the negative results of most previous clinical trials do not necessarily mean that the adjuvant CTx does not work.

Several meta-analyses of adjuvant trials have been published (Earle & Maroun, 1999; GASTRIC Group, 2010; Hermans et al., 1993; Hu et al., 2002; Janunger et al., 2002; Liu et al., 2008; Mari et al., 2000; Oba et al., 2006; Panzini et al., 2002; Zhao & Fang, 2008) in order to overcome the drawbacks of small patient accrual and to assess any potential benefit by adjuvant therapy that may have been missed in the individual trials. Unfortunately, definitive evidence concerning benefits of adjuvant CTx is lacking, with results ranging from an odds ratio for death of 0.56-0.9 to no benefit. However, the first meta-analysis (Hermans et al., 1993), which failed to demonstrate a clear benefit of adjuvant CTx, was later updated by an inclusion of 318 patients who had been erroneously omitted from the initial analysis, resulting in a reduction of the odds ratio for death to a significant 0.82 (Hermans & Bonenkamp, 1994).

Nevertheless, criticism still persists as to the methodologies applied in these meta-analyses that make this interpretation too complicated. For example, there was a great heterogeneity among individual studies in terms of quality of surgery, adjuvant therapy regimens administered, clinical stage, and intervals between surgery and the commencement of CTx. Accordingly, patient recruitment was not uniform within each meta-analysis. Some meta-analyses included studies of palliative resections and curative resections, or studies of CTx, RTx, and immunotherapy together. The quality of surgery could not be evaluated because the scope of LND was not always clarified. Studies with old generation drug(s) and those with newer generation drug(s) were also included together. In addition, one meta-analysis (Hu et al., 2002) included a previous meta-analysis (Hermans et al., 1993).

The most recent meta-analysis (GASTRIC Group, 2010), which was the first patient-level analysis, was published in the year 2010. It demonstrated both mortality and relapse hazard reduction by 18% each by adjuvant CTx. This trend was also reproduced irrespective of the number of CTx drugs (monochemotherapy and polychemotherapies). Furthermore, a survival improvement of 6% by CTx at 5 years postoperatively was maintained during the ensuing 5 years. These consecutive results of the meta-analyses suggest that the benefit of adjuvant CTx change from 'none or inconclusive or borderline' to 'significant'.

Subset metaanalysis have demonstrated inconsistent results, however. When Asian and non-Asian adjuvant trials were grouped together or analyzed separately, survival benefits were seen only in the non-Asian studies (Earle & Maroun, 1999), or only in the Asian studies (Janunger et al., 2002), or in both (Zhao & Fang, 2008). The extant metaanalyses are therefore difficult to interpret due to significant heterogeneity. Interestingly, in the pivotal Japanese RCT for adjuvant CTx (JCOG9206-1) (Nashimoto et al., 2003), the total recurrence rate at 69 months was almost double in the surgery only arm than in the CTx arm (13.8% vs. 7.1%), indicating a possible role by CTx for the prevention of recurrence. Against these backgrounds, several large RCTs of postoperative or perioperative CTx have been conducted in Japan, the United States, and Europe, and each result has been recently published.

The ACTS-GC (Adjuvant Chemotherapy Trial of TS-1 for Gastric Cancer) trial is so far the largest adjuvant RCT conducted in Japan, in which oral S-1, a new generation of fluoropyrimidine derivative, was administered for 1 year as an adjuvant treatment (Sakuramoto et al., 2007). The survival benefit of S-1 at three years postoperatively has also been confirmed at 5 years (Sasako et al., 2010). The results are consistent with the previous metaanalysis demonstrating the survival advantage of oral fluoropyrimidine containing adjuvant regimen (Oba et al., 2006). Of note in this trial is that most of the recruited patients underwent D2 or a wider scope of LND, and even under such circumstances of qualified surgery, the oral fluoropyrimidine derivative alone was able to render benefits in an adjuvant setting. However, caution is required when these results are applied to clinical use. The subanalysis of the ACTS-GC trial revealed that survival benefit was maintained in stages II and IIIA GC but disappeared in stage IIIB. Such stage specific survival differences suggest that oral fluoropyrimidine alone lacks power to exhibit statistically meaningful survival advantages in more advanced stages.

The phase III intergroup-0116 trial (INT0116) is one of the most important adjuvant trials against adenocarcinoma of the stomach or esophagogastric junction ever conducted in North America (Macdonald et al., 2001). The adjuvant treatment in this study was a CRTx consisting of fluorouracil, leucovorin, and extra-beam radiation delivered to the tumor bed with 2cm beyond the proximal and distal margins of resection as well as to the areas of regional draining lymph nodes. Adjuvant CRTx yielded a significant prolongation of disease free survival (DFS) and overall survival (OS) at 3 years postoperatively (Macdonald et al., 2001). More than six years of median follow-up confirmed no deterioration of survival over time (Macdonald et al., 2004), making this regimen optimal as the standard of care in the United States. However, there has also been some criticism directed towards this study. First, the extent of surgery performed in this study has been a focus of much debate. The trial has been criticized for the surgical undertreatment of patients: 54% and 36% of the patients in the trial respectively underwent a D0 and a D1 LND despite a recommendation of a D2 LND in the trial protocol. Such noncompliance clearly undermined survival and led to a high relapse rate of 64% in the surgery only arm after a median follow-up of 5 years, the results contrasting sharply with those of the JCOG9206-1 trial (Nashimoto et al., 2003). Considering that more than two thirds and 85% of the recruited patients respectively had T3/T4 diseases and were node positive, the survival benefit by CRTx seen in this study seemed to be simply a compensation for the control residual disease left by the inadequately limited surgery (D0 or D1), which could otherwise be resected by a D2 LND. Second, usage of 5FU as a radiosensitizer and chemotherapy of 5FU and leucovorin seemed appropriate at the time when INT-0116 was designed (in the 1980's) and executed (in the 1990's); however, new generation agents with a superior antitumor activity and greater radiosensitizing effect continue to be developed (Rice et al., 2003).

As discussed in the General Considerations section, this context raises another important insight that outcomes of adjuvant therapy undoubtedly depend on the quality of surgery, in particular, on the scope of LND. The incidence of locoregional recurrence after curative resection has been higher in the West (Roviello et al., 2003) (45%) than in Japan (Maehara et al., 2000) (22%), suggesting a benefit from the routine performance of a D2 LND for local tumor control. Such a benefit is guaranteed by the safe performance of a D2 LND in Japan, with a 0-2.2% mortality rate (Fujii et al., 1999; Maruyama et al., 1987; Maruyama et al., 2006; Nashimoto et al., 2003; Sano et al., 2002). Indeed, in the JCOG9206-1 trial, 98% of the patients

underwent a D2 or greater LND, with just one (0.8%) postoperative death in the surgery only arm. The number of patients with local recurrence was two (1.6%) in the surgery only arm and none in the adjuvant treatment arm, suggesting a remarkable local control rate by the Japanese style D2 LND. Therefore, convinced of the benefit of a D2 LND, Japanese investigators have always been reluctant to conduct any trial comparing D2 with D1 LND. In contrast, operative mortality remains high within Europe, ranging between 5-16% (Lepage et al., 2010). Under these circumstances, the initial phase III trials conducted in the West demonstrated that a D2 LND provided neither survival improvements nor decreased relapse rates, and that it was even harmful in terms of a 43-46% morbidity and 10-13% mortality (Bonenkamp et al., 1995; Bonenkamp et al., 1999; Cuschieri et al., 1996; Cuschieri A et al., 1999; Hartgrink et al., 2004a; Songun et al., 2010). Such higher D2-associated morbidity and mortality were initially considered to nullify its potential survival benefit by local control; however, a subsequent long-term 15-year follow up of a Dutch trial observed significantly higher local recurrence rates and cancer-related death rates in D1 than in D2 (Songun et al., 2010), leading the authors to conclude that a D2 LND has efficacy for local control and can be recommended for resectable GC, given that a safer, spleen-preserving D2 LND is available. Subsequent studies have supported this procedure-related safety. Several nonrandomized trials have also demonstrated no differences in morbidity and mortality between a D1 and a D2 or wider LND (Bösing et al., 2000; Edwards et al., 2004; Danielson et al., 2007; Zilberstein et al., 2004). The RCTs conducted in the dedicated centers in the West elucidated a safer performance with a D2 or wider LND, with morbidity and hospital mortality being 17-22% and <2%, respectively, if resection of the pancreas and/or spleen was performed for selected patients.

Considering that postoperative CRTx can be a substitution for a D2 LND, adjuvant CRTx constitutes an alternative option for countries where a D1 LND is the primary treatment or at institutions where surgeons are not keen on a D2, whereas its effect is questionable where a D2 dissection is both routine and safe -such as in Japan or in some Western specialized centers (Degiuli et al., 2004a; Degiuli et al., 2004b; Degiuli et al., 2010; Kulig et al., 2007; Sano et al., 2004; C.W.Wu et al., 2004; C.W.Wu et al., 2006). Interestingly, a Korean group (S.Kim et al., 2005) reported the superiority of adjuvant CRTx over surgery alone in gastric cancer patients undergoing a D2 dissection. Since the CRTx protocol in this study was identical to that of INT0116, the results, although observational, suggest that CRTx is promising even for patients undergoing a qualified LND.

Two RCTs assessing the benefits of perioperative chemotherapy have been conducted. The MAGIC trial (Medical Research Council Adjuvant Gastric Infusional Chemotherapy) (Cunningham et al., 2006) was a randomized perioperative CTx trial in United Kingdom for patients with stage II or higher resectable adenocarcinoma of the stomach, esophagogastric junction, or lower esophagus. In this trial, the perioperative CTx consisted of three preoperative and three postoperative cycles of epirubicin, cisplatin, and fluorouracil (ECF). The perioperative CTx significantly improved 5-year DFS and 5-year OS. Another was a French trial (FNCLCC94012-FFCD9703) comparing perioperative cisplatin and 5FU (CF). Again, however, there are several limitations and criticisms regarding these trials. The scope of LND in MAGIC trial was not standardized, with a D1 or a D2 LND being left to the discretion of the surgeons, resulting in only 41% of the patients undergoing a D2 LND. Curative resection rates in the surgery only arm accounted only for 66% (MAGIC trial) or 73% (FFCD trial) of the patients, suggesting that both were not purely adjuvant CTx trials

and likely recruited extremely advanced cases. Perioperative CTx may be harsh because of the relatively higher rate (9%) of not completing the three planned cycles of ECF, and of low rates of commencement (55%) and completion (42%) of the postoperative ECF, predominantly due to toxic effects, early disease progression, patient request, and postoperative complications. In addition, the 30-day mortality rate in both arms of the MAGIC trial was approximately 6%, which is relatively higher than those of a D2 (0-5%) (Degiuli et al., 2004a; Degiuli et al., 2004b; Degiuli et al., 2010; Kulig et al., 2007; Nashimoto et al., 2003; Sano et al., 2004; C.W.Wu et al., 2004; C.W.Wu et al., 2006) or wider scope of LND (0.8-2%) (Kulig et al., 2007; Sano et al., 2004) in Japanese and Western specialized institutions. Pathological T1 disease patients comprised 8% of the surgery only arm, suggesting that preoperative staging was not necessarily accurate and such earlier stage patients received unnecessary CTx, which would be more harmful than beneficial. Finally, the continuous infusion of 5FU that requires an infusion pump and long term infusional access may be associated with a risk of catheter-related complications.

Taking these results concerning benefit and harm of LND into account, a D2 surgery is at least equal to an adjuvant CRTx with D0/D1 surgery, and an adjuvant S-1 improve survivals more than a D2 LND alone -at least in Japan. Encouraged by the positive results of INT0116 and MAGIC trials, a more combined multidisciplinary approach has been investigated in a CRITICS trial, in which adjuvant CRTx (investigational arm) after 3 preoperative cycles of ECC (epirubicine, cisplatin, capecitabine) and D1 surgery (clearance of >15 nodes) was compared with 3 adjuvant cycles of ECC (control arm) after the same preoperative ECC and the same quality of surgery (<http://www.critics.nl>).

3.2 Advanced setting

Evidence of benefits of CTx for patients with advanced or recurrent GC was obtained from the previous RCTs demonstrating a significantly improved survival by CTx as compared with the best supportive care (Glimelius et al., 1994; Murad et al., 1993; Pyrhönen et al., 1995). In Western countries, FAMTX (5FU, adriamycin, methotrexate) then became the standard regimen from the results of the EORTC randomized study, demonstrating the superiority of FAMTX over FAM (5FU, adriamycin, mitomycin C) with regard to median survival time (MST) (42 weeks vs. 29 weeks), RR (41% vs. 9%), and toxicity (Wils et al., 1991). Subsequently, a standard FAMTX regimen was compared with ECF. The RR and MST by ECF (46% and 8.7 months) were both found to be superior to FAMTX (21% and 6.1 months) (Waters et al., 1999). In addition, ECF was less toxic, afforded patients better quality of life, and was more favorable in cost effectiveness as compared with FAMTX (Webb et al., 1997), leading the investigators to propose an ECF regimen as a standard therapy. A recent systematic review revealed that the best survival results are achieved with a three-drug regimen containing 5FU, anthracycline, and cisplatin (Wagner et al., 2006).

In this century, docetaxel, one of the new generation agents, has become available and been recognized as a promising agent, being incorporated in several clinical trials. The non-overlapping toxicity profiles of docetaxel, cisplatin, and 5FU (DCF), as well as synergism among these agents *in vitro* (Maeda et al., 2004) in a schedule dependent manner or in human GC xenografts (Kodera et al., 2005), warrant this combination to be evaluated in treating GC. In Europe, a three-arm, randomized phase II study was conducted to compare DCF (docetaxel, cisplatin, 5FU), DC (docetaxel, cisplatin), and a standard reference regimen ECF (Roth et al., 2007). The RR and MST of the DCF (37% and 10.4 months) were superior to

those of the DC (18% and 11.0 months) and ECF (25% and 8.3 months), leading to the recommendation of DCF as an investigational regimen for further clinical trials. Similarly, in the United States, a randomized phase II trial found that DCF and DC were both active while DCF produced higher RR (43%) than DC (26%) (Ajani et al., 2005b). The DCF was then further positioned as an investigational regimen in a subsequent phase III trial (Van Cutsem et al., 2006), in which DCF was found to be significantly superior to CF with regard to RR (37% vs. 25%, $p=0.01$) and MST (9.2 months vs. 8.6 months, $p=0.02$). In addition, DCF was able to maintain patient performance status longer. However, a higher incidence of grade 3/4 hematological toxicities (82% of neutropenia, 65% of leucopenia, and 29% of neutropenic fever) emphasized a need for a vigilant patient selection and careful patient management and monitoring, which might preclude its more widespread acceptance as a new treatment option.

The requirement for a balance between survival gains and experienced toxicities has prompted several modifications of the DCF regimen to improve tolerability. A weekly administration of DCF may yield an improved safety profile without compromising the efficacy. In lung, breast, and prostate cancers, toxicities were less with weekly taxane than with triweekly taxane, while OS did not significantly differ between the two schedules or was even better in weekly taxane (Bria et al., 2006; Engels & Verweij, 2005; Sparano et al., 2008). There are several trials of DCF modifications (C.P. Li, 2010; Lorenzen et al., 2007; Overman et al., 2010; Park et al., 2005; Sato et al., 2010; Tebbutt et al., 2010). Among these investigations, GASTRO-TAX-1 (Lorenzen et al., 2007) proved modified DCF to have a remarkably prolonged MST (17.9 months) and median time to progression (TTP) (9.4 months), with a reduced incidence of grade 3/4 neutropenia (22%) and febrile neutropenia (5%).

On the other hand, in Japan, JCOG conducted a randomized study comparing mitomycin C (MMC) plus tegafur with MMC plus UFT (uracil and tegafur) (UFT-M). Although MSTs were equivalent in both arms (6 months), significantly higher RR (25%) in the UFT-M arm than in the MMC plus tegafur arm (8%) (Kurihara et al., 1991) led the investigators to recommend UFT-M as a candidate regimen for further clinical trials. JCOG then conducted a three-arm RCT comparing UFT-M, CF, and a continuous infusion of 5FU (JCOG9205) (Ohtsu et al., 2003). In this trial, patient recruitment for the UFT-M arm was stopped due to poor survivals with significant toxicities, and the survival curves of the remaining two arms were overlapping. Taking efficacy and toxicity together into account, a continuous infusion of 5FU monotherapy with a MST of 7.1 months remained as a reference arm for further clinical trials.

At the end of the last century, when S-1 became available, JCOG conducted another three-arm RCT comparing the continuous infusion of 5FU monotherapy (reference regimen) with S-1, or irinotecan plus cisplatin (JCOG9912) (Boku et al., 2009). The investigators observed a noninferiority of S-1 to 5FU, and the convenience of oral administration led to the conclusion that a continuous infusion of 5FU monotherapy could be replaced by S-1 for the first-line CTx.

Encouraged by these results, several randomized trials have been conducted by placing S-1 as a reference arm, and some trials have recently yielded results (Y.H. Kim et al., 2011; Koizumi et al., 2008; Narahara et al., 2011). Taking all these into account, a S-1 plus cisplatin combination (CS) is currently considered as a standard regimen in Japan for the treatment of advanced GC.

Globally, this combination has been investigated in a FLAGS study (First Line Advanced Gastric Cancer Study) (Ajani et al., 2010), in which 1053 patients were randomly assigned to either receive infusional CF or CS in non-Asian patients. Unfortunately, CS failed to prolong MST (8.6 months) as compared with CF (7.9 months). However, significant safety advantages of CS over CF suggested the possibility of the substitution of S-1 for infusional 5FU. The different results between the Japanese SPIRITS trial and Caucasian FLAGS trial are ascribed to the different recommended doses of S-1 between the two studies, presumably due to different metabolic profiles among races. Tegafur, a cytotoxic component of S-1, is converted to 5FU by cytochrome P450 2A6 (CYP2A6). Racial differences for gene polymorphism of CYP2A6 have been identified (Yoshida et al., 2003) (Daigo et al., 2002; M. Nakajima et al., 2006), and the variants are more frequent in Asians than in Caucasians (M. Nakajima et al., 2006). Since such polymorphism accounts for a lower enzymatic activity, Caucasians have a relatively higher enzymatic activity than Asians, leading to a faster conversion from FT to 5FU, more accumulation of 5FU, and consequently less tolerance to S-1. Accordingly, the recommended dose of S-1 in the FLAGS study (50mg/m²/day) was lower than that (80mg/m²/day) for Japanese (Ajani et al., 2005a). Another reason for the different results of the two trials may be the different ratio of patients receiving second-line therapies, which were respectively 74% and 75% in the CS and S-1 arms in the SPIRITS trial (Koizumi W et al., 2008), while they were respectively 30% and 33% in the CS and CF arms in the FLAGS trial (Ajani et al., 2010).

In China, a similar randomized study is now ongoing. In this study, the S-1 dose is the same (80mg/m²/day, twice daily) as that in Japan, but cisplatin is given in four administrations, each being 20mg/m² (<http://www.ClinicalTrial.gov>. NCT 01198392).

Although the results of the FLAGS trial were unfortunately negative, an oral administration route is undoubtedly convenient; its advantages include the alleviation of the requirement for control of a central venous catheter implantation, which in turn may improve the patient's quality of life. Against these backgrounds, there have been attempts to replace intravenous chemotherapy agents with the oral chemotherapy. Two-by-two designed RCTs (REAL-2 trial) have evaluated whether capecitabine (oral fluoropyrimidine) could be an alternative to infusional 5FU (Cunningham et al., 2008) and also whether cisplatin could be replaced by the new platinum compound oxaliplatin. In the REAL-2 study, the incorporated agents were epirubicin (E), cisplatin (C), 5FU (F), capecitabine (X), and oxaliplatin (O). Some 1002 patients were randomly assigned to receive either one of triplet therapies such as ECF, EOF, ECX, and EOX. Interestingly, MST by EOX (11.2 months) was longer than that (9.9 months) of a current European standard regimen ECF. Toxicities of capecitabine and 5FU were similar. Cisplatin requires hydration while oxaliplatin does not. As compared with cisplatin, oxaliplatin was associated with lower incidences of grade 3/4 neutropenia, alopecia, renal toxicity, and thromboembolism, but with higher incidences of grade 3/4 diarrhea and neuropathy. These results, together with the results of the recent RCTs (Al-Batran et al., 2008; Y.K. Kang et al., 2009), suggest the feasibility of substituting 5FU with capecitabine, or cisplatin with oxaliplatin. Indeed, the superiority of capecitabine over 5FU has been confirmed by a recent meta-analysis (Okines et al., 2009)

Trends towards oral administration have prompted researchers to investigate whether 5FU can be replaced by oral fluoropyrimidine S-1 in the DCF regimen. Surprisingly, a combination of docetaxel, cisplatin, and S-1 (DCS) yielded remarkable RR (84%) and MST (23 months) (Sato et al., 2010). The optimal doses of docetaxel and/or cisplatin of this triplet therapy have been investigated in several phase I studies (Fushida et al., 2009; Hironaka et

al., 2010; Nakayama et al., 2008). No requirement of hydration in oxaliplatin has prompted the substitution of cisplatin with oxaliplatin in the DCS regimen, forming a new triplet docetaxel, oxaliplatin, and S-1. However, it achieved modest MST (12 months) with 38% grade 3/4 neutropenia, despite high RR and CR rates (60% and 7.5%) (Zang et al., 2010). Since these studies are preliminary, the efficacy and safety of DCS should be confirmed by large-scale clinical trials. Finally, a multicenter phase II trial has very recently demonstrated that an S-1 and oxaliplatin combination (SOX) can be a substitution for a CS regimen; this has just been indicated as a standard regimen in Japan or a safer regimen than FP in the West. The SOX regimen yielded remarkable MST (16.5 months), while grade 3/4 neutropenia developed in 22% of the patients (Yamada et al., 2010).

Another important concern lies in the fact that the net survival time cannot necessarily be achieved by a single regimen. As discussed earlier, negative results of the FLAGS trial may be attributable to the relatively small number of patients receiving second-line therapies. One should bear in mind that most of the chemotherapy regimens introduced above could yield a median TTP of less than 7 months, suggesting the urgent need for the establishment of effective second-line regimens. The contribution of a second line treatment on survival has been also confirmed by the combined analysis of two large Japanese randomized trials (JCOG9205 and JCOG9912) (Takashima et al., 2010). Because the number of active drugs against GC is increasing, attempts to establish the best second or third line regimen(s) are important, as has been seen for colorectal cancer as well. Currently, several RCTs have been conducted or are now ongoing to provide one answer for this unresolved issue.

3.3 Neoadjuvant setting

Many RCTs for neoadjuvant CTx (NAC) trials have been performed, but the majority have failed to provide evidence concerning the superiority of NAC as compared with controls (Hartgrink et al., 2004b; Imano et al., 2010; Nio et al., 2004; Schuhmacher et al., 2010; Yonemura et al., 1993; C.W. Zhang et al., 2004). The negative results may be accounted for by the small number of recruited patients -usually less than 200, heterogeneous tumor stage- i.e., some studies recruited earlier T1/T2 tumors, heterogeneity in the scope of the LND or drug administration route, and allowance of additional postoperative adjuvant therapies. Therefore, the NAC value remains controversial because of a lack of well-powered trials, and the results of several meta-analyses are conflicting (H. Li et al., 2010; W. Li et al., 2010; A.W. Wu et al., 2007). Although the most recent two analyses revealed benefits of NAC in terms of OS, resection rate, and tumor down staging without increasing perioperative mortality (H. Li et al., 2010; W. Li et al., 2010), the effect of NAC alone on OS remains questionable since individual pure NAC trials, i.e., comparison between surgery alone and NAC without postoperative CTx, failed to demonstrate positive effects on survival (W. Li et al., 2010). Similarly, surgery plus postoperative CTx with or without NAC provided similar survival results (Nio et al., 2004).

As discussed in the General Considerations section, the selection of patients who receive the most benefit from NAC is an important concern (Table). The benefit of NAC was observed only in more advanced (T3/T4) cancers but not in earlier (T1/T2) cancers (W. Li et al., 2010), suggesting that any potential survival benefit may be confined to those patients at greatest risk of relapse (T3/T4). Whether early stage GC received the same benefit of NAC remains unclear since serosa-negative gastric cancer in Japan exhibited 83% DFS and 86% OS by a D2 LND without adjuvant CTx (JCOG9206-1) (Nashimoto et al., 2003). Similar favorable

survival results by qualified surgery in earlier stage GC were also reported from the West (Roukos et al., 2001; Siewert et al., 1998), suggesting that patients with a low risk of recurrence can be cured with adequate surgery alone (Roukos, 2004). The benefit of NAC is also influenced by the quality of surgery. As discussed in the MAGIC trial, positive effects of NAC, if present when the combined surgery is a <D2 LND, can be attributable to a mere substitution for a LND rather than the effects of the NAC itself. When determining the NAC regimen, one providing the highest likelihood of tumor shrinkage is theoretically the best regimen for NAC because subsequent surgery can extirpate the residual disease of the NAC. In this sense, several NAC trials using CS, which provides currently the highest RR (74%) (Koizumi et al., 2003), are ongoing in Japan. First, JCOG0501 is a RCT comparing surgery alone with neoadjuvant CS for type 4 or large type 3 GC (<http://www.ClinicalTrial.gov>. NCT00252161). Second, JCOG0405 is a phase II study investigating the efficacy of neoadjuvant CS for GC with bulky second-tier nodes or positive paraaortic nodes (Kawashima et al., 2008). The third study is a comparison between surgery alone and neoadjuvant CS, both containing adjuvant S-1 for stage III GC (<http://www.ClinicalTrial.gov>. NCT00182611).

4. Esophageal cancer

4.1 Adjuvant setting

In the West, a number of RCTs have been conducted to investigate the efficacy of adjuvant CTx or adjuvant RTx. A subsequent meta-analysis, however, failed to find any survival benefit at three years by adjuvant CTx or at one year by adjuvant RTx (Malthaner et al., 2004). This conclusion concerning the efficacy of adjuvant CTx was, however, drawn from the pooled data of only 2 studies. So far, there have been no RCTs evaluating adjuvant CRTx versus surgery alone (Malthaner et al., 2004); however, several phase II trials have demonstrated that adjuvant CRTx appeared to prolong survival (Bédard et al., 2001; Rice et al., 2003).

In Japan, an earlier RCT of adjuvant CTx consisting of cisplatin plus vindesine conducted in the 1980s failed to exhibit a survival benefit over surgery alone (JCOG8806) (Ando et al., 1997). JCOG then conducted a RCT (JCOG9204) comparing adjuvant CF with surgery alone (Ando et al., 2003). Although 5-year SR did not differ between the two arms ($p=0.13$), adjuvant CF was able to yield significantly improved 5-year DFS ($p=0.04$), which was more evident in node positive patients. In Japan, no adequate RCTs have been conducted to assess adjuvant RTx or adjuvant CRTx as compared with surgery alone.

4.2 Neoadjuvant setting

Multimodal therapies as a neoadjuvant setting have been developed mostly in the West, but their efficacy is conflicting. The significance of survival prolongation by NAC has changed from inconclusive in earlier metaanalyses (mortality hazard=0.88, 95% CI=0.75-1.04) (Malthaner et al., 2004; Malthaner et al., 2006) to just reaching positive (mortality hazard=0.90, 95% CI=0.81-1.00, $p=0.05$) (GebSKI et al., 2007). The significance was more evident in adenocarcinomas by subgroup analyses by histology (mortality hazard=0.78, CI=0.64-0.95, $p=0.014$) (GebSKI et al., 2007). Fortunately, treatment morbidity and mortality did not differ between NAC and surgery alone (Malthaner et al., 2006; Urschel et al., 2002). However, the positive NAC effects on survival (GebSKI et al., 2007) seem to be influenced by one MRC study (Medical Research Council Oesophageal Cancer Working Group 2002) with

the largest sample size ($n=802$), while most of the included RCTs in this meta-analysis in which the number of recruited patients was less than 100 showed no or marginal benefit for NAC. Although the survival benefit of the MRC study has been recently confirmed by a long-term follow-up study (Allum et al., 2009), the interpretation of this study requires caution, because the 5-year SR of the surgery only arm was only 17% despite half of the recruited EC being T3 or less (Medical Research Council Oesophageal Cancer Working Group 2002). This low SR contrasts with that (52%) of the surgery only arm in the JCOG9204 study, in which 65% and 55% of patients had T3/T4 and stage III/IV diseases, respectively. Conceivably, the advantage of neoadjuvant treatment is more likely to be demonstrated when SR in the control (surgery only) arm is lower. In addition, the survival benefit initially observed in adenocarcinoma in the MRC study disappeared in the later analysis (Allum et al., 2009). Finally, although NAC could enhance the chance of R0 resection, the pattern of the first recurrence was similar between the neoadjuvant CTx arm and surgery only arm (Allum et al., 2009), suggesting that NAC showed no clear trend toward fewer patients with distant metastasis as the first site of metastasis. These characteristics were also confirmed in the second largest study (RTOG8911) (Kelsen et al., 1998).

There are several systematic overviews concerning neoadjuvant RTx. These studies have consistently failed to reveal any improvement of survival by neoadjuvant RTx in patients with potentially resectable EC (Arnott et al., 2005; Ask et al., 2003; Malthaner et al., 2004).

In contrast, there are increasing expectations for neoadjuvant CRTx which are also supported by a recent RCT showing that neoadjuvant CRTx resulted in a significantly higher pathological CR rate compared with neoadjuvant CTx (Stahl et al., 2009). This could translate into a marginally significant ($p=0.07$) improvement in 3-year SR from 28% in neoadjuvant CTx to 47% in neoadjuvant CRTx in patients with locally advanced adenocarcinomas of the esophagogastric junction. Although some inconsistencies do exist (Luu et al., 2008), several neoadjuvant randomized CRTx trials have been conducted in the West and elucidated the rates of downstaging (Fiorica et al., 2004), R0 resection (Urschel & Vasani, 2003), and 3-year survival (Fiorica et al., 2004; Urschel & Vasani, 2003) in favor of neoadjuvant CRTx. Such survival benefits in favor of neoadjuvant CRTx were observed in both adenocarcinoma and squamous cell carcinoma (Gebski et al., 2007).

In Japan, the results of JCOG9204 have led to the subsequent RCT (JCOG9907) to determine which timing of the CTx administration is optimal, preoperatively or postoperatively. Preoperative CF was superior to postoperative CF both in progression free survival (PFS) ($p=0.044$) and OS ($p=0.014$), suggesting that neoadjuvant CF is superior to adjuvant CF or surgery alone. Whether the novel active regimen such as DCF or weekly DCF in GC can be extrapolated to EC has been investigated in a phase I/II trial (JCOG0807).

Comparing Japanese trials with Western ones, one should notice the dramatic difference in terms of postoperative mortality and survival outcomes. In Japan, surgery with at least 2-field LND yielded >50% 5-year SR with extremely low mortality, while Western studies demonstrated increased mortality (>5%) with lower 5-year SR. In addition, neoadjuvant CRTx resulted in increased postoperative in-hospital mortality than surgery alone (Fiorica et al., 2004), due to the three most frequent adverse events of respiratory complications, heart failure, and anastomotic leakage (Fiorica et al., 2004). As discussed in the General Considerations section, 3-field LND realizes local control, so that neoadjuvant CTx can afford a most impressive survival advantage and be regarded as a new standard regimen in stage II/III squamous cell carcinoma in Japan.

4.3 Definitive CRTx for resectable EC

CRTx only (definitive CRTx) undoubtedly represents an alternative treatment for patients with EC considered unsuitable for surgery on the basis of comorbidity, poor performance status, and locoregional diseases too extensive for curative resection. For resectable EC, although esophagectomy has still been designated as -at least a part of- a pivotal treatment modality, it is indeed a complex, highly invasive procedure. Operative morbidity and mortality undoubtedly depend on hospital volume; however, reports on this topic from the West, some of which were from high volume centers, documented a near 50% morbidity and 10% mortality (Bailey et al., 2003; Birkmeyer et al., 2002; Jamieson et al., 2004). Since CRTx does have a significant downstaging effect but increases postoperative mortality when combined with surgery, there is growing enthusiasm for the definitive CRTx to treat potentially resectable EC. The choice of CRTx as a definitive treatment option is based on the RTOG 8501 trial, which was instrumental in defining the superiority of definitive CRTx with a 50Gy radiation dose over definitive 64Gy RTx alone (Herskovic et al., 1992). A subsequent metaanalysis has confirmed its promise (Wong & Malthaner, 2006).

Two large RCTs examined whether surgery was necessary after CRTx. A German group demonstrated similar 2-year SR in the neoadjuvant CRTx to a total dose of 40Gy followed by surgery (40%), and in the definitive CRTx with at least 65Gy (35%) in locally advanced squamous cell cancer (Stahl et al., 2005). A subsequent French trial (FFCD9102) (Bedenne et al., 2007; Bonnetain et al., 2006) also confirmed no benefit for additional surgery after CRTx to the responding patients with locally advanced squamous cell cancer. In addition, a nonrandomized comparison revealed the same impact on survival between definitive CRTx and surgery only (without adjuvant treatment) (Hironaka et al., 2003). A single arm phase II study in Japan (JCOG9906) (K. Kato et al., 2010) demonstrated that definitive CRTx in stage II/III esophageal squamous cell cancer could yield a complete response rate of 62%, with 3-year and 5-year SR being 45% and 37%, respectively, comparable to those for esophagectomy (33-47% and 20-52%, respectively) (The Japan Esophageal Society). However, these findings are still inferior to those of the neoadjuvant CF arm in the JCOG 9907 trial. Accordingly, definitive CRTx is not regarded as a standard treatment for stage II/III esophageal squamous cell cancer in Japan.

Nevertheless, these encouraging reports have led to the further activation of several studies to assess the efficacy of definitive CRTx for patients with earlier stage squamous cell cancer. JCOG9708 trial (H. Kato et al., 2009) elucidated 2-year and 4-year SRs of 93% and 81%, respectively, which were comparable to those of the stage I SCC undergoing esophagectomy (The Japan Esophageal Society). Other investigators also reported a high complete response rate (88%) and 3-year SR (79%) in patients with stage I SCC by definitive CRTx (Minashi et al., 2006). However, definitive CRTx is accompanied by several problems.

First, and unfortunately, crude locoregional control rates remain poor, with a respective 23-65% and 13-67% of patients having persistent disease or relapse at the primary site (Coia et al., 2000; Cooper et al., 1999; Minsky et al., 2002; Murakami et al., 1998; Stahl et al., 2005; K.S. Wilson & J.T. Lim, 2000). Tumor recurrence among patients whose treatment results deemed CR is a problem with definitive CRTx because no perfect diagnostic methods currently exist for the evaluation of CR. In the JCOG 9708 trial, although the complete response rate was high (88%), half of the total patients relapsed. In another definitive CRTx trial (Minashi et al., 2006), locoregional diseases were discovered later in 14 (39%) of 36 complete response patients. Although surgery is not intended as part of the definitive CRTx,

salvage surgery that could offer the only chance of cure for patients with recurrent or residual diseases after definitive CRTx should be considered. In addition, definitive CRTx-related local complications such as esophageal stenosis and perforation are also indications for salvage surgery. However, salvage surgery is a highly invasive and complex treatment leading to increased morbidity (50-79%) and in-hospital mortality (7-22%) as compared with those after neoadjuvant CRTx, due to the adverse events of predominantly respiratory complications and anastomotic leakage (Chao et al., 2009; Nakamura et al., 2004; M. Nishimura et al., 2007; Oki et al., 2007; Smithers et al., 2007; Swisher et al., 2002; Tachimori et al., 2009; Tomimaru et al., 2006). These hospital mortality rates are obviously higher than those for esophagectomy in Japan reported from specialized centers (2%) (Tachimori et al., 2009) or the nationwide registry (5%) (The Japan Esophageal Society). Second, definitive CRTx for resectable EC has the merit of preserving the esophagus, though it may increase late toxicity such as pericardial or pleural effusion (Ishikura et al., 2003; Kumekawa et al., 2006). Pericardial and pleural effusion developed in nearly 20% of complete response patients, leading to 10-12% of treatment-related deaths (Ishikura et al., 2003; Kumekawa et al., 2006). Even in the earlier stage squamous cell cancer, definitive CRTx-related mortality was observed in 8% of complete response patients (Minashi et al., 2006). These facts suggest that late toxicities are often progressive in severity and may compromise the long term health-related quality of life of a cancer survivor, leading to nullifying the anticipated treatment benefit from therapy. Since conventional toxicity reporting tends to present the more intensive treatments as less toxic than they really are (Trotti et al., 2007), one should bear in mind that the actual toxicities are likely to be underestimated.

These complications are accounted for by the radiation *per se* that renders risks of pulmonary complications, partly due to the fibrogenic response pathway (Bentzen, 2006), and the radiation induced injury in the thoracic cavity that makes surgical procedures technically more difficult and subsequently increases bleeding. In addition, the irradiated stomach, esophagus, and trachea become fragile with the impaired blood supply that eventually causes anastomotic leakage or conduit necrosis. The incidences of morbidity after salvage surgery were associated with radiation doses rather than clinical factors (Wang et al., 2006), suggesting that a dosimetric aspect should be taken into account in planning a definitive CRTx. On this basis, several attempts have been made to reduce the incidences of postoperative morbidity and mortality of salvage surgery. RTOG94-05/INT0123 elucidated the possibility of total radiation volume reduction (50.4Gy), which was equally effective as compared with higher doses (64.8Gy) (Minsky et al., 2002). A novel radiation technique has been developed to ensure an increased volume of lung unexposed to radiation to deliver large and uniform doses to the tumor while sparing nearby normal tissues (X. Zhang et al., 2008). In Japan, based on the phase I study results (T.E. Nakajima et al., 2009), a phase II study of definitive CRTx with a radiation dose of 50.4Gy for stage II/III esophageal squamous cell cancer is ongoing (JCOG0909). In planning a definitive CRTx, therefore, a higher radiation dose is not recommended because it does not improve survival but would presumably increase the risks of salvage surgery if needed.

Given the highly invasive and formidable procedures of salvage surgery, patient selection of those who would receive the most benefit or who would be unfit for surgery is a major concern in the clinical field. Several factors have been proposed for patient selection for salvage surgery. First, there is some evidence that patients with recurrence after CRTx had a significantly better survival after salvage surgery than those with persistent disease (Swisher et al., 2002). Salvage surgery could be avoided in complete response patients, but the

diagnosis of complete response by imaging is not always reliable and is possible merely by resected specimen. Indeed, 10-13% of patients undergoing salvage surgery were proved to have pathologically complete response (Murakami et al., 1998; M Nishimura et al., 2007; Tachimori et al., 2009). Unfortunately, positron emission tomography using 2-[fluorine-18]-fluoro-2-deoxy-D-glucose (FDG-PET) (Klaeser et al., 2009) or its combination with other imaging modalities such as computed tomography and/or endoscopic ultrasonography (Swisher et al., 2004) failed to distinguish between patients with >10% viable cells and those with <10% viable cells, resulting in a false negative rate of 16-31% by each modality (Swisher et al., 2004). In this study, the accuracy rates decreased dramatically when an attempt was made to distinguish microscopic residual disease (1% to 10% viable cell) from the “true” pathological complete response (0% viable cells), implying that these modalities have limited value for response assessment for patients receiving preoperative treatment. Consequently, patients whose tumor response is deemed complete response after CRTx could have residual diseases and not be ascribed a reason to preclude further additional treatment. To solve these drawbacks, recent research has focused on the gene expression that can predict CRTx response (Eschrich et al., 2009; He et al., 2009; Maher et al., 2009). Second, multivariate analysis revealed that the most significant factor associated with long-term survival was a R0 resection (Chao et al., 2009; Tomimaru et al., 2006). No patients left with gross or microscopic residual tumors after salvage surgery (R1/R2 resections) survived more than 24 months in any series (Chao et al., 2009; Nakamura et al., 2004; Oki et al., 2007; Swisher et al., 2002; Tachimori et al., 2009; Tomimaru et al., 2006). However, the R1/R2 resection rate has been substantially high, ranging from 15-50% (Chao et al., 2009; Nakamura et al., 2004; Oki et al., 2007; Swisher et al., 2002; Tachimori et al., 2009; Tomimaru et al., 2006), and the resection status cannot be confidently predicted before surgery or even during surgery because of the indistinct planes between tumor and fibrotic masses within the irradiated mediastinum. Therefore, FDG-PET or other imaging modalities are used to select patients who are absolutely unfit for salvage surgery. There is an urgent need for the development of more reliable, accurate diagnostic tools for the assessment of response and resection status prediction.

5. Molecular targeting therapy

Despite the many challenges for establishment of more active multimodal therapy regimens, the mean average survival benefit remains only slight in GC and EC. The MST remains consistently around or less than 12 months in metastatic GC and EC (Ishida et al., 2004; Koizumi et al., 2008; Y. Nishimura et al., 2002; Ohtsu et al., 1999), underscoring a need for more active new agents and regimens. Against these backgrounds, a new generation of therapies designed to target epidermal growth factor receptor (EGFR) and subsequent cellular responses, or angiogenic processes, which both involve and promote tumor growth and survival, have been very recently introduced.

There are several approaches to target EGFR or angiogenic processes. First is monoclonal antibodies against EGFR, including cetuximab (a chimeric monoclonal immunoglobulin G1 antibody), panitumumab (a fully human monoclonal immunoglobulin G2 antibody), and trastuzumab (a monoclonal antibody against human epidermal growth factor receptor-2 (HER2)). Second is an inhibition of the tyrosine kinase (TK) domain and subsequent signal cascade; the molecules which play the role include gefitinib, erlotinib (both are inhibitors of EGFR-TK), lapatinib (a dual inhibitor of HER2-TK and EGFR-TK), sunitinib (inhibitor of TK

of various kinds of proteins), and everolimus (RAD001) (inhibitor of mammalian target of rapamycin). Third is an inhibitor of tumor vascularization to anticipate the prevention of eventual tumor invasion and metastasis such as bevacizumab, a monoclonal antibody developed to target vascular endothelial growth factor (VEGF). There are several planned or ongoing RCTs incorporating molecular targeting therapies, and some trials have provided encouraging results.

5.1 Anti-EGFR antibody

A positive HER2 protein or amplified HER2 gene was observed in approximately 20% of GC patients (Jørgensen, 2010). An efficacy of trastuzumab for GC has been very recently demonstrated by a global RCT (ToGA trial; NCT01041404) which has revealed a significantly ($p < 0.005$) prolonged MST (13.8 months) and RR (47%) by adding trastuzumab to 5FU (or capecitabine) and cisplatin as compared with those (11.1 months and 35%) without trastuzumab (Bang et al., 2010). However, it should be noted that the RR by adding trastuzumab to CTx was at best 50% even among the HER2 positive GC patients. Furthermore, the improvement of MST (13.8 months) was -although promising- only marginal as compared with those of a CS in SPIRITS trial (Koizumi et al., 2008). Furthermore, subanalysis by region revealing the efficacy of adding trastuzumab was observed in Europe and Central/South America but not in Asia. Considering that second-line treatment was performed more in Asia than in Europe or Central/South America, the power of second-line therapy in the control (without trastuzumab) arm may bring the treatment results of the two arms closer, leading to non-significant results in Asia.

5.2 Anti-VEGF antibody

The AVAGAST trial (NCT00548548) is the first RCT investigating the efficacy of bevacizumab, in which GC patients were randomized to 5-fluorouracil (or capecitabine) and cisplatin with or without bevacizumab (Y. Kang et al., 2010). Adding bevacizumab achieved a longer PFS (6.7 months vs. 5.3 months) and higher overall RR (38% vs. 30%); however, it failed to produce significant MST prolongation (12.1 months vs. 10.1 months). Several explanations are possible for these negative results. The results appeared to differ among subgroups according to geographic region. As was seen in the ToGA trial, adding bevacizumab proved to be effective in the pan-American region and in Europe but not in Asia, reflecting the role of a second-line treatment (van Cutsem et al., 2010). Alternatively, a potential disadvantage of the AVAGAST trial is a lack of a specific target that would allow for the optimal patient selection that was possible in the ToGA trial.

6. Future perspectives

While many regimens incorporating multimodal therapies have been investigated, it is also true that there is a great variability in tumor response and patient survival among regimens. In addition, even among patients receiving the same regimen, a given regimen may prove too active or too toxic for an individual. Unfortunately, however, it is difficult to predict perfectly the efficacy and toxicity prior to therapy. Therefore, there is a pressing need to explore the molecules and genes that could help explain the interindividual differences in drug response and toxic events. Such a discovery and validation of predictive biomarkers could allow us to develop a model for selecting the optimal therapy on an individual basis

and reduce morbidity and reduce health care costs by avoiding potentially unnecessary or futile treatment, ultimately allowing treatment to be individualized (Shimoyama, 2009).

One example is a predictive role of a biomarker in the usage of anti-EGFR therapy. Since mutant K-Ras or mutant B-Raf causes cells to escape from adequately controlled cell proliferation and consequently confers resistance to anti-EGFR therapies, K-Ras (Lièvre et al., 2008) or B-Raf (Tol et al., 2009) is considered a negative predictor for the efficacy of anti-EGFR therapy such as cetuximab and panitumumab in colorectal cancer (Allegra et al., 2009; Amado et al., 2008; Jiang et al., 2009). In contrast, incidences of K-Ras mutation were 3-21% in GC (Hongyo et al., 1995; I.J. Kim et al., 2003; Lee et al., 2003; Yoo et al., 2002; W. Zhao et al., 2004) and 0-9% in EC (Janmaat et al., 2006; Lorenzen et al., 2009), both relatively low as compared with those of colorectal cancer (Andreyev et al., 1998). In addition, the predictive value of the K-Ras mutation concerning the efficacy of anti-EGFR therapy has not been clearly established in esophagogastric cancer (Park et al., 2010). Furthermore, incidences of B-Raf mutation in GC were also low (2.2-3%). Therefore, whether mutations of K-Ras or B-Raf as negative predictors seen in colorectal cancer can be extrapolated into gastric and esophageal cancers requires further study. Actually, current phase III trials of cetuximab or panitumumab in GC and EC allow the inclusion of patients irrespective of K-Ras mutation status or EGFR immunohistochemical positivity. In addition, since racial difference does exist in some drug metabolizing enzymes (Shimoyama, 2010), the usefulness of predictive biomarkers may differ between Western and Eastern hemispheres as well as between tumor types.

Another perspective is the incorporating of molecular targeting therapy or other agents into conventional therapy. As discussed in the INT0116 trial, new generation agents with radiosensitizing effects should be continuously incorporated into future clinical trials. Accordingly, several promising results have been reported by the use of new generation agents in combination with molecular targeting therapy, or with radiation, or both (Gaast et al., 2010; Knox et al., 2010; Pinto et al., 2007; Safran et al., 2008; Spigel et al., 2010; Syrigos et al., 2008). Furthermore, non-cytotoxic agents such as statins are theoretical candidates for overcoming current problems of molecular targeting therapy (Shimoyama, 2011).

It is impossible to conduct RCTs for exhaustive drug combinations. There must be continuing efforts to obtain knowledge on specific drug interactions that could bypass clinical trials from one administration schedule to another or from one tumor type to another. This stance may most efficiently facilitate the establishment of the best multimodal therapies.

7. Conclusion

In GC, recent RCTs have elucidated the promising efficacy of multimodal therapies in an adjuvant and advanced settings, where S-1 plays a pivotal role in these settings. This is in agreement with the recent stance in which oral administration takes advantage over the intravenous administration. In EC, CRTx in neoadjuvant or definitive setting has gained the most intensive research topic; however, the latter setting is inevitably associated with highly morbid salvage surgery. Furthermore, researches in novel targeted therapies against growth signal transduction cascade have just begun and their efficacy has been anticipated.

For the treatment of GC and EC, we should say “good-by” for the surgery only treatment era while the “multimodal treatment era” is welcomed. It is hugely encouraged to consider

multimodal therapies on the adjuvant, neoadjuvant, and advanced settings, as well as by the usage of conventional treatment (CTx, RTx, and CRTx) and targeted therapies, alone or in combination. Recent attempts have continuously clarified the molecular profiles or genetic events to stratify patients who receive the best benefit, which realizes maximization of the treatment effects instead of “one-regimen-fits-all” stance.

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Evidence-Based Usefulness of Physiotherapy Techniques in Breast Cancer Patients

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1. Introduction

Breast cancer is one of the most frequent causes of death among women, with high incidence in both developed and developing countries. When it is diagnosed at an advanced stage, including cases of metastatic lymph node invasion, the treatment becomes more aggressive and expensive (Johansson et al., 2002; Parkin & Fernandez, 2006), with the implication that the post-treatment complication rate will be greater (Kwan et al., 2002; Szuba et al., 2003; Goffman et al., 2004; King & Difalco, 2005).

The complications may be short or long-term, and the commonest of them include hemorrhage, infection, seroma, axillary web syndrome (AWS), chronic pain, paresthesia, reduction of the range of motion, muscle weakness in the shoulder homolateral to the surgery and lymphedema (Fig. 1). Of these, lymphedema is the most important morbid condition (Lee et al., 2008; Paskett, 2008; Fourie & Robb, 2009; Stanton et al., 2009).

Aggressive surgery, such as radical axillary dissection, may interrupt the main lymphatic drainage route in the arms, and this is the most important factor in edema formation (Bourgeois et al., 1998). Associated complementary radiotherapy induces fibrosis in lymphatic vessels in 20 to 30% of the cases, thus worsening the lymphatic flow (Paci et al., 1996; Bumpers et al., 2002; Carpentier, 2002; Kwan et al., 2002; Van Der Veen et al., 2004; Moseley et al., 2007).

This modification to the lymphatic flow induces alteration of the homeostatic equilibrium of absorption and transportation of interstitial fluid, which triggers lymphedema. This is a progressive condition characterized by four pathological factors: excess protein in tissues, edema, chronic inflammation and fibrosis. When the arm remains untreated, its volume progressively increases, as does the frequency of complications relating to this condition (Weissleder & Weissleder, 1988; Carpentier, 2002; Didem et al., 2005; King, 2006).

It is difficult to diagnose lymphedema, especially in its initial stages. Without a proper diagnosis, it always takes time to institute therapy. When the treatment is immediate, the improvement occurs quickly and progression of the condition is prevented (Szuba et al., 2003; Linnitt, 2007).



Fig. 1. Lymphedema following axillary dissection.

The pathogenesis of post-mastectomy lymphedema associated with axillary dissection is mainly attributed to greater numbers of dissected lymph nodes (Filippetti et al., 1994; Glass et al., 1999). The more extensive the axillary dissection is, the greater the risk of complications will be (Glass et al., 1999).

The desire to prevent lymphedema has led to intraoperative techniques that take a more conservative approach to the axillary chain, such as investigating the sentinel lymph node. Through this, selective, safe and less mutilating resection has become possible, with satisfactory results. Nevertheless, this is limited to patients without evidence of lymph node macrometastases (Clodius et al., 1981; Bourgeois et al., 1998; Bumpers et al., 2002; Goffman et al., 2004; Rietman et al., 2004; Ronka et al., 2004; Sakorafas et al., 2006).

When lymphedema becomes established, it is incurable. However, it can be avoided, treated and controlled with daily preventive measures (Linnitt, 2007). Studies have demonstrated that surgical and drug treatments are unsuccessful (Roucoute & Oliveira, 1999; Didem et al., 2005). The aim of physical rehabilitation is to prevent and minimize the sequelae caused by oncological treatment or by the disease itself, and to improve patients' quality of life, in both its physical and its psychological aspects.

2. Physiotherapeutic approaches

Physiotherapeutic approaches for lymphedema can be divided into two parts: prophylactic and therapeutic approaches, with the aim of preventing sequelae and improving the patient's physical condition to face up to the treatment (Bergmann et al., 2006).

2.1 Prophylactic physiotherapeutic approaches

It should be started preoperatively or in the immediate postoperative period, with the aim of preventing sequelae and improving the patient's physical condition to face up to the treatment (Bergmann et al., 2006).

The focus of this evaluation aimed to identify risk factors for developing complications and morbid conditions relating to the axillary approach and to implementation of strategies for minimizing preexisting symptoms, with the aim of achieving better postoperative functional recovery (Paskett, 2008; Springer et al., 2010).

2.1.1 Preoperative physiotherapy

Limitations on shoulder range of motion, pain and diminished muscle strength are the main focuses of the assessment, since these may lead to morbid conditions that relate directly to axillary manipulation.

In the presence of limitations on shoulder range of motion and/or loss of muscle strength, passive exercises, assisted active exercises, active exercises of the scapular belt and neck relaxation exercises are indicated (Lauridsen et al., 2005; Rezende et al., 2006; Springer et al., 2010). For patients with locoregional pain, transcutaneous electrical nerve stimulation (TENS) can be applied.

Improvement of respiratory capacity through the use of apparatus to incentivize the respiratory flow or volume (incentive spirometry) is also indicated for diminishing general morbid conditions, when present (Bergmann et al., 2006).

Although preventive interventions have shown good results, the numbers of patients referred for preoperative assessments and preventive care are still very small.

2.1.2 Immediate postoperative intervention

The second step of preventive intervention starts immediately after the surgical procedure, on the first day after the operation, with guidance about positioning in bed (with the operated arm above the head), functional exercises on the limb homolateral to the surgery as shown in Fig. 2, and respiratory physiotherapy if necessary.

Guidance regarding care for the limb that underwent manipulation, with prevention of trauma and lesions that might trigger inflammation and infection in the arm homolateral to the axillary dissection, avoidance of using clothes that restrict the superficial lymphatic circulation, skin hydration and provision of kinesiotherapy limited to 90° of range of shoulder movement are administered to patients who undergo radial axillary dissection and sentinel lymph node biopsy, before hospital discharge (Roucout & Oliveira, 1999; Andersen et al., 2000; Camargo & Marx, 2000; Huit, 2000; Bergmann et al., 2006).

Additional guidance regarding early lymphatic self-massage should also be provided for patients after axillary dissection (Sarri et al., 2010).

2.2 Postoperative follow-up

A new physiotherapeutic reassessment is undertaken immediately after removal of the patient's surgical stitches and suction drain, focusing on reorientation regarding preventive measures against lymphedema and assessment of the need for early physiotherapeutic intervention, before the start of radiotherapy.

Physiotherapeutic treatment concomitant to radiotherapy is very important, because it minimizes the side effects, such as subcutaneous fibrosis, limitations on shoulder range of motion, muscle weakness and pains (Lee et al., 2008).

In outpatient rehabilitation, in addition to individualized attendance, it is also undertaken in collective groups. This has the benefit of promoting interaction among the patients, with exchanges of experiences, thus making the session more agreeable and providing encouragement towards doing the exercises (Fig. 3). Patients should be advised to continue with the treatment at home.



Fig. 2. Postoperative functional exercises.

In the following, we will describe the main physiotherapeutic interventions relating to specific symptoms of complications following surgical manipulation of the axillae.



Fig. 3. Group of mastectomized patients.

3. Lymphedema

Early diagnosis and intervention, such as skincare (Kwan et al., 2002; Williams et al., 2002), kinesiotherapy and self-massage (Glass et al., 1999; Williams et al., 2002; Rietman et al., 2004), may significantly reduce the incidence of complications (Williams et al., 2002). The search for better quality of life has indicated that prevention of lymphedema is the best strategy among this group of patients. Prior knowledge of the normal lymphatic circulation and the changes that it undergoes in the presence of obstruction directs the techniques for physiotherapeutic stimulation. Several lymphatic drainage techniques are used to stimulate the lymphatic flow and treat lymphedema, such as the techniques proposed by Vodder, Leduc and Földi. Recently, we studied 22 women with breast carcinoma who underwent surgical treatment and axillary lymph node dissection. We performed early homolateral inguinal and contralateral axillary lymph node stimulation for up to 60 days after the operation, thereby simulating self-massage according to the Földi technique, and used lymphoscintigraphy to identify the immediate improvement in lymphatic flow following the stimulation (Sarri et al., 2010).

The main objective of manual lymphatic drainage is to increase lymphokinetic activity in healthy areas, before stimulating the edematous areas. There are several physiological effects, which include increased contraction of the lymphatic vessels and increased protein reabsorption, thereby reducing the microlymphatic hypertension, increasing the collateral lymphatic drainage among the lymphatic areas of the skin and improving the drainage capacity in order to direct the lymph away from the edematous area and towards lymph nodes in areas that are unaffected by lymphedema (Fritsch & Tomson, 1991; Araujo et al., 1997; Andersen et al., 2000; Huit, 2000; Williams et al., 2002; Moseley et al., 2007). As a strategy for stimulating the lymphatic circulation in cases of obstruction of the normal lymphatic flow, the collateral routes and anastomoses of the lymphatic capillaries should be taken to be the peripheral circulation. These routes deviate the lymph flow in a direction contrary to the usual flow, through the lateral cephalic lymphatic bundle and continuing over the deltoid muscle, thereby bypassing the axillary lymph node chain and draining the lymph directly to the supra and infraclavicular lymph nodes (Stanton et al., 2009; Sarri et al., 2010). Within this context, physiotherapy to treat lymphedema that has already become established and to prevent this and other comorbidities is of paramount importance for diminishing the surgical sequelae from breast cancer treatment.

3.1 Manual lymphatic drainage

The treatment for lymphedema that has already become established is based on techniques that are well accepted and described in the worldwide literature. In Brazil, treatment also known as lymph therapy or complex physical treatment is the type most used. Complete decongestive physiotherapy (CDP) is composed of four approaches: manual lymphatic drainage (MLD), compressive bandaging, skincare and lymph myokinetic exercises (Bergmann et al., 2006; Leal et al., 2009).

Lymph therapy is carried out in two phases. The first has therapeutic aims and the second consists of maintenance. The therapeutic phase aims to mobilize the accumulated protein-rich fluid and reduce the fibrosclerotic tissue, using MLD and compressive bandaging (Foldi, 1998; Camargo & Marx, 2000; Leal et al., 2009). MLD can be carried out in regions with or without edema, depending on the aim at the time of treatment. It is done in two stages: evacuation and uptake.

The evacuation maneuvers begin with a series of gentle circular movements with the therapist's palms in contact with the patient's skin. Firstly, the contralateral axillary lymph node chain is stimulated and then the inguinal region homolateral to the surgical manipulation. After these areas have been stimulated, wavelike movements are made across the anterior region of the chest, towards areas adjacent to the axilla that underwent the operation, thus stimulating first the contralateral quadrant until reaching the ipsilateral quadrant, and then making the same movement going from the ipsilateral inguinal region to the axilla, as shown in Fig. 4. These movements can be carried out both in the anterior and in the posterior region of the trunk.

After finishing the evacuation phase, the uptake process is started. This is always performed from proximal to distal regions, with wavelike maneuvers in the upper arm region and then the forearm and hand, until reaching the fingers (Fig. 5) (Foldi et al., 1985; Camargo & Marx, 2000; Williams et al., 2002; Linnitt, 2005).

The direction of lymphatic drainage should respect the anatomy of the lymphatic system, and it is very important to take into consideration the deviation in the region of the deltoid muscle (Stanton et al., 2009; Sarri et al., 2010).

After completing the manual lymphatic drainage, the arms should be hydrated using neutral cream so that compressive bandaging can be started.

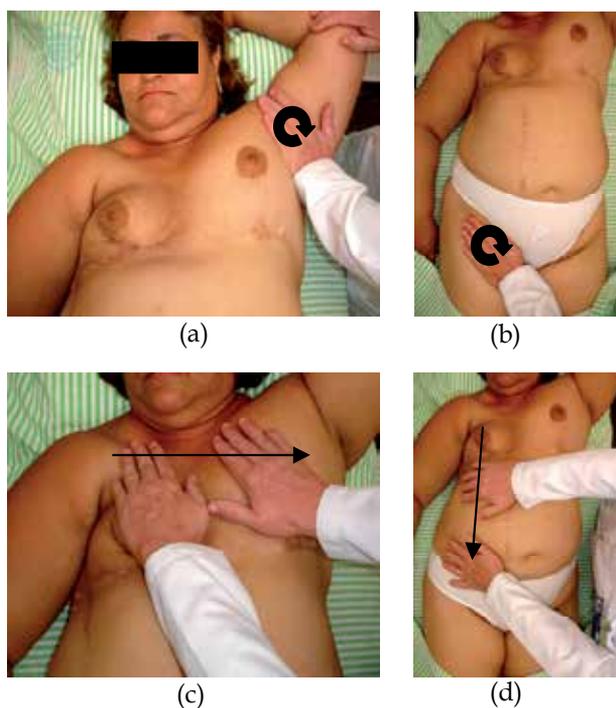


Fig. 4. Evacuation maneuvers on a patient who underwent radical right-side lymphadenectomy: a) axillary lymph node chain stimulation; b) inguinal lymph node chain stimulation; c) wavelike maneuver directing the lymphatic flow towards the contralateral axillary region; d) wavelike maneuver directing the lymphatic flow towards the ipsilateral inguinal region.

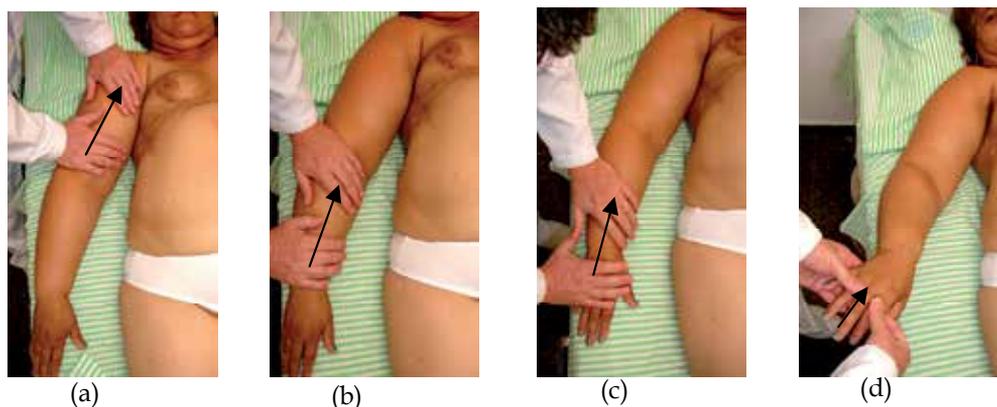


Fig. 5. Uptake maneuver on a patient who underwent radical right-side lymphadenectomy: wavelike maneuvers directing the lymphatic flow from the proximal to the distal region: a) upper-arm region; b) forearm region; c) hand region; d) finger region.

3.2 Compressive bandaging

One of the consequences of lymphedema is that the elastic fibers are destroyed. Evacuation of the lymphatic fluid by means of manual lymphatic drainage diminishes the pressure on the tissue and increases the effective ultrafiltration pressure. Elastic bandages increase the tissue pressure and counterbalance the elastic insufficiency, thereby avoiding recurrence of lymphatic fluid accumulation in the interstices (Foldi et al., 1985).

Compressive bandaging is an important technique, because it boosts the effects achieved through the preceding lymph drainage. It should always be functional, thereby enabling all day-to-day movements and guided kinesiotherapy (Fig. 6).



Fig. 6. Functional compressive bandaging, such that the patient was able to make movements.

After hydration, the skin and bone prominences should be protected using cotton gauze and foam, with the aim of filling in any anatomical spaces. The compressive bandaging is done using low-elasticity bandage rolls. It is started on the fingers and then the forearm and the upper arm. The pressure exerted by the bandaging is greater in the distal region and diminishes towards the root of the limb (Fig. 7). It is also important to respect the trophic conditions of the skin. The compressive bandaging is kept in place until the next physiotherapy session, i.e. for two days.



Fig. 7. Functional compressive bandaging: a) hydration; b) placement of cotton gauze; c) protection with cotton gauze; e) bandaging, starting with the fingers; f) bandaging on forearm; g) bandaging on upper arm.

The second phase, called the maintenance phase, has the aims of keeping the interstitial pressures in balance and optimizing the results obtained from the first phase of the treatment. It consists of using compression garments, skincare, hydration, kinesiotherapy and manual self-massage (Foldi et al., 1985; Camargo & Marx, 2000; Bergmann et al.; Leal et al., 2009; Sarri et al., 2010).

3.3 Compression garments

The aim of using compression garments is to maintain and optimize the results obtained from the first phase of lymphedema treatment and avoid recurrences, through keeping the interstitial pressures in balance. They should be used continuously and only be removed for personal hygiene. The model of garment and the compression used depend on the patient's needs (Fig. 8). Concomitantly with using the compression garments, the patient should also comply with the guidance previously given regarding limb care, hydration and kinesiotherapy. (Foldi et al., 1985; Roucout & Oliveira, 1999; Andersen et al., 2000; Camargo & Marx, 2000; Huit, 2000; Hampton, 2003). In our institution, we use compression garments in conjunction with self-massage and kinesiotherapy in cases of initial lymphedema, with significant improvements achieved.

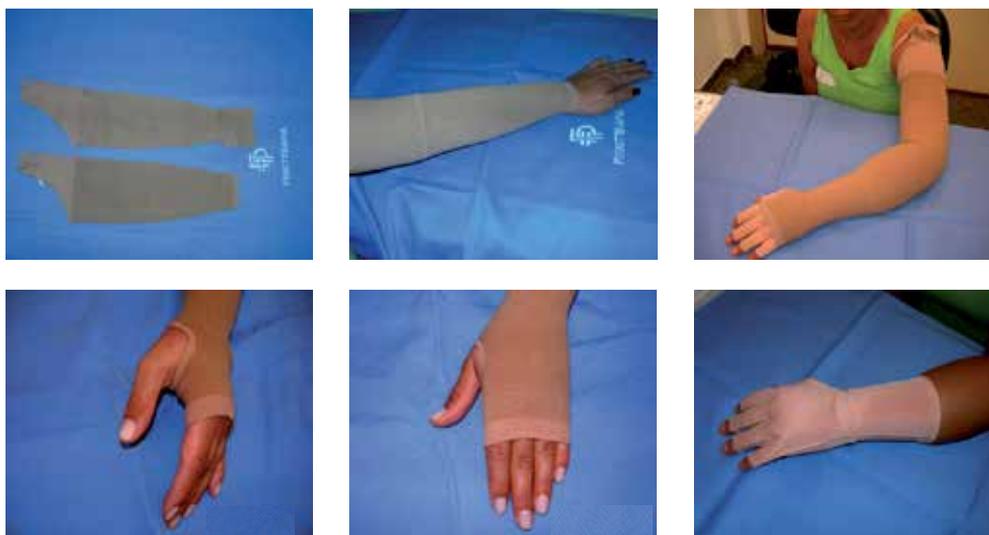


Fig. 8. Garments and gloves for elastic containment.

3.4 Lymphatic self-massage

Lymphatic self-massage, also known as simple manual lymphatic drainage, is a version of manual lymphatic drainage. The technique for this procedure is taught to the patient, who can then perform this alone, at home every day. It involves a series of gentle circular movements that begin with stimulation of the contralateral axillary lymph node chains and the inguinal chains homolateral to the surgical manipulation, followed by gentle movements starting at a place distant from the congested area and moving towards the edematous limb (Foldi et al., 1985; Camargo & Marx, 2000; Williams et al., 2002; Linnitt, 2005).

The patient should begin the self-massage with circular movements in the contralateral axillary region, with the palms in the axilla, lightly and gently moving the skin 30 times, and

then repeating this in the inguinal region homolateral to the axillary dissection. After the axillary and inguinal lymph node chains have been stimulated, hand movements are made with the aim of shifting the lymph from the operated axilla to the contralateral axilla, in the region above the surgery. The same movement is then made to shift the lymph from the manipulated axilla to the homolateral inguinal region. The movement in each region is repeated 30 times (Fig. 9).

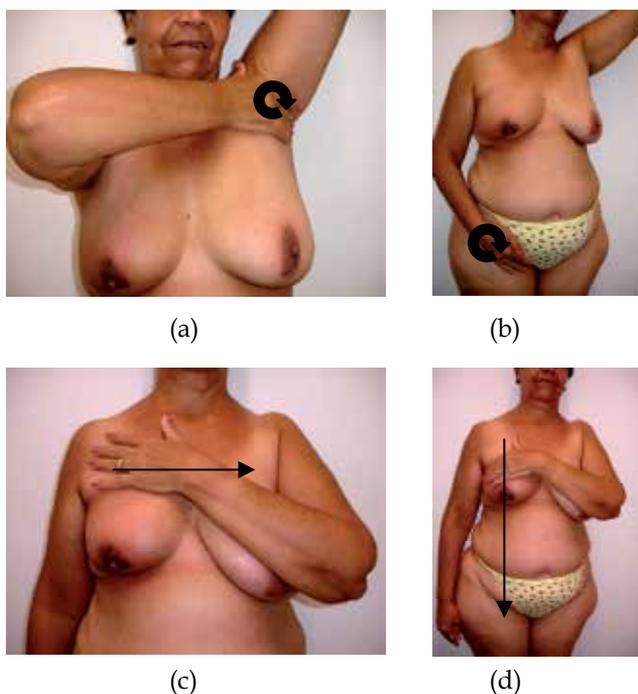


Fig. 9. Lymphatic self-massage on patient who underwent right axillary dissection: a) axillary lymph node chain stimulation; b) inguinal lymph node chain stimulation; c) wavelike maneuver directing the lymphatic flow towards the contralateral axillary region; d) wavelike maneuver directing the lymphatic flow towards the ipsilateral inguinal region.

3.5 Pneumatic compression

This consists of mechanical compression with air pumps, on the edematous limb. There are basically two types of compression pumps: the segmental or sequential type and the dynamic type. Individual use of pneumatic compression has not shown satisfactory results, and high incidence of complications has been found. Several studies have combined its use with other types of treatment for lymphedema, such as the use of lymph therapy (Dini et al., 1998; Leduc et al., 1998; Camargo & Marx, 2000; Szuba et al., 2002; Leal et al., 2009).

4. Axillary web syndrome

Axillary web syndrome, also known as cording (Box et al., 2002), axillary strings (Lauridsen et al., 2005), or vascular strings (Johansson et al., 2001), is a sequela of breast cancer

treatment (Fourie & Robb, 2009). It is developed between the first and fifth week after axillary dissection, as tense and painful strings under the skin of the axilla. It may extend as far as the cubital fossa or the medial face of the upper arm (Tilley et al., 2009). It is always associated with pain and limitation of shoulder and elbow movement (Lauridsen et al., 2005). Its incidence and predisposing factors are not well defined in the worldwide literature (Moskovitz et al., 2001). It is believed that interruption of the axillar lymphatic vessels has an important role in the development of this syndrome. Lymphovenous lesions with stasis, thrombophlebitis, aseptic lymphangitis and lesions in the lymphatic ducts also seem to be involved (Johansson et al., 2001; Moskovitz et al., 2001; Lauridsen et al., 2005). The literature is deficient with regard to the approach to be taken in cases of AWS. Some papers have shown that this syndrome resolves within three months, without specific treatment (Moskovitz et al., 2001; Leidenius et al., 2003). Other studies have shown benefits from implementing active range-of-motion exercises, stretching exercises and manual manipulation techniques (Moskovitz et al., 2001; Leidenius et al., 2003; Fourie & Robb, 2009; Tilley et al., 2009).

In our service, we have used string stretching with very favorable results. The maneuver consists of stretching the string with the thumbs, while applying pressure from central to distal regions, as shown in Fig. 10. This maneuver triggers tolerable local pain that is relieved as soon as the maneuver ends, thereby enabling movements that had previously been limited (Fig. 11). We have also used active and passive kinesiotherapy on the limb and light stretching.



Fig. 10. Axillary Web Syndrome maneuver: a) string in anterior region of elbow; b) axillary web syndrome maneuver; c) anterior region of forearm after maneuver, without string.

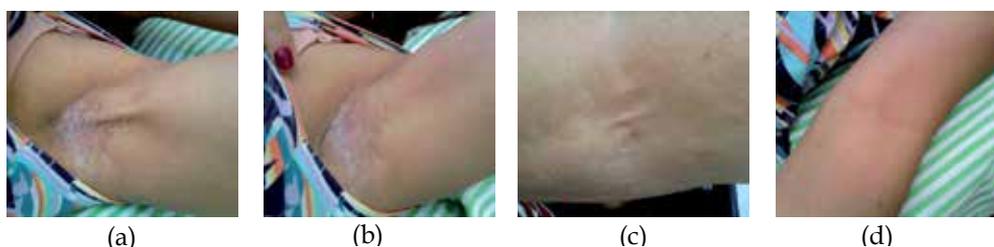


Fig. 11. Axillary Web Syndrome: a) axillary region before the maneuver; b) axillary region after the maneuver; c) anterior region of the elbow before the maneuver; d) anterior region of the elbow after the maneuver.

5. Limitation of shoulder movement

Restriction of shoulder movement, which is one of the complications following axillary lymph node dissection, may occur because of tissue and nerve lesions, with prevalence of 7-36%. This musculoskeletal disorder of the shoulder results in considerable joint debility and pain. The symptoms generally diminish within three months, but they may become chronic, thus interfering with these patients' quality of life (Kärki et al., 2001; Beurskens et al., 2007). Early physiotherapeutic treatment is effective for this disorder and promotes faster functional recovery (Kärki et al., 2001; Lauridsen et al., 2005; Beurskens et al., 2007). The treatment should be progressive, taking care in manipulating the limb so as to avoid tissue or muscle injuries. Passive exercises, assisted active exercises, active exercises of the scapular belt, neck relaxation and postural guidance should be undertaken.

6. Painful post-mastectomy syndrome

Chronic pain secondary to surgical treatment for breast cancer is of neuropathic origin or results from muscle and ligament injuries. Intercostal brachial neuralgia has been correlated most frequently in axillary treatments, in which the nerve may become injured because of its proximity (Poleshuck et al., 2006; Labreze et al., 2007; Couceiro et al., 2009). Such pain may be continuous or intermittent, with varying intensity, and it can be located in the anterior wall of the chest, the axilla or the medial face of the upper arm (Vecht et al., 1989; Caffo et al., 2003; Burckhardt & Jones, 2005). The physiotherapeutic treatment consists of specific analgesia techniques, such as transcutaneous electrical nerve stimulation (TENS), cryotherapy and kinesiotherapy. It can also be implemented in association with the use of analgesic medications.

7. Conclusion

In conclusion, physiotherapeutic interventions on patients who have undergone axillary lymph node manipulation can be implemented at any postoperative stage. However, it is increasingly certain that early intervention significantly minimizes the emergence of lymphedema.

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Negative Impact of Paclitaxel Crystallization on Hydrogels and Novel Approaches for Anticancer Drug Delivery Systems

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1. Introduction

Paclitaxel, better known as Taxol® its original name and now trade mark, and its direct derivatives, has been one of the most effective drugs used against tumors and cancer (Rowinsky et al., 1990; Rowinsky & Donehower, 1995) due to its ability to stabilize microtubules in the mitotic spindle and thus stopping the cell cycle in eukaryotic cells (Wani et al., 1971). The stabilization of microtubules is due to the specific bond between Taxol® and beta tubulin preventing microtubule depolymerization (Schiff et al., 1979). It has also been reported that Taxol® inhibits the process of angiogenesis (Wang et al., 2003). These Taxol® qualities have been taken as an advantage for the usage of this drug in killing carcinogenic cells in tumors.

One of the main drawbacks of this molecule is its high hydrophobicity; making it practically insoluble in water which hinders their use in treatments inside the human body. In order to overcome such inconveniences, several strategies have been developed such as the use of adjuvants like Cremofor/Etanol (CrEL), solubilizant agent that facilitates the intravenously administration of the drug, process termed as chemotherapy. This technique has brought many disadvantages such as the fact of reducing considerably the drug's diffusion capacity, making that a limited quantity of the drug be reached into the target site (Marupudi et al., 2007), and therefore limiting the concentration level needed to eliminate tumors. To solve these inconveniences it has been resolved to increase the delivery cycles. The usage of Taxol® in CrEL and the high doses have produced undesired consequences with a numerous of hypersensitivity reactions and side effects including nausea, vomiting, urticaria, abdominal pain, diaphoresis, and other (Weiss et al., 1990; Wiernik et al., 1987). Looking for minimizing such secondary reactions, new options in the field of drug delivery are being explored. In the last decade a variety of drug delivery systems have been proposed as carriers of hydrophobic anticancer drugs. The list of these systems includes

drug loaded hydrogels, drug loaded nanoparticles, functionalized nanoparticles and some combined systems. Through this chapter we review the current trends for the transport and delivery of the most common anticancer drugs. Based in facts we point a delicate issue, Taxol crystallization in hydrogels and its negative impact, and finally we make a discussion about novel approaches for drug delivery against cancer.

2. Hydrogels for drug delivery systems

Currently the development of smart hydrogels that can respond to external stimuli such as variations in temperature, pH, and electric fields or hydrogels with controlled biodegradability has been used in biomedical applications. As such hydrogels are used in a wide range of applications including tissue engineering and regenerative medicine (Lee & Mooney, 2001), diagnosis (Van Der Linden et al., 2003), cell immobilization (Jen et al., 1996), bimolecular and cell separation (Wang et al., 1993), barriers to regulate the biological adhesion (Bennett et al., 2003). Hydrogels can be made, in theory, from any water-soluble polymer, encompassing a wide range of chemical compositions and physical properties. The polymer forms aggregates that form a three-dimensional matrix with interconnected pores in which the solvent (usually water or aqueous solutions) and other particles can diffuse (Figure 1). Some hydrogels have a high capacity to contain compounds that can be released in a controlled manner for therapeutic purposes. Its porosity allows the loading of drugs within the polymer matrix and their subsequent release at a rate that depends on the coefficient of diffusion of drug through the gel matrix in the case of hydrophilic drugs. For hydrophobic drugs, the rate of release depends on the rate of degradation of the gel. Besides their biodegradability can be designed to be via enzymatic, hydrolytic, or environmental (pH, temperature or electric fields).

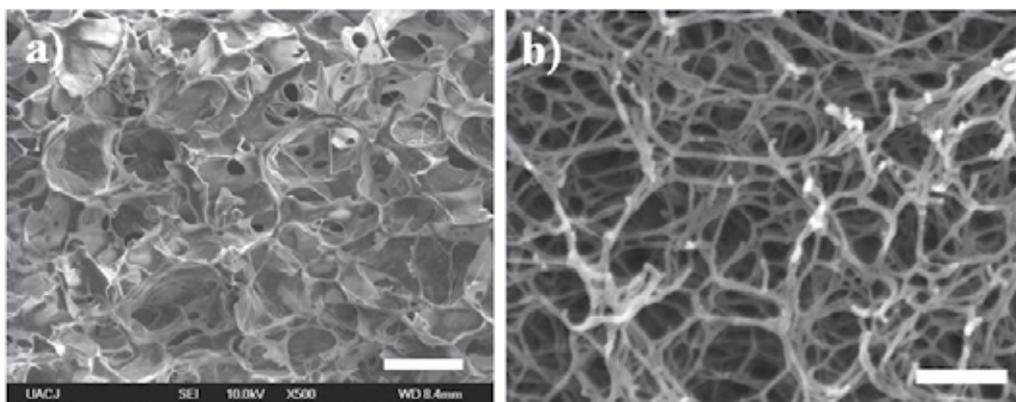


Fig. 1. Hydrogel structures observed by FESEM (Field Emission Scanning Electron Microscope). a) Chitosan-Glycerophosphate thermosensitive hydrogel. Bar 40 μ m. b) Agarose hydrogel. Bar 250 nm.

2.1 Thermosensitive hydrogels for anticancer drug delivery

Commonly hydrogels gelatinize when their temperature drops; in other words they are present in a liquid state at higher temperatures, and they solidify when it drops. However,

there are hydrogels that exhibit the phenomenon of reverse thermal gelation, also known as thermally induced hydrogels. In this case they are liquid at lower temperatures, but they solidify when their temperature rises (Klouda & Mikos, 2008). This property has been exploited to create hydrogels that remain liquid at temperatures below 30 ° C, but they solidify at temperatures close to physiological temperature (37 °C). Therefore, they are better injected subcutaneously as a liquid and solidify when they reach body temperature. Some of these hydrogels are tri-block copolymer synthesized by open-ring polymerization such as poly (ethylene glycol)-poly(epsilon-caprolactone)-poly (ethylene glycol) (PEG-PCL-PEG) (ChangYang et al., 2009; Yu, 2009), tri-block copolymer poly(ethylene oxide)-poly (oxidopropileno) -poly (ethylene oxide) (PEO-PPO-PEO) (Li & Li, 2008), and biopolymer chitosan (C) neutralized with b-glycerophosphate (GP) (Ruel-Gariépy et al., 2004). Most of these hydrogels are studied for drug delivery and some have already been used successfully for this purpose. For example, PEO-PPO-PEO-name Poloxamer 407 has been used to prolong the release of lidocaine (Chen et al., 2004). Also, some natural polymers exhibit the phenomenon of reverse thermal gelation such as those based on chitosan, which have reported positive results; for instance, a solution of chitosan with 40% weight of PEG has been tested on the release of serum bovine holding a sustained release of at least 70 hours (Bhattarai et al., 2005). Other organic hydrogels with this property are hydroxypropyl cellulose (Cai et al., 2003; Uraki et al., 2004) and methyl cellulose (Kumar et al., 1993).

Among the new methods aimed to replace intravenous chemotherapy for the treatment of tumors there are certain proposals which emphasize the use of temperature-sensitive hydrogels loaded with anticancer drugs. They suggest the injection of the thermosensitive hydrogels containing drug directly into the affected area (tumor), due to its reverse thermal behavior it solidifies inside the body and then the drug is released gradually. The most common proposed systems for Taxol® delivery have been those that offer direct incorporation of the drug into the thermosensitive hydrogel (Chun et al., 2009; Dai et al., 2006; Kasala et al., 2008; Livnat et al., 2005; Marupudi et al., 2007; Ruel-Gariépy et al., 2004; Shi & Burt, 2004; Woo Sun et al., 2007). Just to mention some examples, the alginate hydrogel with polyethylene glycol (PEG) (Livnat et al., 2005), the hydrogel Dx-g-PCL result of the synthesis of polycaprolactone (PLC) and Dextran (Dextran70 and Dextran 500) (Shi & Burt, 2004), so as the thermosensitive chitosan-based hydrogel BST-Gel liquid (Marupudi et al., 2007).

2.2 Paclitaxel crystallization in hydrogels

We have studied Taxol® crystallization in aqueous solutions and hydrogels (Castro et al., 2010). In both environments Taxol® forms needle-like crystals that grow concentrically forming complex morphologies such as spherulites, in the case of heterogeneous nucleation, or sheaves (axialites) when they are formed by homogeneous nucleation. In solution or gel these crystals can be observed by DIC (Differential Interference Contrast) microscopy or by fluorescent microscopy when they are labeled with a fluorescent dye such as rhodamine, as described in (Castro et al. 2009). Dried they can be analyzed by scanning electron microscopy (Figure 2). solution by heterogeneous nucleation with a Taxol concentration of 50 µM. Sample was prepared by drying a drop in a TEM (Transmission Electron Microscope) cooper grid and then metalized by sputter coating with a gold-palladium target. a) Bar 2.5 µm, b) bar 10 µm.

The process of crystallization in aqueous solutions and hydrogels is quite similar and we can describe it in few words, Taxol crystallization follows the classical homogeneous nucleation theory. For low supersaturation (low Taxol concentrations), only a small number of crystals nuclei may form whereas for high supersaturation ($>20 \mu\text{M}$) the number of nuclei increases drastically with increasing Taxol® concentration.

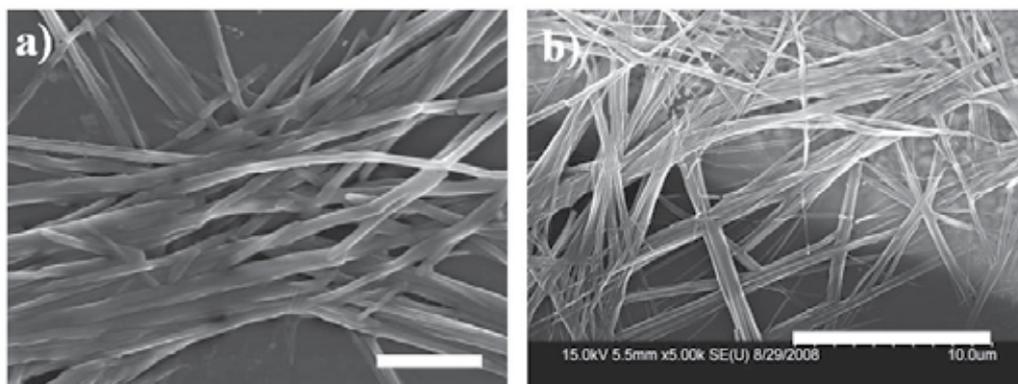


Fig. 2. Taxol crystals observed by FESEM. These crystals were obtained in aqueous solution.

As we mentioned before, the most common Hydrogel-based Taxol® delivery systems reported in literature suggest the direct incorporation of the drug into the thermosensitive hydrogel (Chun et al., 2009; Dai et al., 2006; Kasala et al., 2008; Livnat et al., 2005; Marupudi et al., 2007; Ruel-Gariépy et al., 2004; Shi & Burt, 2004; Woo Sun et al., 2007). But nobody, except Shi & Burt (2004), has reported Taxol crystallization in their systems. We claim that this phenomenon is present in most of these works, unfortunately this problem has been a neglected topic by many researchers and may go unnoticed because Taxol® crystals are not detectable by commonly used techniques for the characterization of the hydrogels. Perhaps, researchers have been concerned primarily in designing a good delivery system, neglecting other aspects of the drug, such as its crystallization. In studies reported in the literature that follow this line, no one takes into account, neither they mentioned nor reported studies of crystallization of Taxol® in hydrogels, despite that the use of the drug concentrations is greatly exceeded in its limit of solubility in aqueous solutions ($0.77 \mu\text{M}$) (Shi & Burt 2004).

In order to provide evidence of Taxol crystallization in these hydrogels we have performed several experiments following similar conditions to that reported in the papers mentioned before. For instance we used similar hydrogels such as agarose, chitosan, poly (L-lactic acid) PLLA and thermosensitive Chitosan-Glycerophosphate and we also used Taxol concentrations similar, or even lower, to that reported in that works. As result we have found that Taxol® crystallization is always present in hydrogels and thermosensitive hydrogels (Figure 3). According to our observations Taxol® crystallization follows the behavior of the classical homogenous nucleation theory, as mentioned before. That means that at low Taxol concentrations ($<30 \mu\text{M}$) few but big ($15\text{-}25 \mu\text{m}$) axialites are present, while at higher many and smaller axialites are observed (Castro et al., 2011). Moreover paclitaxel crystals are very stable in aqueous environments. We have observed these crystals up to two months of formation without any noticeable change. Hence we can speculate that Taxol crystals inside the body probably would last for long time activating immune reactions.

Direct incorporation of paclitaxel into hydrogels can lead to crystallization of the drug which can dramatically decrease the therapeutic effects and effectiveness of paclitaxel delivery systems. Supporting our theory, we find in the work of Chun (2009) who reported that *in vivo* experiments with rats, contrary to what would be expected, the hydrogel with a lower concentration of Taxol® was more effective at tumor inhibition than hydrogel with the highest concentration. Although the authors did not provide explanations for such unexpected results, for us a good reason of this fact is the phenomenon of Taxol® crystallization, which is enhanced at higher concentrations. All this experimental evidence could be enough reason to believe that these systems do not meet the desired expectations and require major rethinking.

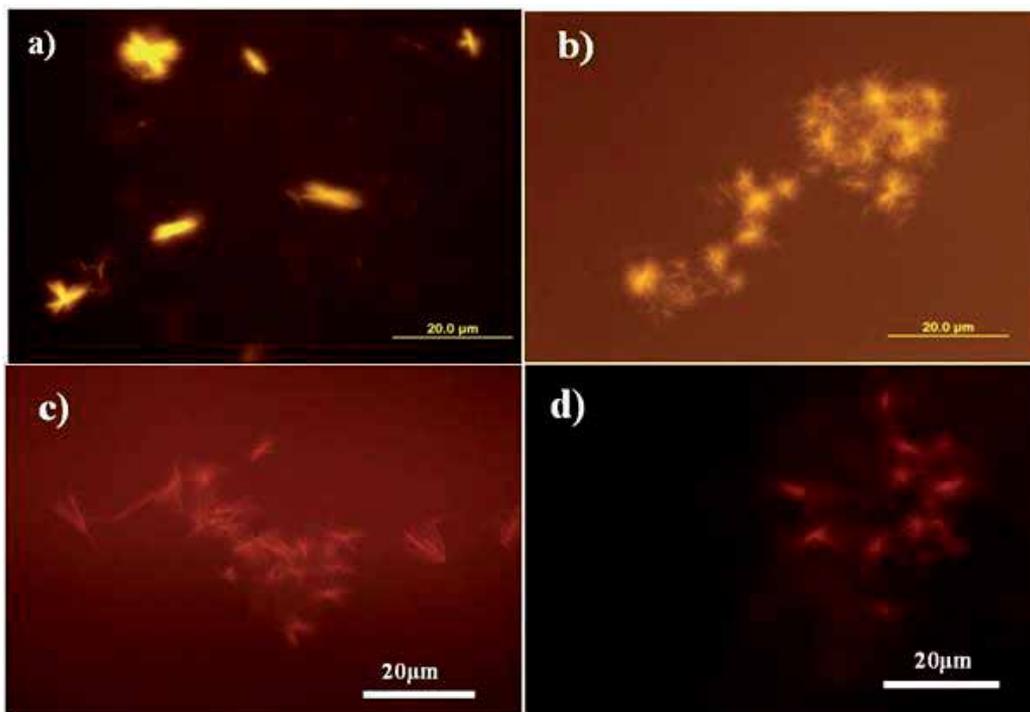


Fig. 3. Taxol crystals formed in hydrogels. a) in aqueous solution, b) in agarose hydrogel, c) in poly (L-lactic acid) hydrogel, d) Chitosan-Glycerophosphate hydrogel. All samples prepared at a paclitaxel concentration of 100 μ M.

3. Nanoparticles for drug delivery

In order to minimize the inconvenient of the hydrophobicity many authors have proposed the encapsulation of hydrophobic drugs in nanoparticles, this would allow increasing the amount of drug administered without using nasty solvents such as CrEL in the case of paclitaxel. Thus it is being explored new types of delivery systems based in nanocarriers such as micelles (You et al., 2008), nanoparticles (Liang et al., 2006), thin films (Shi & Burt, 2004), and microspheres (Liu et al., 2007) among others (Ferrari & Downing, 2005), whose effectiveness is related to its size, modification of pharmacokinetics, biodistribution,

delivery control and toxicity reduction (Jun et al., 2008). Each one of these particles has unique characteristics that should be carefully analyzed taking into account the type of drug and the desired interaction to obtain the adequate system delivery. The liposome usage for delivery agent of the anticancer drugs is very broad, such as Doxil®, this drug is commercially distributed and used in several ovarian cancer treatments (Gordon et al., 2001). However, the liposomes present several disadvantages due to their high permeability and stability *in vivo* (Conlin et al., 2009). Dendrimers are macromolecules characterized by their structural monodispersivity and symmetry. They consist of a central nucleus and its diverse ramifications. It contains different functional groups generally located on the exterior, which play an important role in their properties (Caminade et al., 2005). A disadvantage is its difficulty to be synthesized (Tomalia et al., 1990). The dendrimers are used in research studies for the prevention of sexually transmitted diseases (Zolnik & Sadrieh, 2009), as contrasting agents in Magnetic Resonance Imaging (Zolnik & Sadrieh, 2009), combined with anticancer drugs (Malik et al., 1999), among other usages. The nanocapsules are vesicular systems with colloidal size, in which the drug is located inside and surrounded by a polymeric membrane (Soppimath et al., 2001). In this system, the nucleus consists of oily liquid and a simple polymeric layer covers it, this system has shown to be effective in encapsulating and releasing certain types of hydrophobic drugs (Ameller et al., 2003). Nanospheres are solid colloidal particles in which the drug can be dissolved, encapsulated, conjugated or absorbed (Gref et al., 1995; Soppimath et al., 2001). They are generally larger than micelles and although its elimination is slow due to its hydrophobicity, are susceptible to renal filtration and the mono nuclear phagocyte system (MPS), It is therefore necessary to modify its surface (Letchford & Burt, 2007). Good stability has been achieved in nanospheres using amphiphilic copolymers (Gref et al., 1995), so it is possible the encapsulation of the bioactive molecule at the core of the nanosphere (Yanasarn et al., 2009), for example, it has been observed that the nanoparticles synthesized from emulsion of lecithin / water loaded with paclitaxel are more effective in attacking cancer cells than pure paclitaxel (Hu et al., 2009). In another study, gold nanoparticles encapsulated in nanospheres of chitosan and poly (acrylic acid) CS-PAA-Au have been used as delivery system drugs as well as for observation of cells (Latere et al., 2002).

Within this range of particles could not miss the micelles, which have had a great interest in the issue of drug delivery and location into tumors (Lee et al., 2003; Yokoyama et al., 1999). Micelles are amphiphilic molecules consisting of hydrophilic and hydrophobic segments. Its outer layer, which is highly hydrated, provides stability in aqueous environments, while the hydrophobic core allows the incorporation of water-insoluble drugs. Moreover, if the polymeric micelles have a diameter ranging from 10 to 100nm allows long circulation in the bloodstream avoiding defenses of the mono nuclear phagocyte system and suppressing renal clearance. They can be large enough to avoid being excreted by the kidneys but small enough to pass the interendothelial cell filtration (Yang et al., 2009).

As a result, drug activity continues after a single application over a long period of time. In addition, these micelles remain intact at levels below the critical micelle concentration (CMC), so they retain their structure and preferentially accumulate in solid tumors via the permeability and retention effect (EPR) (Nakayama et al., 2007; Saez et al., 2004). Its amphiphilic nature gives them a central place in the area of the release of hydrophobic drugs, due to its excellent drug storage capacity in its core.

The hydrophobic core also has other important features such as protecting the drug from being deactivated by enzymes or other bioactive species from the aqueous medium (blood fluid) (Wilhelm et al., 1991; Yokoyama et al., 1990). Also, this medium affects the rate of drug release, in many cases declining it, as the release rate is controlled both by the stability of the micelles as the hydrophobicity of their core and the chemical species used to attach the drug to the polymer backbone. These factors may be independent of the properties of the drug.

While each system has its advantages and disadvantages, micelles and nanospheres formed by amphiphilic copolymers have greater flexibility in their synthesis and uses, and they have had good results with hydrophobic drugs. The difference lies in the method of preparation and length of the hydrophobic segment. Although for some authors the nanospheres are systems that perform *in vivo* by presenting better retention properties of the drug, they have a burst effect when the drug is readily released; in this sense, the nanospheres are overcome by the micelles who exhibit more stable behaviors (Gaucher et al., 2010; Kwon, 2003). We can mention some examples of micelles made of different polymers used to carry drugs. We cite a few micelles synthesized from copolymer PEG-PASP-DMEDA loaded with ammonium glycyrrhizate (AMG) poorly water soluble drug used against Hepatitis C (Yang et al., 2009). Copolymer polylactide / polyethylene glycol is synthesized by polymerization of ring-opened was used to encapsulate paclitaxel without the presence of an organic solvent (Kim et al., 2009). Also, some micelles are sensitive to changes in pH, this feature can be used to control drug release as self-assembled micelles of copolymer PEG-*b*-PMA with divalent metals loaded with doxorubicin (Li et al., 2009). Tamoxifen and paclitaxel have also been charged in micelles with a hydrophilic block of poly [2 - (ethyl methacryloyloxy) phosphorylcholine] (MPC), hydrophobic block and a pH sensitive poly [2 - (diisopropylamine)ethyl methacrylate] (DPA) (FA-MPC-DPA) (Licciardi et al., 2008). Doxorubicin was loaded using thermosensitive blocks such as poly (N-isopropylacrylamide-co-N, N-dimethyl-acrylamide) in which the hydrophobic part was synthesized from poly (D, L-lactide), poly(epsilon-caprolactone) or poly (D, L-lactide-co-epsilon-caprolactone) (Masamichi et al., 2006). Another type of thermosensitive micelles have been synthesized from poly (N-isopropylacrylamide) and poly (butyl methacrylate) also loaded with doxorubicin (Chung et al., 1999). Similarly, micelles have been developed and modified in the copolymer as synthesized from poly (ethylene glycol)-poly (aspartate ester groups heptyl, nonyl, phenyl propyl benzyl and for greater stabilization of the drug N-(4 - hydroxifenil) retinamide (Tomoyuki et al., 2008).

Encapsulation of paclitaxel in nanoparticles is very promising and results have been favorable, judging by the reports in the literature. However, most authors suggest the use of nanoparticles to be injected into the bloodstream, following the route of traditional chemotherapy, which somehow still has the disadvantage of low specificity in which the problem is attacked. But that's not all, perhaps the main problem is not the lack of specificity of the method, but the risk involved in the use of nanoparticles in the bloodstream. Currently, no one knows for sure the adverse effects of the use of nanoparticles in the bloodstream, since its size could pass biological membranes or barriers and it has unknown effects on areas of the body, becoming a health risk factor, as noted by experts on the subject of the risks of nanotechnology in medicine (Scott et al., 2008). To this, we must add that the nanoparticles contain a highly cytotoxic drug.

3.1 Functionalization of nanoparticles

Particle functionalization with specific ligands for cancer cells has gained considerable attention for those looking for more specificity in their systems carrying the drug. Work is being currently done to prove their effectiveness with different ligand-receptor systems in a variety of cancer cell lines to test the efficiency of the functionalization of these systems. The effectiveness of the functionalization of nanoparticles lies in the premise of the existence of certain molecules in tumors are different from those found in normal tissues and can be identified as biomarkers of tumorigenesis (Mohd & Mohammad, 2009). Depending on its test site, they may be biomarkers of tissue or circulatory. Tissue biomarkers have different categories such as membrane receptors, oncogenes, tumor suppressor genes, nucleic antigens, growth factors and components of degradation. Circulatory biomarkers include a broad category of tumor-associated antigens (TAA). Selective biomarkers can identify risks and help tumor detection, and an early diagnosis allows appropriate therapeutic interventions for an effective treatment. In addition to these biomarkers, it is also common to find an over expression of certain proteins membrane in cancer cells, which has been used for the use of specific ligands or molecules to these proteins, such as folic acid to folate receptors, to obtain greater interaction of nanoparticles to cancer cells. In this case, the effectiveness of the functionalization is more a matter of statistics than specificity by itself, since having the over expression of these receptors will increase the likelihood of attracting a greater number of drug-loaded nanoparticles to diseased cells.

Micelles have been recently reported to have been functionalized with folic acid as a specific ligand, which is strongly attracted to its receptor in the cell membrane. The folate receptor is a protein that is generated in large quantities during cell proliferation in several types of cancer cells such as ovarian, breast, brain and lung (Ross et al., 1994).

Also, peptides have been used for the functionalization of micelles as locators of cancer cells. The nature of polypeptides allows performance optimization of specific ligands by adjusting the sequence or conformation of the peptides. One example is the peptide cRGD (cyclic Arg-Gly-Asp-D-Phe-Lys) specific for $\alpha\beta_3$ receptor produced in large amounts in tumor endothelial cells (Nasongkla et al., 2004). These receptors are a cell membrane protein that is affected with the growth of tumors, local invasiveness and potentially in metastasis, but it is not detected in quiescent vessels (Rueg et al., 2002; Teti et al., 2002; Vamer & Cheresch, 1996). This membrane receptor increases its levels in vascular angiogenesis, thus making tumor treatment specific for the generation of new blood vessels (Wermuth et al., 1997). Carbohydrate functionalized micelles, asialoglycoprotein receptor (ASGPR) is a recipient of lecithin membrane commonly found in liver cells (Ashwelland & Harford, 1982). In hepatocellular carcinoma are high levels of (ASGPR), which helps the specificity of chemotherapy for liver (Wands & Blum, 1991). Carbohydrate molecules such as galactose and mannose are specific ligands of this receptor (Goto et al., 1994; Jansen et al., 1991).

The use of antibodies as specific ligands is promising as they are able to bind to a range of specific antigens in cancer cells. The combination of a brain-specific antibody increased to 5-fold the neuroleptic action in charged micelles than in non-functionalized micelles, and it is 20 times greater than for the free drug (Kabanov et al., 1989). Micelles have been reported (PEG-PE) functionalized with 2 antibodies, a monoclonal anti-cancer antibody (mAb2C5) and anti-myosin (mAb2G4). Both antibodies have great ability to bind to the substrates after conjugation of the micelles. The antibody 2C5 in micelles loaded with paclitaxel increased 4 times the drug accumulation in the tumor after 2 hours (Torchilin et al., 2003). Furthermore, there is the functionalization of micelles with aptamers. The aptamers are DNA and RNA

oligonucleotides that can identify a large number of specific molecules (Torchilin et al., 2003; Tuerkand & Gold, 1990). We have seen that PEG-PLA micelles with an RNA aptamer specifically bind to an antigen on the membrane of prostate tumor (PSMA). Nanoparticles with the induction of aptamer showed 77 times more specificity to PSMA receptor than non-functionalized particles (Ellingtonand & Szostak, 1990). The use of ligands has yielded promising results for the location of cancer cells. This will allow drug release systems based on nanoparticles using different ligands that recognize different types of cancer. There is further research to look for future and further specific treatments with low or no side effects. Instead of using a single ligand, nanoparticles' specificity can be potentiated using multiple ligands in a single particle. Sooner or later, these systems will have the ability to attack different cancers at once or they will be designed to exchange specific ligands in an easy way, having a generic nanoparticle system capable of receiving any desired ligand presenting a universal binding system.

4. Combined systems based on hydrogels and nanoparticles

There are few studies that have integrated particles or micelles loaded with Taxol® in thermosensitive hydrogels. As good examples we can mention the system proposed by Jiang Liu et al. (2007) (Liu et al., 2007). This system consists of a biodegradable gelatin sponge containing PLGA-PTX microspheres (polylactide-co-glycolic acid-paclitaxel) in order to provide continuous local release of PTX (paclitaxel). They obtained a more prolonged release rate for up to 19 days, in contrast to simple systems where the paclitaxel was released within a range less than one hour. In another study presented by Yang Yang et al. (2009) joined Docetaxol (DTX) in a pluronic gel F127(PF127) injectable thermosensitive mixed micelles prepared with the same material (PF127) and loaded with Taxol® (system GMM). During a test of 156 hours, it was found that the proposed system maintains a prolonged release of DTX compared with other control systems (Yang et al., 2009).

The development of combined drug delivery systems based on efficient and innovative drug encapsulation in functionalized nanoparticles to detect and attack cancer cells, using thermosensitive hydrogels as vehicles that can be injected locally can be an excellent option for the treatment of cancerous tumors, thereby eliminating the supply of intravenous drugs and consequently the severe side effects. Specifically for Taxol®, its encapsulation in nanoparticles would prevent crystallization when deposited in the hydrogel. In addition, the release rates could be controlled in a double form, both by the action of the degradation of gel particles released and at the same time by the degradation of the particle containing the Taxol®. Achieving prolonged drug release would increase the therapeutic effects for longer periods. An added value to these systems is the functionalization or even multifunctionalization of nanoparticles with ligands or antibodies for high specificity to cancer cells, which should increase the efficiency and the therapeutic effectiveness of the system. Even today very few researchers have worked with Taxol® delivery systems with the characteristics listed above; making this theme of the project attractive and innovative for study.

5. Conclusions

Researchers are currently exploring new methods for transport and controlled release of drugs for cancer treatment, thereby seeking to reduce the side effects the of currently

applied chemotherapy as well as looking for battling more efficiently and accurately such disease. Although there are a number of published research focused on this issue, the fact is that so far no systems have emerged to prove conclusively its efficacy, although always positive progress has been shown, each proposed system suffers from certain drawbacks that slows down the progress towards real applications. The disadvantages found in the systems of transport and release of anticancer drugs proposed in recent years can be overcome by taking advantage of each method and combining them into drug delivery systems composed with more sophistication and complexity than their predecessors. This idea has already begun to be implemented by some researchers who have integrated drug-loaded nanoparticles on thermosensitive hydrogels (Liu et al., 2007), but these authors have not exploited the alternative of functionalization or multi-functionalization of nanoparticles. So, there is still much room for much improvement in these drug delivery systems and it is where researchers could find novel ideas. For example we can think in to make functionalized or multifunctionalized nanoparticles loaded with a anticancer drug, then incorporate them into a thermosensitive hydrogel. With this proposed system, the hydrogel may be injected into the tumor area (in the case of solid tumors) and gradually functionalized nanoparticles will be released. Due to its functionalization, they will be attracted mainly to diseased cells, nanoparticles will be biodegraded and drug will be released surrounding the cell. There are several advantages of using combined systems. For example, the highly localized application by avoiding the bloodstream, in the case of solid tumors; an improved release profile with a slow and steady dose that highly favored the drug's effectiveness in eliminating cells. The effectiveness of the functionalization of nanoparticles will be favored by the close application to malignant cells; in general, we will expect to acquire treatments where the typical collateral damage observed in common chemotherapies would be considerably lower as well as the required dose.

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The Evolving Role of Tissue Biospecimens in the Treatment of Cancer

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1. Introduction

The field of surgical pathology is diagnostic in nature and since its inception, has always supported the treatment of cancer. The manner by which tissues are processed may vary between institutions, but the vast majority utilize formaldehyde as the fixative of choice. This fixative allows for excellent architectural tissue preservation thereby enabling optimal microscopic examination, the foundation from which surgical pathology is based upon. From fixed tissue, cancers have been categorized, sub-typed and features enumerated to help elucidate the differences that exist between them. Classification schemes that include grading and staging systems have been incorporated in their examination and, when coupled with the patient's clinical data, have been useful in prognostication and guidance of follow-up treatment. The development of immunohistochemistry has only served to further ingrain the importance of proper tissue fixation with cancer sample preparation. Yet, despite providing the basis for these invaluable contributions, the approach of fixing tissue biospecimens with formaldehyde has, in recent years, undergone criticism as being a suboptimal means of preservation when molecular analysis is desired from these specimens. Added to this are other pressures to develop novel approaches by which tissue biospecimens can be interrogated. Within the field of surgical pathology, it is becoming recognized that various pre and post fixation factors may contribute to negatively impact the overall integrity of the tissue biospecimen. Outside surgical pathology, the continually decreasing size of core biopsy specimens are minimizing the amount of tissue present for architectural evaluation. In addition, newer treatments are continually being sought in order to further personalize cancer treatment based on the type of tumor present in the tissue specimen. These forces are necessitating a re-evaluation of the entire established process of fixation, and whether alternative methods may exist or be developed that may be more amenable for both diagnosis and biospecimen integrity. In this chapter, the central role of tissue biospecimens for the support and guidance of cancer care are discussed, with an examination on how newer technologies and minimally invasive approaches will change the landscape by which these specimens will be processed.

2. The history of tissue biospecimens in cancer treatment

The examination of tissue through the microscope falls within the modern day purview of surgical pathology. Although this examination is now performed by pathologists, the

emerging field of surgical pathology in the 19th century began with surgeons. Before the widespread acknowledgement that microscopic examination of tissue was compulsory for an adequate diagnosis, surgeons were limited to the gross examination of specimens and their personal assessment and experience as to whether the extirpated specimen was benign or malignant (Gal, 2001). At that time, malignant tumors often ran their course, and their examination in the living patient limited to only observation at the gross, not microscopic level. In 1856 Rudolf Virchow published the first book on histopathology, introducing the world of microanatomy to the medical community. Observations from this book and those that followed slowly led to a newfound understanding that diseases of the human body could be correlated to findings at the cellular level. A number of discoveries and inventions in the 19th century also proved instrumental in providing the basis from which tissue could be reliably and widely examined by others under the microscope. These advances, like the invention of the microtome for cutting thin tissue sections and the discovery of natural and synthetic dyes like hematoxylin for the staining of nuclei and eosin for the cytoplasm of cells, allowed for the reproducible examination of tissue and the subsequent classification of organ structure. With these advancements, excised tissue was no longer discarded, but examined and documented. The first observations between malignant and benign tissues were recorded, with malignant cells described as being different in size and shape, not connected at their margins, possessing nuclei that varied in size and number and forming irregular edges at their juxtaposition with the adjacent normal tissue. In contrast, the cells of benign epithelial growths resembled each other in size and shape, had fairly homogenous appearing nuclei, were more cohesive along the edges of their cell borders and formed straighter lines (Rosai, 1997). With time, specimen processing and evaluation of the specimen became more standardized. Through correlation over time with the clinical outcome of the patient and with observations of similar tumors from other patients, these tissue biospecimens became the first to influence the treatment of cancer in patients. With improvements in surgical techniques, anesthesia and the application of antiseptic precautions, surgeons began to broaden the degree of surgical intervention they performed on patients. The development of the frozen section, wherein a portion of the specimen was excised and sent to the pathologist for evaluation intra-operatively, burgeoned and soon became integral to the cancer patient's care. The rapid freezing and hardening of the tissue specimen enabled the cutting of thin sections that could subsequently be stained and evaluated under the microscope. The surgeon now could know if the excised margins of a tumor specimen still had tumor cells in them, allowing for an intra-operative decision as to whether additional sections were needed or not. For certain other tumors, determining the depth of invasion through the frozen section became integral in planning out the remainder of the surgical plan for the cancer patient. The processing of the biospecimen would evolve to include frozen section evaluation on some, and fixation with paraffin embedding on the majority of excised tissue specimens sent to the pathology laboratory. Through the microscopic examination of all the subsequent slides, the diagnosis that is formulated by a pathologist is based on the resemblance and similarities to an archival history of previously diagnosed specimens that have been correlated with clinical outcome. This largely empiric system has served the medical community well, with the diagnostic information rendered by the pathologist enough to guide the cancer patient's care and estimate their prognosis (Kufe, 2003).

2.1 Early tools in the assessment of tissue and tumor biospecimens

Along with hematoxylin and eosin, a number of other stains were specifically developed to help identify cell structure and determine cellular function, but in the process aided to further classify tissues and categorize tumors. Their development was spurred by the fact that, despite the enumeration of histologic criteria published and disseminated about specific diseases and tumors, reproducibility among the growing numbers of physicians that practiced pathology remained a problem. In order to engender consensus, a number of special stains were created through experimentation that would eventually serve to better delineate cellular structure, content and properties. One of these stains, the Periodic Acid Schiff stain, allowed for the detection of the presence of carbohydrate macromolecules, a feature that would prove to be a useful ancillary aid to morphology in the differentiation between different types of tumors. For example, the identification of the Periodic Acid Schiff reaction in tumors known as small round blue cells can be helpful in leading to the correct diagnosis. Positive staining material in rhabdomyosarcomas, Ewing's sarcomas, malignant peripheral neuroectodermal tumors and germinomas can help distinguish them from other tumors with similar histologic features such as desmoplastic small round cell tumors, small cell mesothelioma, and a number of other tumors with small round blue cell morphology (Leuschner, 1996). This stain is particularly helpful in identifying Ewing's sarcoma, due to the fact that over 80% of these tumors have been reported to contain glycogen within their cytoplasm. Other special stains, like the Mucicarmine and Alcian blue stains, were originally used to help characterize cells that secreted mucin, aid in the early differential diagnosis of tumors and determine if they were useful in identification of tumor origin for metastatic tumors (Johnson, 1963). These stains have persevered and have modern day utility in defining poorly differentiated tumors as being or not being adenocarcinoma and in distinguishing adenocarcinomas from poorly differentiated squamous cell carcinomas in the evaluation of lung cancers for the former stain, and as an aid in the detection of intestinal metaplasia in the medical condition known as Barrett's esophagus for the latter stain (Wallace, 2009). Yet another special stain, the reticulin stain, showed initial promise for staining specific extracellular matrix constituents, namely the collagen type III fibers that comprise the stromal network in many different organs. It can be particularly prominent in the liver, as these fibers invest the hepatocytes that make up the hepatic plates, with normal hepatic plates being two cell layers thick or less. Reticulin stains help to define hepatic adenomas and hepatocellular carcinomas from normal liver, subtle changes not so obvious when examined by hematoxylin and eosin stains alone. In hepatic adenomas, they show an expansile growth pattern whereas in hepatocellular carcinoma they demonstrate an increase in trabecular thickness. To this day, the reticulin stain continues to be recommended as part of the evaluation of nodules within the liver (Lennerz et, 2009).

Later, the development of the electron microscope, with its ability to probe sub-cellular structure, proved a boon to tissue diagnostics as it related to cancer treatment. Delivery of the correct diagnosis enabled a treating physician the opportunity to give the most clinically relevant care. Electron microscopy allowed for the visualization of subcellular organelles that could otherwise not be discerned using traditional light microscopy. When tumor tissue was examined at the electron microscopy level, the presence or absence of organelles known to be specific to certain cell types enabled the identification of poorly differentiated tumors whose cell of origin could not, at the time, be properly classified at the morphologic level. The electron microscope provided more definitive characterization of tumor subtypes than

histology and special stains. In the case of Ewing's sarcoma, although it was known to be one of the tumors that possesses cytoplasmic glycogen, variability in the degree of differentiation could mean that their abundance may also be variable, and hence it was advised that the diagnosis of Ewing's sarcoma should not be based solely on the presence of glycogen detected by the Periodic Acid Schiff stain in tissue sections with the appropriate morphology. One factor may have played a role in this ambiguity, was the type of fixative being used. Eventually it was determined that the best fixative for detecting glycogen in tissue sections was alcohol, and that fixation in formaldehyde resulted in variable preservation of this macromolecule (Llombaart-Bosch, 1996). Additionally, it was also learned that specimens that were poorly fixed could lead to a complete absence of detectable glycogen. In these cases, ultrastructural evaluation would prove to be helpful in establishing the correct diagnosis. In the case of Ewing's sarcoma, examination at the ultrastructural level revealed the presence of two cell types, the primary cell referred to as the light or principal cell, and a secondary cell referred to as the dark cell. The principal cell was characterized as having homogeneously sized nuclei and ample cytoplasm, sparse numbers of organelles and abundant glycogen. The dark cells possessed elongated to ovoid nuclei with condensed chromatin, and likened to involuting principal cells. The identification of these cells have helped to characterize Ewing's sarcoma as different from other tumors possessing a similar histologic appearance, particularly olfactory neuroblastomas (Trump, 1983). Another instance where electron microscopy has impacted cancer care and proven integral to the proper identification of a tumor is in the case of poorly differentiated tumors of the pleural cavities. In these situations the differential diagnosis revolves between poorly differentiated adenocarcinoma versus mesothelioma. An incorrect diagnosis of adenocarcinoma, when in reality a tumor is a mesothelioma, can result in an expensive and time consuming work-up and legal issues. For quite a while, electron microscopy was considered the gold standard in the diagnosis of mesothelioma. The presence of long, slender, sinuous, branching and bushy microvilli found on the cell surface at the ultrastructural level were reported as being pathognomonic features for diagnosing mesothelioma (Velez, 2002). Taken altogether, the development of special stains and electron microscopy aided in leading to the further subclassification of tumor tissue biospecimens. These tools were readily incorporated into the surgical pathology community and contributed to the early attempts to tailor patient cancer care. Despite the advances these tools brought to patient cancer care, their limitations would eventually become apparent with the development of a newer tool that would be introduced to the armamentarium of surgical pathology, immunohistochemistry.

2.2 Immunohistochemistry and the beginning of the end for empiric medicine

In the latter half of the 20th century the ability to exploit the specificity of the antibody-antigen reaction was successfully transferred from the experimental laboratory to clinical specimens. This application began with the immunofluorescence technique. However, the major liability with this approach was three-fold: the need for a specialized microscope with fluorescence capabilities; the pre-requisite for fresh frozen tissue samples; and poor morphologic resolution (Taylor, 1994). Over the period from the mid 1970's to the early 1990's these obstacles were eventually addressed with the development of alternative, non-fluorescence labels and the discovery of antigen retrieval. In the latter, the abolishment of the interfering formalin induced cross-links in fixed tissue specimens led to the widespread application of this technique, now called immunohistochemistry, to the vast archive of fixed tissue specimens banked in pathology departments. A period in the surgical pathology

community began anew, similar to the advent of special stains and electron microscopy eras, wherein panels of recently developed antibodies were tested against series of tissue and tumor specimens with the intent to identify and again better characterize human disease. This period, as with the introduction of all new tools in the fields of diagnostic pathology, was met with initial skepticism. However, one key difference that this new tool brought would emerge that would distinguish it from that of its' predecessors in the care of cancer patients. The previous tools only enabled the observation of cellular organelles and cytoplasmic or nuclear constituents. The technique of immunohistochemistry, with the specificity of the antibody-antigen relationship, allowed unprecedented access to the macromolecules integral to the functions of the cell, proteins. Through subsequent investigations of an assortment of proteins by a myriad of different investigators, the era of Personalized Medicine, in terms of its current day namesake, was unceremoniously ushered in. It now became possible to identify the presence or absence of proteins in specific cell types and tumors. In contrast to traditional empiric medicine, immunohistochemistry allowed for the identification of a specific target molecule in specific cell types. In empiric medicine, not all patients will respond to a specific drug based on the absence of the knowledge if the cancer cells in a patient contained those proteins acted upon by the drug, or had only low levels of those targeted proteins. With the advent of immunohistochemistry, proteins that were involved in or acted to drive the process of oncogenesis could now be identified. This identification allowed for rational drug treatment, with therapy based on the presence of a target protein or molecule in a cancer patient identified by immunohistochemistry on the tissue specimens. This approach represents the potential to significantly improve cancer care, taking into account the fact that the efficacy of pharmacotherapy in oncology is less than 50% (Jorgensen, 2009). In the very least, this approach will help eliminate the administration of certain therapeutic agents to those cancer patients who would not benefit from a drug, based again on the absence of the targeted molecule or protein in the patient's tissue specimen.

Possibly the first successful implementation of Personalized Medicine can be attributed to the steroid receptor estrogen in human breast cancers. The observations in preceding decades by physicians and scientists that the growth of certain reproductive organ related tumors appeared dependent on sex steroids led to further direct investigations. Eventually it was borne out that certain tumors, like those of the breast, possessed large numbers of the estrogen receptor and thus could be targeted for endocrine therapy. Later it became apparent that the amount of estrogen receptors in these tumors could be variable, and that patients with estrogen receptor positive tumors tended to have a better clinical course than those patients with estrogen receptor negative tumors. It thus became imperative to be able to determine the estrogen receptor status in these patient's tumors. The method that became the initial mainstay to assess estrogen receptor status was the steroid ligand binding assay and involved the homogenization of tumor tissue into a lysate that was then exposed to labeled estradiol. This assay however, lacked adequate specificity and sensitivity for the clinical setting. The major drawback of this assay was the fact that it was based on a tissue homogenate and was therefore without any correlative histologic picture. Thus the proportion of tumor cells to stromal cells, or the amount of necrosis present in the sample submitted could not be accounted for in the sample. Additionally, improper collection of the sample, that is, prolonged procurement time leading to artificial loss of this labile receptor, could bias the final results. With the concurrent progress in immunohistochemistry, the development of an antibody suitable to test on frozen, and then ultimately formalin fixed and paraffin embedded tissue became available that ultimately

showed suitable concordance with the steroid based assay (Ottestad, 1988). Immunohistochemistry, because it enabled visualization of the receptor status on glass slides, was relatively inexpensive compared to the ligand binding assay and could evaluate small tumors, gradually gained acceptance as a means to evaluate steroid receptor status in breast cancer patients. Currently, immunohistochemistry is the standard by which estrogen and progesterone receptor status are assessed. Their assessment in the breast cancer patient is integral in guiding that patient's direction of clinical care, as endocrine therapy has proven to be of benefit only to those that have tumors that are estrogen receptor positive. However, recent emphasis on the importance of proper tissue handling has been raised again bringing back into focus the importance of the tissue biospecimen's role in cancer care. A recent collaborative effort by the American Society of Clinical Oncology and College of American Pathologists has brought to light the startling finding that up to 20% of estrogen or progesterone receptor findings by immunohistochemistry may be inaccurate, either being falsely negative or falsely positive (Hammond, 2010). These findings were determined by taking the original results and testing the same tissue blocks at an experienced immunohistochemistry central laboratory for comparison. In order to rectify this problem, this collaborative group published recommendations for the optimal handling of extirpated breast cancer specimens that under present day methods of tissue processing, would result in reproducible inter-laboratory steroid receptor studies. In this instance, breast cancer tissue biospecimens play a continuing role in the guidance of clinical care and the quality assessment of diagnosis.

The story of the Her-2 gene and the development of the humanized monoclonal antibody trastuzumab represents a case wherein tissue biospecimens aided in the rapid identification and confirmation of a targeted cancer therapy. Through the use of tissue biospecimens, researchers were able to identify a subset of patients that overexpressed the gene product of the Her-2 gene. With clinical correlation, this subset was determined to be associated with a worse overall prognosis and a relative resistance to endocrine therapy (Press, 1993). But more importantly, the presence of this overexpressed protein meant that it defined a particular group of breast cancer patients who might benefit the greatest by the creation of an anticancer agent directed specifically at that amplified gene product. The biotechnology company Genetech eventually developed a recombinant monoclonal antibody that fit within a specific extracellular cleft and effectively abrogated any further tyrosine kinase activity. An antibody was also subsequently developed that could be used on tissue biospecimens to detect this proteins presence in breast cancer cells. Through the use of banked tissue, this antibody was tested on breast cancer tissue biospecimens, the results from which produced an FDA approved assay. The publication of these results led to the ability for pathology laboratories, community in addition to academic, to incorporate this immunostain into their diagnostic regimen. The broad use of this immunostain led to the findings that the prevalence of the HER2 gene amplification in the breast cancer population was consistently between 20 to 30 percent. Similar to estrogen and progesterone receptors, continued research in the field of biospecimen science led to the conclusion that approximately one fifth of all breast cancer tissue specimens immunostained for the Her-2 protein could be inaccurate (Wolff, 2007). A number of sources for variation were identified, ranging from preanalytic (time to fixation, method of tissue processing, time of fixation, type of fixation), analytic (assay validation, equipment calibration, use of standardized laboratory procedures, training and competency assessment of staff, type of antigen retrieval, test reagents, use of standardized control material, use of automated laboratory methods) and postanalytic (interpretation criteria, use of image analysis, reporting elements, quality

assurance procedures). Due to the number of sources of variation and the influence they could have on the tissue biospecimen, a number of situations were enumerated that were grounds to not use immunohistochemistry to assess Her-2 protein levels. These exclusionary criteria included the use of any fixatives other than formalin, needle biopsies fixed for less than 1 hour, excisional biopsies fixed for less than 6 hours and longer than 48 hours, core needle biopsies with edge or retraction artifact affecting the entire core or with crush artifact, tissues with strong membrane staining on internal normal ducts or lobules and tissues with controls that showed unexpected results. The initial and continued use of tissue biospecimens to assess Her-2 overexpression have been integral in creating appropriate standards aimed at establishing a uniform, reproducible result. In turn, these results continue to help further refine the clinical decision process in the care of breast cancer patients.

The use of tissue biospecimens also played a significant role in expanding the utility of certain drugs in the treatment of cancer. Imatinib mesylate (Gleevec), originally designed to target the chronic myeloid specific protein BCR-ABL, was later found to show some activity with another tyrosine kinase, notably the gene product KIT. Using tissue biospecimens, investigators were able to document that a relatively rare gastrointestinal tumor, called a Gastrointestinal Stromal and Tumor, expressed the KIT gene (Hirota, 1998). Treatment of Gastrointestinal Stromal patients with this therapeutic agent produced remarkable results. These previous examples illustrate how tissue biospecimens have helped shape the development of therapeutic agents for the treatment of cancer. A known protein that can be targeted is identified, a potentially therapeutic agent created or already is in existence, and assays developed that can identify those protein(s) on tissues. The changing paradigm is to treat a patient's tumor with an agent that is antagonistic to a protein or molecule that can be documented to be present in the tumor tissue. This is in contrast to treating the tumor based on a histologic classification and previous experience of response in a certain percentage of patient's with that tumor type. In this new era of Personalized Medicine, therapies will be given specifically to those individuals most apt to respond to them. Therefore, those who will benefit from a targeted therapy will be appropriately selected for treatment, and those who will not benefit will be treated by another regimen. Tissue biospecimens will continue to play a significant role in the new medical paradigm, however their traditional role may evolve. Whereas the current means of evaluating tissue biospecimens is after fixation and processing, with the end result a paraffin embedded specimen that is stained for visual examination either through traditional hematoxylin and eosin stains complimented by immunohistochemistry, tomorrow's biospecimen may be subjected to molecular assays. A representative scenario is the case of CD-117 positive tumors. Initially, Gastrointestinal Stromal Tumors were found to express this protein in abundance. By similar reasoning, it was assumed that other CD-117 positive tumors could also be treated by this tyrosine kinase inhibitor. A number of CD-117 positive tumors were rapidly identified that included colorectal cancers, renal cell carcinomas, thymic epithelial tumors, seminomas, Merkel cell cancers, endometrial stromal sarcoma and aggressive fibromatosis (Quek, 2009). However, the clinical community soon came to the realization after much scientific investigation that the underlying molecular alterations in Gastrointestinal Stromal Tumors, namely the gain of function mutations in the C-Kit gene in specific exons, were the underlying reason for their therapeutic responsiveness. Although other tumors may express the C-Kit gene product and can be readily identified as being CD-117 positive, without the specific mutations seen in GIST tumors, these other tumors turned out to be non-responsive to Gleevec administered

therapy. This example highlights how the oncoming molecular age in medicine will play a role in the analysis of biospecimens for the rational delivery of targeted therapy.

3. Achieving personalized medicine through improved diagnostics

Although conventional light microscopy has been the stalwart of diagnostic pathology for decades, it continues to have problems with reproducibility among members of its collegium. As a continually evolving field, newly described entities go through the academic rigors of debate before consensus is reached. This can take some time, with consensus established after many years. Several examples showcase the limitations of diagnosis based solely on morphology. In the colon, preneoplastic polyps have traditionally been classified as either hyperplastic or adenomatous. In the late 1990's, two new entities were described called the serrated adenoma and the admixed polyp. Despite a decade where these entities were studied and further described, some confusion still remains regarding their correct diagnosis. When specialist gastrointestinal pathologists were shown a number of cases of these two entities, only a moderate degree of concordance was noted, and only fair inter-observer agreement evident (Wong, 2009). When cells alone are examined, that is, in the form of a cytology specimen, molecular diagnosis can be an improvement. The detection of atypical cells in sputum cells prepared for cytologic examination for the screening of lung cancer is a proven method, but suffers from low sensitivity. The addition of genomic markers can raise the sensitivity of cancer detection to 86% and specificity to 93% (Jiang, 2010). When it comes to actual tumors, interobserver agreement has had a history of similar problems with at best, modest reproducibility. One of the more difficult tumors to classify are those epithelial tumors originating from the ovary. Through decades of examination, diagnostic reproducibility based on morphology has steadily increased (Kobel, 2010). Aiding this improvement has been immunohistochemistry and molecular analysis. There are now considered to be five major subtypes of ovarian carcinoma: high grade serous, clear cell, endometrioid, mucinous and low grade serous carcinoma. The importance of this new paradigm based classification again is treatment based, with high grade serous being responsive to neoadjuvant chemotherapy whereas the others are not. From continued research utilizing all available data from the patient, the clinical outcome and most importantly the tissue, consensus in diagnosis is being achieved with diagnostic algorithms to achieving reproducible results being reported (Kalloger, 2011).

These examples demonstrate the limitation of histologic diagnosis and the increasing role molecular analysis will play in identifying, classifying and prognosticating tumors. Assays that will be developed to achieve this will most likely be used to interrogate nucleic acids, whether DNA or RNA, but may also analyze proteins. The arrival of these assays may be tied directly to therapy and hence have been given the designation companion diagnostics (Papadopoulos, 2006). The implications of using these molecular approaches are that they may eventually yield more informative data than conventional hematoxylin and eosin stained microscopic slides, even when the tissue is further worked up with immunohistochemistry. The goal is to be able to identify subgroups within a given patient population whose histology may appear similar, but end up having a different clinical course or outcome. It is hoped that through the molecular evaluation of tissue specimens, molecular signatures can be identified and assays developed that can recognize and separate these subsets of patients from the general cancer population. A realistic future scenario where molecular studies may eventually take precedence over histology is with

Stage II and III adenocarcinoma of the colon. Although histologically these tumors may appear morphologically similar and stage similarly, it is known that a certain proportion of these patients do benefit from adjuvant chemotherapy whereas for the other proportion of this group it is unnecessary. The identification of a biomarker or biomarkers capable of clearly delineating these two subpopulations would save time, effort and benefit the patients having to be and those not having to be treated. Another reason molecular analysis may be more informative than traditional hematoxylin and eosin stained sections is its objective nature. There are a number of tissue types from which human bias has been known to exist. The classification of dysplasia in the oral-pharyngeal space has for years been known to suffer from inter- and even intraobserver variability (Karabulut, 1995 and Abbey, 1995). For gliomas of the brain, histologic classification, which guides subsequent therapy, suffers from interobserver variability (Kros, 2007). Even with an updated classification scheme, diagnostic variability persists now due to the addition of newly added entities and variants (Tremblath, 2008). It would appear logical then, that another mechanism of diagnosing these types of tumors would be attempted. In fact, a recent study did find that molecular analysis did perform better than traditional histology, albeit in the realm of survival prognostication (Gravendeel, 2009). Thus it appears that with the molecular era steadily encroaching the clinical realm, the dedication of a proportion of tissue from excised tumor biospecimens may be needed as part of the standard of care for the cancer patient. The tissue biospecimen will still be integral in patient care, but the manner by which it is examined will evolve.

4. Barriers to the molecular profiling of clinical specimens - formalin fixation

Several barriers exist that limit the application of molecular techniques to tissue biospecimens. Since the main goal of extirpated tissue is the establishment of a diagnosis, the priority for such specimens is the optimal preparation of a tissue section for morphologic evaluation. In order to do this, tissue preparation involves fixation and processing, followed by sectioning and staining for microscopic examination. In the vast majority of community and academic pathology departments, the fixation used is 10% neutral buffered formaldehyde. Whereas this fixative results in a reliable and reproducible end product for histologic examination, it has been shown to be detrimental to the recovery and examination of cellular molecules. Formaldehyde interacts with DNA to create hydroxymethyl groups, forms methylene bridges between amino acids, generates apurinic and apyrimidic sites that lead to highly unstable cyclic carboxonium ions that hydrolyze into 2-deoxy-D-ribose, and cause the slow hydrolysis of phosphodiester bonds that result in short chains of polydeoxyribose with intact pyrimidines. In short, the chemical reaction between formaldehyde and deoxyribose nucleic acids leads to the denaturation of these molecules as well as the formation of cross links with proteins. The result is the recovery of shortened segments or fragmented DNA and decreased recovery. For certain high throughput downstream molecular based assays, like array comparative genomic hybridization that are designed to evaluate the copy numbers of genes from the entire genome, the absence of gene segments may lead to erroneously biased conclusions relating to genetic deletions. Several reports have noted that DNA extracted from formalin fixed, paraffin embedded tissue blocks and run on an array comparative genomic hybridization platform, tended to yield data prone to spurious changes in genetic copy number in addition to copy number loss when compared to matched fresh frozen tissue (McSherry,

2007). To complicate matters worse, the presence of formaldehyde results in the presence of high background 'noise' on array comparative genomic hybridization data, making the determination of legitimate significant molecular alterations problematic (Johnson, 2006 and Mojica, 2008). At the nucleotide level, formaldehyde has been documented to result in random changes in amplified sequences relative to the original DNA sequence. Formalin fixation is thought to cause base damage, but the overall template can still be read by polymerases. When PCR is performed using *Taq* polymerase, errors in translesional synthesis can occur (Quach, 2004). Although this error rate is low, it can be a factor when small amounts of originating material are used. This has grave implications at the clinical level, as the reporting of non-existent mutations may severely impact a patient's care altering the treatment regimen from which the fixed tissue originated from.

RNA is another potential biomarker that can be assayed from tissue biospecimens, and in the past decade has already influenced the realm of pathologic diagnostics in the field of breast cancer. Through expression array analysis, breast cancers now are considered to comprise a number of subgroups (normal-like, luminal, basal-like, HER-2(+)) within the broad spectrum of what was originally only considered part of one entity, ductal adenocarcinoma. Work is currently progressing on finding the most appropriate therapeutic regimen for each subtype. Based on the clinical success of this molecular based classification, other tumors are being probed for their molecular signature. However, it must be noted that the original work that defined these breast cancer subtypes was based on fresh frozen material. Any subsequent discoveries on such tissue must be able to be translated to clinical material, which again routinely undergoes formalin fixation and processing. Similar to DNA, mRNA recovery from FFPE tissue is encumbered by the crosslinking of molecules and poor recovery (Cox, 2006). But since mRNA is a more labile molecule than DNA, degradation of the RNA molecule impacts the overall recovery more with significantly reduced quantities and poor quality obtained from fixed tissue specimens when compared to matched frozen material (Specht, 2001). This negatively impacts the consideration of using FFPE tissue biospecimens for gene expression on cancer samples. RNA extracted from FFPE tissue is partially degraded resulting in gene expression data with low signal intensity data. In one study, only a quarter of unselected samples that were FFPE provided enough starting material for subsequent gene expression assays (Penland, 2007). In another study, gene expression values differed by a value greater than two fold in almost 20% of the genes studied between matched FFPE and frozen tissue samples (Mojica, 2007). This rather significant discrepancy can be accounted for by either a decrease in gene expression due to degradation or an increase in gene expression due to changes in the tissue microenvironment and the cells subsequent reaction prior to fixation. The major implication however, in regards to human tissue that could be potentially assayed for mRNA expression levels that may influence clinical care, is to determine beforehand which mRNA transcripts are stable, and remain stable. It is these transcripts that would be of clinical value, as opposed to those more labile ones prone to either degradation or biased due to *ex vivo* conditions (Lee, 2005). This obviously would require an in depth investigation into which transcripts are most stable and least resistant to the external pressures associated with the tissue fixation process, so that those that are susceptible to degradation during the process of fixation are excluded from further consideration and studies (Opitz, 2010).

Proteins are the last major molecule to be mentioned here that are routinely assayed from clinical tissue biospecimens. The formalin-fixed, paraffin-embedded tissue specimens

routinely examined in surgical pathology departments currently undergo proteomic evaluation in the form of immunohistochemistry. However, this analysis is often limited to evaluating only one, or at most two proteins in the tissue section. When tissue biospecimens are exceedingly small, the tissue may get exhausted, limiting the number of proteins that can be examined through immunohistochemistry. An approach that may see increased use, especially when a number of proteins will need to be evaluated in a tissue biospecimen, is mass spectrometry. Currently, mass spectrometry is finding initial success as a diagnostic modality for amyloidosis, and its integration into other pathologic conditions most likely will soon follow suit. However, as with DNA and RNA, the analysis of the traditionally formalin-fixed, paraffin-embedded tissue biospecimen by mass spectrometry will present with problems once again associated with formaldehyde. The cross links formed in proteins increase the complexity of data analysis and peptide identification through the addition of 12 and 30 Dalton changes in the peptide mass (Metz, 2006). Adding to the complexity of this reaction is the finding that with time, more methylene bridges (cross-links) are formed creating increases in the molecular weight of a peptide by multiples of 12 Daltons (Toews, 2008). Chemical reactions that occur with cross-linked peptides can result in incomplete fragments upon collision-induced dissociation, requiring additional targeted experiments to correctly identify those peptide fragments (Sutherland, 2008). This necessitates the creation of specialized software that takes into consideration these mass effect changes before correct peptide identification can be made, a daunting task considering the multitude of combinations possible due to fixation (Leitner, 2010). The presence of cross links may also result in intra-protein peptide combinations as well as portions of peptides between different proteins, making an already difficult task even more complicated. Research into the mass spectrometry analysis of formalin-fixed, paraffin-embedded tissue has shown that comparable data with matched fresh frozen material can be done using principles learned from antigen retrieval and immunohistochemistry. The original findings that compared the numbers of proteins identified by mass spectrometry analysis between matched formalin fixed, paraffin embedded and frozen tissue sections showed the former to consistently be quantitatively less than the latter (Crockett, 2005, Bagnato, 2007 and Guo, 2007). Despite claims touting the feasibility of mass spectrometric analysis on formalin-fixed paraffin-embedded tissue, complete concordance of protein inventories with matched frozen tissue has yet to be achieved, leading to speculation that material not detected from formalin-fixed, paraffin-embedded tissue may be due to incomplete lysis of cross links and either incomplete or biased protein extraction (Nirmalan, 2008). Research on fixed biospecimen material continues with the goal that either a mechanism of improved recovery will be found, a more molecular friendly method of fixation developed, or an alternative means of diagnostics created that is compatible with both surgical pathology and molecular assays.

4.1 Barriers to the molecular profiling of clinical specimens - preanalytical variables

Complicating the implementation of molecular analysis on fixed tissue biospecimens is the growing awareness that a number of preanalytical factors may unduly influence the characteristics of certain molecules prior to fixation. Since the intent of assaying a tissue biospecimen is the characterization of molecules reflective of a cancer cells' *in vivo* state, it stands that any factor that alters that state is undesirable. Unfortunately, a number of factors have now been recognized that may introduce unintended molecular variation to the biospecimen and include the type of surgical procedure, warm and cold ischemia, time to fixation, tissue thickness and rate of fixative penetration. These variables predominantly

affect the most labile molecules, namely mRNA and protein phosphorylation status (Sprussel, 2004 and Espina, 2008) and occur early in the procedure (Miyatake, 2004 and Schlomm, 2008). The first of these variables, the type of surgical procedure, can influence a cell's molecular signature due to the initiation of hypoxia. As the specimen is being excised, vessels are sequentially ligated before the entire specimen is ready to be extirpated. The type of procedure can influence the molecular signature, as the shorter, quicker procedure will have less of an influence than procedures that take longer, time wise. An approach that is becoming more popular because it is less invasive and therefore results in a shorter hospital stay, is laparoscopic surgery. For resections of colon cancer specimens, the durations of surgery increases 55 minutes from that of an open surgical resection (COSTG, 2004). With the introduction of robotic assisted surgery, a procedure rising in popularity because of benefits like less blood loss for the patient, the duration of ischemia can increase between 30 minutes to 1.5 hours in prostatectomy specimens. These short changes in time may not be significant enough to change the levels of proteins, but can unduly influence the expression profile signatures of the more labile mRNA transcripts (Ricciardelli, 2010). This increase is attributable to a reversal in the sequence of vessel ligation, where in robotic assisted prostatectomy procedures they are done earlier as opposed to later, as in open prostatectomies. Whereas these changes occur prior to the acquisition of the tissue by the department of pathology, other variables influence the molecular signature of cells within tissue post-acquisition. Cold ischemia, or the time the tissue is outside of the body and either frozen or fixed, is the major factor in determining the adequacy of the tissue for further analysis. Although not incorporated into many protocols, the time from receipt to freezing has a strong negative impact on the molecular profile of the tissue specimen (De Cecco, 2009). For tissue that undergoes fixation, the cold ischemia time is longer. For these specimens, once they are received within the department of pathology, they are immersed in 10% neutral buffered formalin. The cells within the tissue however, will remain viable for a limited time, most likely until they become fixed. The cells are now enduring a loss of their blood supply, accumulation of lactic acid with the subsequent decrease in their cellular pH, and changes in temperature (room temperature vs. *in vivo* body temperature), all resulting in biologic stress. In response to this biologic stress, they will react, and in the process their molecular signature will be altered to some degree to this stress. The obvious conundrum is to determine what of the molecular signature can be considered artifactual, i.e., as a complication of this artificially induced stress, and what can be characteristic of the neoplastic state. An added layer complicating these changes is the fact that tissue thickness can lead to regional differences in the specimen's molecular profile. Formalin infuses into tissue at 1 mm an hour. If specimens are not sectioned before they are placed in a container of formalin, the tissue at the center of the specimen will be fixed last relative to the exterior of the specimen. Since the cells in the tissue are still viable until fixation, the cells within the center of the specimen will be responding to their new environment (Stan, 2006). Depending on their distance from the fixative, these cells will experience progressively hypoxic, acidic and nutrient depleted conditions over time (Espina, 2008 and van Maldegem, 2008). As viable cells now under biologic stress, their molecular profile may alter leading to falsely elevated or decreased levels of a putative biomarker relative to those cells in direct initial contact with formaldehyde. The basic tenet when working with clinical specimens is to realize that excised tissue is viable and not only vulnerable but reactive to *ex vivo* stressors, and that an understanding as to their location within the specimen with respect to exposure to fixative should be considered in the data analysis (Espina, 2008).

These preanalytical factors have the potential to lead to significant variability in the molecular signature of cells within a tissue specimen. Although there exists numerous tissue biorepositories within the United States, a major problem with each is the wide variation in tissue collection, processing and storage of these samples and an absence of standardized procedures for each step (National Biospecimen Network Blueprint, 2003). The development of a sample preanalytical code proposed by the International Society of Biospecimen and Environmental Repositories represents a good start towards standardization (Betsou, 2010). Through the compilation of data from tissue biospecimens, and correlation with this proposed grading system of specimen integrity, the factors that play into macromolecule integrity can be identified. Each organ may exhibit differences in the stability of the molecules within their cells, with those possessing digestive type enzymes (e.g., the pancreas) more labile than those without (e.g. skeletal muscle). A previous study indicated that the biopsy, based on the smaller tissue size and exposure to the shorter periods of warm or cold ischemia, to be the optimal tissue biospecimen for molecular analysis. The small size of the biopsy allows for even exposure to fixative, while the actual procedure of acquiring small pieces of tissue are not encumbered by extensive periods of surgically induced ischemia required to excise a diseased organ nor intraoperative procedures that are deemed clinically imperative to the needs of biospecimen collection (Schlomm, 2008 and Espina, 2008).

4.2 Barriers to the clinical profiling of clinical specimens - tissue heterogeneity

Another barrier to molecular profiling of clinical tissue biospecimens is tissue heterogeneity. Human tissue specimens are increasingly being used as the primary source of investigational material for cancer related studies. They offer advantages over cells lines because they are more representative of the diagnosed condition and reflective of the *in vivo* condition, not having undergone numerous passages and the resultant phenotypic and genetic drift. Properly collected and annotated, they can avert the problems of misidentification, a relatively widespread situation wherein certain cell lines actually correspond to other cell types than what they are designated to be (Buehring, 2004)). Despite extensive work on cell lines, the realization that the cell of origin may actually be a contaminant would be disastrous for any investigator. However, using tissue itself is fraught with problems. The integrated architecture of tissue means that the targeted cell of interest will vary with respect to the overall cell volume, even in normal tissue (Figure 1). In tumor samples, the same problem exists (Enkemann, 2010). Depending on the type of tumor, the percentage of non-tumor cells can also be less than 50%. Although in grossly solid areas of tumor tissue colon cancer, lung cancer and breast cancer can compromise over 80% of the tumor, prostate cancer is notable for interdigitating between normal glands, possibly biasing any subsequent findings based on such a tissue biospecimen (Figure 2). The presence of contaminating normal cells in a tumor sample can have the affect of dampening a signal or mask the detection of a potential biomarker molecule. The presence of segments of nucleic acids originating from contaminating normal cells can have the untoward affect of decreasing the amplitude of a deleted gene in a tumor sample (Mojica, 2007). Ideally, any work done on a sample should ensure that it is a pure, or close to pure cell population of the desired cell type, so that retrospective analysis trying to deconvolute the data does not have to be performed (Tureci, 2003 and Shen-Orr, 2010). The development of the laser capture micro-dissection tool has provided an answer to tissue heterogeneity. With this machine or one of its congeners, specific cell types can be visualized, identified and then procured,

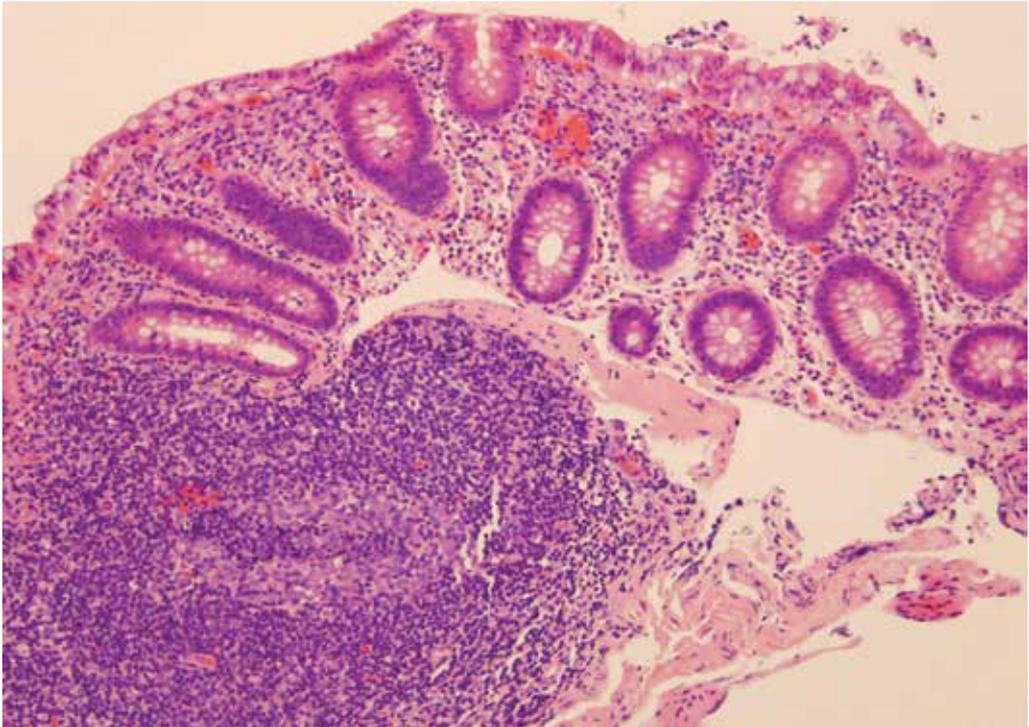


Fig. 1. Histology of normal colonic mucosa. The glands and surface consist of epithelial cells, while the lamina propria contains chronic inflammatory cells. Interspersed in the mucosa are lymphoid aggregates (left side of figure), which cannot be readily discerned at the gross examination level. Without microscopic examination, the proportion of targeted cells, in this case colonic epithelial cells, could account for less than 50% of the cells in sample. Hematoxylin and Eosin stain, 10X

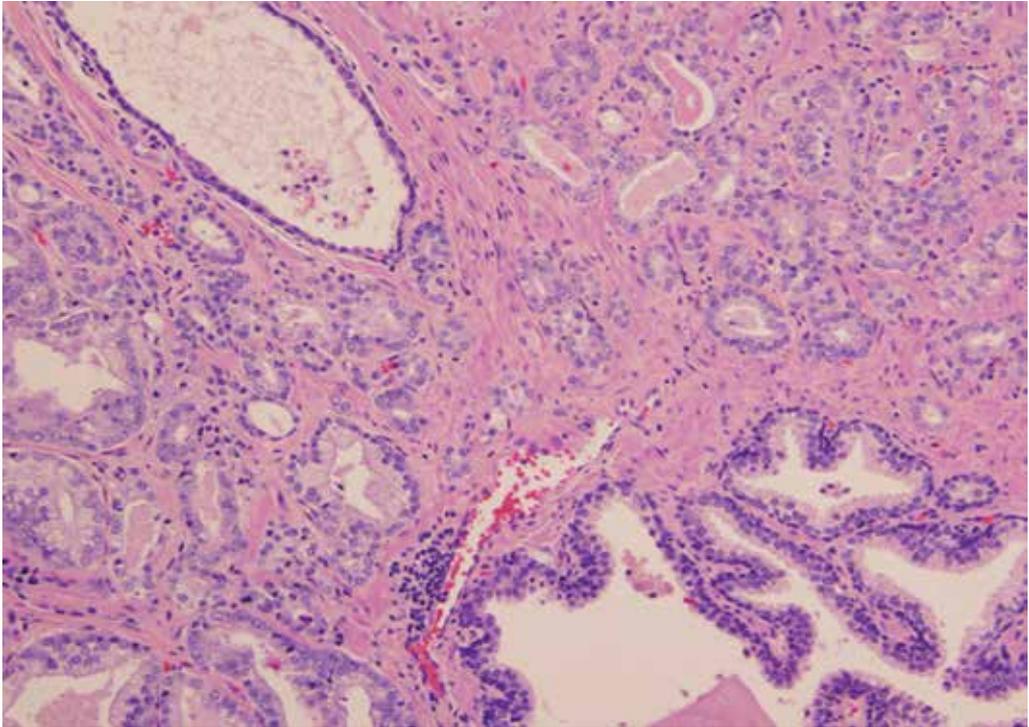


Fig. 2. Histology of prostate cancer. Tumor is in the upper right hand corner, while normal glandular prostatic cells and stroma make up the remainder of the specimen. Hematoxylin and Eosin stain, 10X.

leaving the unwanted contaminating cells with the tissue. It works for both frozen tissue specimens and formalin fixed paraffin embedded tissue. A major drawback with its use however, is the time consuming nature of manually procuring the wanted cells. This issue has been addressed with the development of automated programs, that coupled with cell recognition software, have alleviated the overall time needed to select cells from tissue specimens. The other major drawback with this instrument has been its high start-up cost. Most machines have a six figure price tag, but newer, cheaper versions have been developed. An alternative method has been the use of immunomagnetic beads for the recovery of targeted cells (Mojica, 2006). This approach starts with fresh tissue specimens, that is, before they are fixed, and after a series of manipulations, recovers a highly enriched collection of cells. This method is cost effective, and does not involve any significant expenditure. It has advantages over using straight (un-enriched) biopsy samples in that it can enrich for a targeted cell population. Simple adjustments to the procedure can

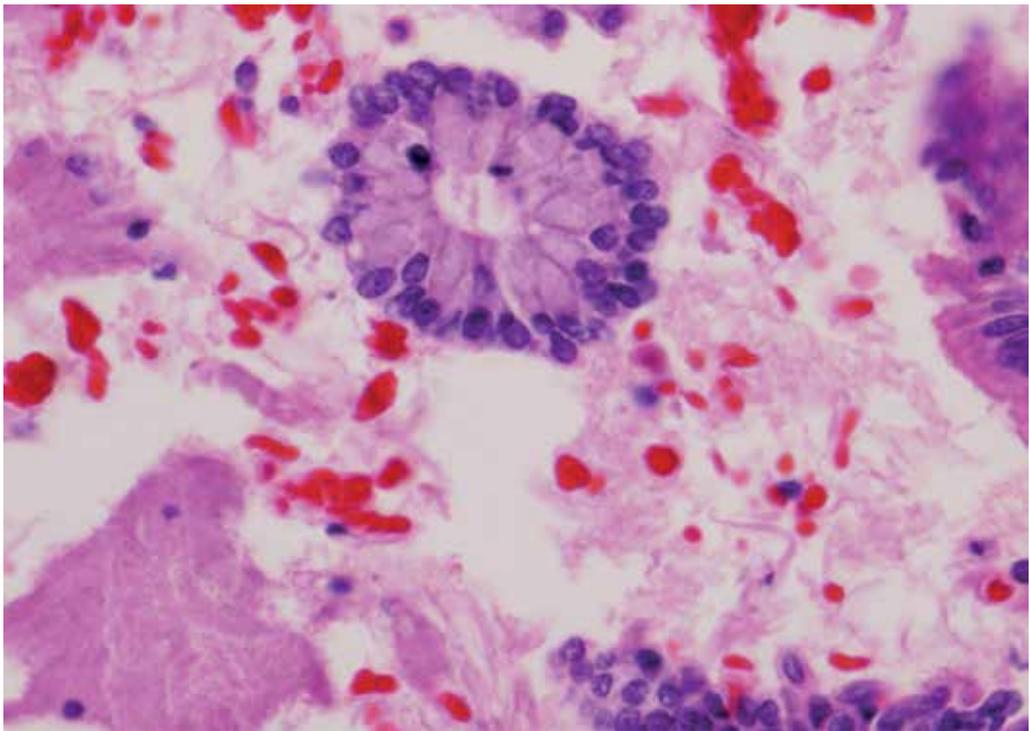


Fig. 3. Cells exfoliated from colonic tissue with no enrichment. Note presence of red blood cells and mucus. Hematoxylin and Eosin stain , 40X.

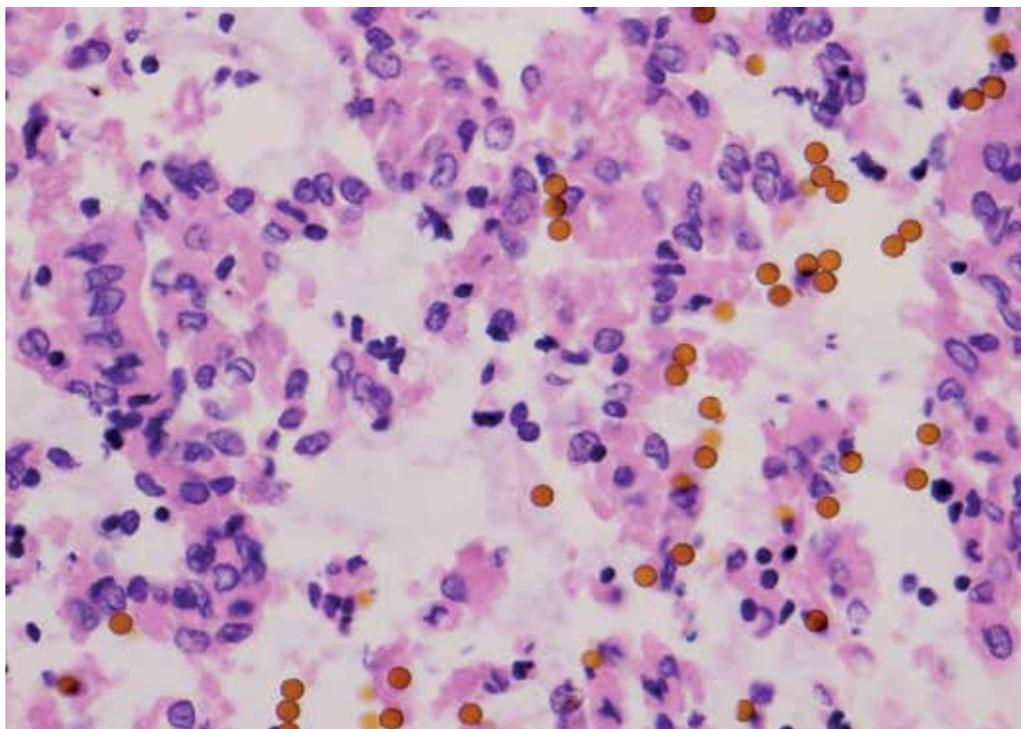


Fig. 4. Recovered cells from enrichment described in text. Yellow dots are immunomagnetic beads. Hematoxylin and Eosin stain 40X

targeted cell population. Simple adjustments to the procedure can be made to eliminate the presence of red blood cells, whose presence is significant in tissue biospecimens (Figure 3). This is particularly important for proteomic, and not so much for nucleic acid studies, as red blood cells have a number of proteins within their cytosol that may contribute to complicate any subsequent un-enriched lysate (Pasini, 2006, and D'Alessandro, 2010). The result of such enrichment is an expansion in the detection of the proteins from the targeted cell (dePetris, 2010). The use of several washes helps eliminate the presence of other contaminating substances, using as an example, excess mucus from colon specimens. Mucus itself is not detrimental to downstream analysis, since it is the secreted product of certain types of colonic epithelial cells. It is however, the location whereby commensal organisms like bacteria habitate, and their inclusion in any sample for downstream analysis could lead to confounding genetic or proteomic results (Qin, 2010). Since the technique can be done on fresh tissue specimens, the problems associated with formalin enumerated earlier are not encountered. Finally, the use of the ber-Ep4 immunomagnetic beads are commercially

available and have been for several years. As ber-Ep4 recognizes an epitope in epithelial cells, this method works nicely as a means for their procurement from specimens with the intent to isolate epithelial cells from the underlying tissue (Figure 4). The overall technique however, is not restricted to ber-EP4, as other antibodies with specificities to other cell surface membrane proteins can be conjugated to the magnetic beads. One of the limiting factors however, is the relative paucity of antibodies available to select from that may be used to positively enrich for a specific cell type from a heterogeneous population. This dearth is attributable to the limited knowledge of the proteome of the plasma membrane, which in turn is due to the difficulties associated with examining molecules with hydrophilic and hydrophobic properties. When the plasma membrane proteome is not known for a specific cell type, an alternative but reportedly equally effective approach would be through negative enrichment, where contaminating cells are targeted and removed from the sample population. This has been used for the examination of sputum with respect for lung cancer diagnosis (Qui, 2008). In cytology, the presence of pulmonary macrophages is essential to document that the material has cells from the lung, and not just the oral cavity. These same cells however, would contribute to the heterogeneity of the sample for any subsequent molecular analysis. Their selection, and that of neutrophils using anti-CD-14 and anti-CD-16 immunomagnetic beads can lead to a significant improvement in the numbers of bronchial epithelial cells recovered. An alternative to using antibodies are aptamers. Aptamers are short single stranded nucleic acid oligomers that can take on a variety of three dimensional shapes, thus allowing them to bind to a wide variety of molecules, with binding affinities similar to monoclonal antibodies (Kim, 2009). One advantage of using aptamers over antibodies is their relative stability. Another is the ability to work with cells without knowing specific proteins. This approach would thus be an alternative to the extensive plasma membrane profiling that would be required in order to identify differentially expressed proteins between normal and tumor cell populations.

5. The advent of molecular analysis in cancer tissue specimens

Despite all these roadblocks to using clinically derived tissue specimens for molecular analysis, their use in clinical cancer care already exists. A perfect example is the use of monoclonal antibodies created to antagonistically bind and inhibit the plasma membrane protein Epidermal Growth Factor Receptor (EGFR) in colorectal cancer and non-small cell lung cancer (Plessec, 2009 and Wang, 2010). Although the EGFR receptor can be recognized by immunohistochemistry in tissue specimens, this assay has been found to not be a reliable means of determining those likely to respond to this type of therapy. However, the identification of those patients most likely to respond is imperative in order to avoid any unnecessary side effects, treatment related costs and delays in the administration of a more effective therapy. Since immunohistochemistry was deemed unreliable as a mechanism that could select patients for EGFR therapy, downstream components of the EGFR signaling cascade were examined. The result of these investigations was the finding that mutations in the KRAS gene could identify those patients who would not be responsive to monoclonal antibody therapy directed at the EGFR receptor. In unselected patients, the number of patients that responded to cetuximab, the antibody directed against EGFR, was between 10-20%, but when patients were selected for treatment based on the criteria of no KRAS mutations, this percentage rose to 60%. The detection of these mutations can be accomplished by a number of different methods, the common denominator being that they

evaluated the DNA sequence of the KRAS gene within tumor cells. The issues previously described associated with DNA and formalin fixation now have practical clinical relevance. Again, these issues relate mostly to template degradation and tissue purity. For the former, the amplified product must be designed to be small enough to account for degradation yet long enough to enable specificity. For tissue purity, most laboratories require the tumor component in the tissue to be equal to or greater than 75%. The presence of contaminating normal cells raises the possibility that the DNA from these cells may be assayed instead of the tumor cell's DNA, resulting in a normal sequencing electropherogram or false negative finding for the relevant mutations. In some specimens the amount of contaminating normal cells is significant, and although laser capture microdissection can be used, the transference of this predominantly research tool to the clinical setting is prohibitive due to its labor intensive nature. A promising approach is the technique of "cold-PCR", wherein mutant alleles in tumor cell can be amplified and subsequently sequenced when specific alleles from these tumor cells comprise only a minority population in a tissue sample (Li, 2008). A recent report documented the ability to detect mutations in tumor cells when they constitute only 20% of the tissue sample using the technique of Cold-PCR (Yu, 2011). If no other developments in tissue preparation are introduced, this method may represent an approach that may become a standard assay for tissue biospecimens.

6. Changes in the clinical arena warranting changes in biospecimen preparation

Although the technique of cold-PCR will prove very useful in the evaluation of tumor specimens for specific mutations, its application or modification to whole genome profiling is unknown. Despite an ever expanding database of information that is characterizing oncogenesis, there still remains a significant amount of knowledge to be gained. A recent initiative to correlate the molecular signatures of tumor tissue specimens with the corresponding cancer patient's clinical parameters underscores the need to identify biomarkers that will aid in cancer care. As such, the tissue biospecimen will continue to play a significant role in cancer diagnosis and treatment. One lesson that is becoming increasingly more apparent is that material that is not fixed serves as the best medium from which to start (De Rienzo, 2010 and Jimeno, 2010). This is a reiteration of what has previously been documented, but applied to actual biospecimens. The nature of the unfixed specimen in the form of fine-needle aspirates however, has one major drawback. The technique of fine needle aspiration involves inserting a needle into a mass, and pulling the needle in and out of the mass so that tissue fragments are obtained. Since the tool is a needle, the tissue fragments are minute. Obtaining a diagnosis based on cytology requires extensive training in pathology and its subspecialty, cytopathology. Without the architecture, the presence of malignant cells can be diagnosed, but the diminutive nature of the specimen sometimes precludes assigning what type of tumor it is. This is particularly true for tumors of the lung, where a process similar to fine needle aspiration, in terms of quantities of cells recovered, is performed. Whether the procedure is a bronchoscopy with material obtained as washings or brushings, or a trans-thoracic needle biopsy, the material recovered will be diminutive. Again, the diagnosis of malignancy can be made, but for poorly differentiated tumors, the classification into adenocarcinoma, squamous cell carcinoma or large cell undifferentiated carcinoma, which is now often requested to help guide therapy, will be extremely difficult if next to impossible without a large enough piece

of tissue to showcase architecture (Wallace, 2009). For adenocarcinoma, the presence of five or more vacuoles in two consecutive high power fields is required to differentiate adenocarcinoma from large cell undifferentiated carcinoma. However, the diminutive nature of these specimens may not even fulfill this size criterion. If not enough material is available to make a definitive diagnosis, the patient may require another invasive procedure that may aim to get tissue instead of cells. A similar situation is occurring with the biopsy of small kidney tumors where cryoablation instead of resection will be performed. Although attempts to obtain tissue so that a histologic diagnosis can be made, often times the specimen is too small and not recovered after tissue processing. The absence of an established diagnosis means that should another mass lesion arise in a patient treated by cryoablation, it will not be known if the mass represents a metastatic or primary tumor. Although tumors of unknown origin can be worked up through conventional immunohistochemistry, the absence of a previous diagnosis incurs additional costs for this work-up. Thus, at today's medical environment, a histologic diagnosis is still imperative, and thus in order to optimally process any specimens in the near future, tools will need to be developed that are capable of allowing the evaluation of these cells at both the cytologic and molecular level.

In order to circumvent the problem of enabling a diagnosis on small biopsy specimens all the while providing some material for molecular analysis, investigators have begun designing platforms capable of recovering specific subsets of cells from clinical specimens (Weigum, 2010, Wan, 2010, and Sun, 2010). These platforms are ideal for taking on the problems of small amounts of cells or tissues. Although immunohistochemistry is an extremely useful diagnostic tool in the laboratory, the amount of tissue used per assay is significant. When a battery of stains is needed to be performed, each 4 or 5 micron thick section may end up exhausting the amount of recoverable tissue. Precious tissue can also be lost at the microtome step, where some material may be lost just due to the process of aligning the block and blade. Flow cytometry has been used extensively for years for hematologic malignancies, but its requirement for ample amounts of starting material negate its consideration as a tool for biopsy specimens. Thus, platforms based on microfluidics appear to be best suited to interrogate these small biopsy specimens. Using techniques similar to the one previously described above that help enrich for targeted cells, these enriched cell populations can then be introduced to a microfluidic platform for subsequent evaluation. One noticeable limitation of these platforms however, is the absence of the ability to visually evaluate these cells. Although the enrichment approach previously described is capable of selecting for epithelial cells from the heterogeneous cell population that comprises tissue, the epitope recognized by the ber-EP4 antibody can be present in both non-neoplastic and malignant epithelial cells. Thus, until plasma membrane proteins are identified and characterized that are specific for either normal or neoplastic epithelial cells, enrichment is limited to epithelial cells. This is not so much a limitation in certain tumors like colonic adenocarcinomas, where the tumor mass effaces the surrounding tissue so that it is composed of tumor epithelial cells and surrounding stroma, but will not be as effective in other tumors like the prostate, where the tumor cells infiltrate between normal prostatic glands and stroma. For microfluidic platforms, the need to visualize cells will allow investigators whether to attribute molecular findings appropriately to neoplastic cells or not. At the clinical level, the ability to visualize cells allows for a diagnosis, and determination whether the biopsy contains tumor cells and thus is representative of a mass versus no tumor cells and therefore not representative of a mass.

In the development of such a platform, cytologic examination plays the vital role of stratifying whether the cells are tumor or not. Identifying the type of tumor is not so vital as the cells, or specifically their contents, can be interrogated through a variety of downstream assays. The platform needs to be designed such that the cells are not permanently immobilized on the glass slide. Once visualized, they should either be able to be eluted out as intact cells, lysed so that the molecules from them can be assayed, or have a lab-on-a chip assay integrated to a portion of the slide. Thus, such a microfluidic platform should allow for conventional cytologic based examination for screening purposes, followed by molecular interrogation for tumor designation. A period of molecular annotation will take place within the next decade wherein reliable biomarkers will be discovered and validated for specific tumor types. The transition to molecular identification of tumor tissue has already begun and has shown early success in helping determine the site of origin in several instances (Ismael, 2006, Greco, 2010, and Monzon, 2010). Molecular tumor classification is not yet a reality, with histologic examination still the gold standard for diagnosis (Kotsakis, 2010). However, in the next decade, when molecular analysis of tumor specimens will occur concurrent with histologic examination, a gradual shift in emphasis may occur. With Personalized Medicine, the identification of target molecules may eventually become more important for cancer treatment than histology.

6.1 An integrated approach to optimize the biospecimen for cancer care

Taking into consideration all the factors that may bias the macromolecular profiles of a specimen, a tentative approach can be postulated that would recover cells bearing the most similar molecular disposition to their *in vivo* counterparts from clinical material. To address preanalytical bias, the specimen most suited for analysis may be the biopsy. Clinically, the patient undergoing a biopsy of a tumor would not be subjected to the depth of anesthesia that the patient undergoing a surgical resection would be for the same tumor. The degree of preparation of the patient is less, with the process most often an out-patient procedure. Therefore, confounding issues that potentially could influence, but are hard to confirm, the molecular profile of a cell, like administration of intravenous fluids, antibiotics and anxiety to name just a few, are not introduced (Compton, 2006). For issues related to warm ischemia, the short time required to obtain a biopsy is also preferred to the comparatively longer time required to excise a portion of organ with tumor (Schlomm, 2008). Once the biopsy is obtained, the next step would be sample stabilization, an attempt to immediately try to preserve the molecules within the cells. For the more labile molecules like RNA, this can be achieved by immersing the specimen in RNA Later (Mutter, 2004) or for proteins, through heat denaturation (Svensson, 2008). For the latter, the use of heat inactivation helps to preserve post-translational modifications, a form of intercellular signaling with a transient time frame within proteins that can be lost or altered during cold ischemia, or the period after extirpation (Rountree, 2010). Once stabilized, the cells should be disaggregated from the tissue and unwanted elements and contaminating normal cells eliminated through some form of enrichment. It should be noted that at no point in this proposed procedure fixation with formaldehyde is involved. The cells then should be examined cytologically, to confirm that the targeted cells are indeed present, that they are enriched and to what percentage they constitute the sample. This should be done on some fluidic based platform, so that these same cells can be further examined at the molecular level after microscopic diagnostic evaluation. The cells are then eluted and their contents extracted for downstream

molecular analysis. Alternatively, other methods may develop wherein diagnostics can be performed on the immobilized cell. Through the development of the above protocol, the maximal amount of informative data can be extracted from the biospecimen, with a combination of traditional anatomic (cytologic) related diagnostics and future molecular interrogation for treatment guidance obtained from an optimally recovered specimen.

6.2 Biospecimens as the focal point of cancer care

The development of a platform capable of enriching unfixed, preserved tumor cells from tissue specimens will enhance clinical research. For some years now it has been known that one of the major obstacles to progression in the post-genomic era has been the absence of high quality biospecimens. An attempt in the previous decade to obtain 500 cases each of specific types of brain, lung and ovarian cancer for gene sequencing initially failed at the accrual stage. Although archives in pathology departments have numerous amounts of preserved tissue specimens, issues with percentage of tumor content, necrosis or even proper consent have become factors preventing their use at a national level. Differences in the procurement, handling and storage of samples may also play a role in the differences noted between similar tumors from different institutions. Nevertheless, the National Cancer Institute has embarked on an initiative to address those issues related to poor biospecimen procurement and to rectify them. The need for high quality biospecimens is reflected in their incorporation in innovative study designs for clinical trials, like the new phase 0 trial. In this new type of study, the need for patient tumor tissue is necessary in order to determine the biological effectiveness of the therapeutic agent being tested. In phase 1 trials the need for high quality biospecimens is also warranted, to match the administration of specific agents to patients possessing a specific molecular signature, deemed through previous experimentation, to be the target of those agent(s). This approach identifies useful therapeutic agents in a subset cohort from the general cancer population, where if an empiric approach had been used, the agent may not have resulted in a significant enough number of patients to warrant further testing. Molecular testing should also be able to identify patients who will be resistant to certain agents, thus guiding the clinician to alternative approaches and avoiding any unwarranted side effects and unnecessary costs. In the future, the tissue biospecimen will serve many roles, with two being the basis of future molecular, proteomic and/or metabolomic research and the other being the starting point for the treatment of the cancer patient. The development of newer biotechnological tools will certainly result in a continuing evolution of the tissue biospecimen's role in the treatment of cancer.

7. Conclusions

The role of tissue biospecimens in cancer care has evolved over time from observation and little impact, to anatomic and molecular diagnostics with high impact on guidance of clinical treatment. This evolution has come about based on the changes in the evaluation of the specimen, from humble beginnings wherein only the gross anatomic features were noted in malignant tissue and the patient's clinical course observed, to the biotechnological revolution and the high throughput capabilities created that can profile molecular alterations within cells, identify therapeutic targets and selectively lead to the administration of reagents best suited for each individual cancer patient. Advances in

science continue to influence the evaluation of the biospecimen, with an urgent need to develop and adapt newer approaches capable of maximizing the amount of informative data that can be derived from these samples. The importance of the tissue biospecimen in the care of the cancer patient has grown dramatically. It is safe to assume its role in the care of the cancer patient of the near future will become even more vital than it is today.

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Currently there have been many armamentaria to be used in cancer treatment. This indeed indicates that the final treatment has not yet been found. It seems this will take a long period of time to achieve. Thus, cancer treatment in general still seems to need new and more effective approaches. The book “Current Cancer Treatment - Novel Beyond Conventional Approaches”, consisting of 33 chapters, will help get us physicians as well as patients enlightened with new research and developments in this area. This book is a valuable contribution to this area mentioning various modalities in cancer treatment such as some rare classic treatment approaches: treatment of metastatic liver disease of colorectal origin, radiation treatment of skull and spine chordoma, changing the face of adjuvant therapy for early breast cancer; new therapeutic approaches of old techniques: laser-driven radiation therapy, laser photo-chemotherapy, new approaches targeting androgen receptor and many more emerging techniques.

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