IntechOpen

Breast Cancer

Current and Alternative Therapeutic Modalities

Edited by Esra Gunduz and Mehmet Gunduz

BREAST CANCER – CURRENT AND ALTERNATIVE THERAPEUTIC MODALITIES

Edited by **Esra Gunduz** and **Mehmet Gunduz**

Breast Cancer - Current and Alternative Therapeutic Modalities
http://dx.doi.org/10.5772/1746
Edited by Esra Gunduz and Mehmet Gunduz

Contributors

Rita Nahta, Sylvia Shabaya, Zhiwei Hu, Romulus Scorei, Ben O. de Lumen, Chia-Chien Hsieh, Blanca Hernández-Ledesma, Weena Jiratchariyakul, Tanawan Kummalue, Joaquín M. Campos, María C. Núñez, Ana Conejo-García, Olga Cruz-López, Gopal Kundu, Reeti Behera, Mansoor Ahmed, Vinit Kumar, Kathrin H. Kirsch, Joerg Kumbrink, Danuta Drozdowska, Chong-Zhi Wang, Guang-Jian Du, Chun-Su Yuan, Yohei Miyagi, Shiro Koizume, Weimin Fan, Meihua Sui, Donghai Jiang, Peter Fedorocko, Peter Ferenc, Ján Koval', Peter Solár, Jaromír Mikeš, Peter Crooks, Narsimha Reddy Penthala, Thirupathi Reddy Yerrmreddy, Vijaya Kumar Sonar, Nikhil Reddy Madadi, Stan Kubow, Chujian Chen, Hing Man Chan, Fatemeh Rahbarizadeh, Fatemeh Rahimi Jamnani, Farnoush Jafari Iri-Sofla, Sung-Woo Kim, Grey Atteridge Wilkinson, Adam Elwi, Rhonda Rosengren, Mélanie Di Benedetto, Amanda Harvey, Rajpal Burmi, Vicente Micol, María P. Menéndez-Gutiérrez, Enrique Barrajón-Catalán, Alberto Falco, Angela Catania, Miguel Saceda, Martin Berger, Michael Zepp, Franz-Paul Armbruster, Tobias Bäuerle, Victoria Elazar, Gershon Golomb, Rafat Siddiqui, Jeffrey Altenburg, Graziella Joanitti, Sonia Freitas, Ricardo Azevedo, Masa-Aki Shibata, Junji Morimoto, Eiko Shibata, Mariko Harada-Shiba, Shigekazu Fujioka

© The Editor(s) and the Author(s) 2011
The moral rights of the and the author(s) have been asserted.
All rights to the book as a whole are reserved by INTECH. The book as a whole (compilation) cannot be reproduced, distributed or used for commercial or non-commercial purposes without INTECH's written permission.
Enquiries concerning the use of the book should be directed to INTECH rights and permissions department (permissions@intechopen.com).
Violations are liable to prosecution under the governing Copyright Law.

CC BY

Individual chapters of this publication are distributed under the terms of the Creative Commons Attribution 3.0 Unported License which permits commercial use, distribution and reproduction of the individual chapters, provided the original author(s) and source publication are appropriately acknowledged. If so indicated, certain images may not be included under the Creative Commons license. In such cases users will need to obtain permission from the license holder to reproduce the material. More details and guidelines concerning content reuse and adaptation can be foundat http://www.intechopen.com/copyright-policy.html.

Notice

Statements and opinions expressed in the chapters are these of the individual contributors and not necessarily those of the editors or publisher. No responsibility is accepted for the accuracy of information contained in the published chapters. The publisher assumes no responsibility for any damage or injury to persons or property arising out of the use of any materials, instructions, methods or ideas contained in the book.

First published in Croatia, 2011 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019.
IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

Additional hard and PDF copies can be obtained from orders@intechopen.com

Breast Cancer - Current and Alternative Therapeutic Modalities
Edited by Esra Gunduz and Mehmet Gunduz

p. cm.

ISBN 978-953-307-776-5

eBook (PDF) ISBN 978-953-51-6557-6

We are IntechOpen, the world's leading publisher of Open Access books

Built by scientists, for scientists

4,100+
Open access books available

116,000+
International authors and editors

120M+
Downloads

Our authors are among the

151
Countries delivered to

Top 1%
most cited scientists

12.2%
Contributors from top 500 universities

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Meet the editors

Dr. Esra Gunduz is Associate Professor of Medical Genetics at Fatih University Medical School, Ankara, Turkey. She completed her PhD program in 2005 at Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Japan. She was awarded by the Japan Society for the Promotion of Science (JSPS) as a post-doctoral fellow in 2006. In 2009 she was appointed as Assistant Professor at Fatih University, and in 2010 she was promoted to Associate Professor at the same faculty. Her research focuses mainly on tumor suppressor genes. In her lab she leads various ongoing projects for her research teams. She contributed to the literature with forty articles in peer reviewed journals and over sixty presentations both in national and international meetings.

Dr. Mehmet Gunduz graduated from the Faculty of Medicine of Hacettepe University in Ankara, Turkey in 1990. From 1995 until 2009 he studied and worked in Japan, both at Wakayama Medical University and Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences at various academic levels (from PhD candidate to Assistant Professor). During this period, he led a discovery and identification of roles of several cancer-related genes such as ING1 and 3. He has also been a visiting scientist in MD Anderson Cancer Center University of Texas, Houston, USA. Since March 2011, he is holding the position of a Professor at the Departments of Otolaryngology and Medical Genetics in School of Medicine Fatih University in Ankara. He has over 150 publications in internationally indexed journals, and over 160 presentations at various levels in national and international meetings. He also served as a reviewer for a number of journals.

Contents

Preface

Cancer is the leading cause of death in most countries and continues to increase mainly because of the aging and growth of the world population as well as habitation of cancer-causing behaviors such as smoking and alcohol. Based on statistics of the GLOBOCAN 2008, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 (Siegel et al. Ca Cancer J Clin 61:212-236, 2011). Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths. Thus cancer researches, especially breast cancer, are important to overcome both economical and physiological burden. The current book on breast cancer aims at providing information about recent clinical and basic researches in the field. The book includes chapters written by well-known authors, who are worldwide experts in their research areas and mainly covers therapeutic applications in breast cancer. Other topics covered in this book are: therapeutic modalities targeting signaling pathways, coagulation factor VII as well as extracellular matrix, use of anti-tumor compounds, use of herbal medicine and derivatives as well as application of alternative medicine, and recent novel therapies including gene therapy, nanoparticles as well as other experimental methods, and finally, the issue of chemoresistance is also discussed. We hope that the book will serve as a good guide for the scientists, researchers and educators in the field.

Assoc. Prof. Dr. Esra Gunduz
Prof. Dr. Mehmet Gunduz
Fatih University Medical School
Turkey

Part 1

Targeting Signaling Pathways and Extracellular Matrix

1

Novel Therapeutic Strategies and Combinations for HER2-Overexpressing Breast Cancer

Sylvia Shabaya and Rita Nahta
Emory University,
USA

1. Introduction

Approximately 20-30% of breast cancers show increased expression of the HER2 receptor tyrosine kinase. Elevated levels of HER2 are associated with aggressive disease, high metastatic potential, and reduced survival versus other breast cancer subtypes (Slamon, 1987). Trastuzumab (Herceptin) is a monoclonal antibody targeted against an extracellular region of HER2 (Carter, 1992). Clinical trials have shown that 15-30% of patients with HER2-overexpressing metastatic breast cancer respond to single-agent trastuzumab for a median duration of approximately 10 months (Baselga, 1996; Cobleigh, 1999). Response rates improve when trastuzumab is combined with chemotherapy in patients with HER2-overexpressing metastatic breast cancer (Esteva, 2002; Slamon, 2001). A subset of trastuzumab-resistant breast cancers respond to the dual EGFR/HER2 kinase inhibitor lapatinib, although the majority (70% or more) show primary resistance (Geyer, 2006). Similar to trastuzumab treatment, clinical trials with lapatinib indicated that the median duration of response to lapatinib in a heavily pre-treated, trastuzumab-refractory population was less than one year (Geyer, 2006). Hence, resistance to clinically available HER2-targeted agents is a major concern in the treatment of patients with HER2-overexpressing metastatic breast cancer.

2. HER2 and breast cancer

The human epidermal growth factor receptor 2 (HER2) is overexpressed in approximately 25% of invasive breast carcinomas. HER2 is a member of the epidermal growth factor receptor (EGFR) family, which also contains two other receptors, HER3 and HER4 (Fig. 1). Each of these cell surface receptors has an extracellular ligand-binding domain and a transmembrane-spanning domain (Nielsen, 2008). All HER family receptors except HER2 bind specific ligands that induce conformational changes and receptor homo- or hetero-dimerization. Several HER family ligands have been identified including transforming growth factor alpha (TGFa), epidermal growth factor (EGF), and the heregulins (Nielsen, 2008). In addition, all except HER3 contain an intracellular tyrosine kinase domain. Receptor dimerization activates the kinase function of receptors, leading to receptor auto- or trans-phosphorylation. The phosphorylated tyrosine residues serve as docking sites for SH2 and PTB-domain containing proteins, which links the receptors to multiple cell survival and proliferation pathways including the phosphatidylinositol-3 kinase (PI3K) and mitogen-

activated protein kinase (MAPK) cascades (Spector, 2009; Graus-Porta, 1997). HER2 is the preferred dimerization partner for the other HER family members, as HER2 heterodimers have increased ligand binding affinity and increased catalytic activity relative to other heterodimer complexes (Spector, 2009; Graus-Porta, 1997). In particular, the HER2-HER3 heterodimer has the strongest kinase activity and transforming ability, as HER3 possesses multiple PI3K docking sites in its cytoplasmic tail.

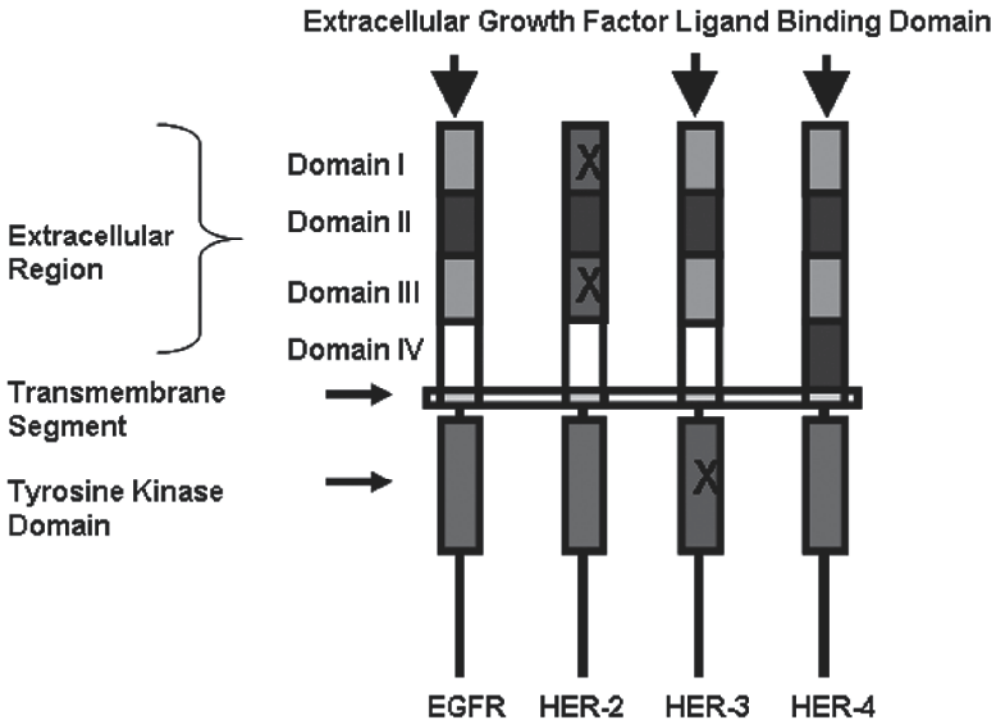


Fig. 1. HER/erbB family of growth factor receptors. The four members of the EGFR family are illustrated. The inactive ligand-binding domains of HER2 and the inactive kinase domain of HER3 are denoted with an X. Trastuzumab binds to domain IV of the extracellular region of HER2.

2.1 Targeting HER2 in breast cancer

Patients who are diagnosed with HER2-overexpressing breast cancer have a poor prognosis, and shorter progression-free and overall survival compared to patients with other subtypes of breast cancer (Eccles, 2001). HER2-overexpressing tumors have been found to be larger in size, and higher in nuclear grade, S phase fraction, and aneuploidy (Nielsen, 2008). Traditional cancer treatments have targeted DNA replication or cell division, leading to nonspecific cytotoxicity (Oakman, 2010). The identification of abnormal signaling from HER2 led to the development of trastuzumab (Herceptin) (Genentech, San Francisco, CA, USA), which is the first drug to target the genetic lesion or oncogenic addiction found in patients with HER2-overexpressing breast cancer. Clinically, trastuzumab was found to significantly enhance the effectiveness of conventional chemotherapies. However, the median duration of response was less than one year, indicating rapid development of

resistance. The precise mechanism of action of trastuzumab is unclear, but it is thought to involve HER2 downregulation (Cuello, 2001; Gajria, 2011), selective inhibition of HER2-HER3 heterodimerization (Junttila, 2009; Gajria, 2011), prevention of HER2 extracellular domain proteolytic cleavage (Molina, 2001; Gajria, 2011), and activation of an immune response including antibody-dependent cellular cytotoxicity (Sliwkowski, 1999). As a single agent, trastuzumab achieved an overall response rate for a median duration of about nine months (Baselga, 1996; Cobleigh, 1999; Nielsen, 2008; Slamon, 2001). The low response rate indicates that many patients with HER2-overexpressing breast cancer have primary resistance to trastuzumab, while the short duration of response indicates rapid development of acquired resistance. Multiple mechanisms contributing to trastuzumab resistance have been proposed, resulting in multiple approaches to potentially treat resistant cancers (Table 1).

Target	Role in trastuzumab resistance
PI3K	Increased PI3K signaling due to *PIK3CA* mutations or PTEN loss was reported in trastuzumab-resistant cancers
mTOR	As a downstream molecule of PI3K, mTOR has become a target of inhibition in resistant cancers; multiple mTOR inhibitors are in advanced phases of clinical development
IGF-IR	Increased expression of IGF-IR has been shown to reduce response to trastuzumab; increased IGF-IR overexpression was associated with lower response to neoadjuvant trastuzumab; IGF-IR/HER2 interaction and crosstalk were associated with acquired resistance
Src	Trastuzumab-mediated inhibition of Src activity appears to be important to its anti-cancer activity; resistance to trastuzumab was associated with PTEN loss and increased Src activity; targeting Src with dasatinib or genetic knockdown blocked growth of resistant cancers
Cdk2	Reduced p27kip1 levels or amplification of *cyclin E* gene have been reported to result in increased cdk2 activity in trastuzumab-resistant cancers

Table 1. Potential pharmacologic targets in trastuzumab-resistant HER2-positive breast cancers.

3. Targeting PI3K/mTOR signaling in HER2-overexpressing breast cancer

HER2 signaling is initiated upon receptor dimerization, which induces phosphorylation of tyrosine residues within the receptor cytoplasmic domain. The phosphorylated residues serve as docking sites for adaptor proteins and link the receptor to downstream survival pathways including the PI3K/Akt/mTOR axis (Spector, 2009). The PI3K pathway is frequently hyper-activated in many cancers. An association between oncogenic PI3K mutations and trastuzumab resistance was found in a study examining HER2-overexpressing tumors from patients with trastuzumab-refractory disease (Berns, 2007). About 25% of tumors analyzed had PIK3CA mutations, and reduced phosphatase and tensin homolog (PTEN) expression was present in 22% of the tumors. Immunohistochemistry studies performed in a retrospective analysis of *HER2*-amplified breast tumors treated with trastuzumab plus taxanes showed a postive correlation between PTEN down-regulation and tumor response (Nagata, 2004). To evaluate the role of PI3K

post-trastuzumab exposure, tumors that had progressed on trastuzumab were analyzed for changes in PI3K signaling. The findings demonstrated that PI3K mutations and PTEN loss were identified in patients who had initially responded to trastuzumab; reduced PTEN expression was identified in tumors that had developed trastuzumab resistance, but had not been identified before trastuzumab treatment. This finding indicates that PI3K mutations can occur as a result of trastuzumab treatment in some tumors (Kalinsky, 2009; Sakr, 2010; Gajria, 2011). Thus, there is ample rationale for co-targeting PI3K and HER2 in breast cancer.

Activated Akt regulates several downstream signaling molecules including mTOR, a highly conserved 289-kDa serine/threonine kinase that plays roles in cell proliferation, survival, and motility (Lang, 2010). mTOR activation is initiated when phosphorylated PI3K/Akt inhibits the TSC1/TSC2 complexes, thereby preventing Rheb from inhibiting mTOR. mTORC1 (mTOR, Raptor, mLST8/GBL and PRAS40) and mTORC2 (mTOR, RICTOR, mLST8/GBL, SIN1, and PROTOR/PRR5) are the two distinct complexes through which mTOR exerts cellular effects. The complexes have different functional roles, with mTORC1 having been implicated in cell cycle progression, motility, and protein biosynthesis, while mTORC2 regulates cytoskeleton organization, and regulates cell growth and survival (Wullschleger, 2005; Van der Heijen, 2011).

Preclinical *in vivo* studies in which mice were treated with single agent trastuzumab, the mTOR inhibitor rapamycin, or a combination of trastuzumab plus rapamycin showed that the combination was more effective at inducing tumor regression than either of the single agent treatments (Miller, 2009). In cell culture experiments using the rapamycin analogue RAD001, a greater amount of growth inhibition was observed with combination mTOR inhibition plus HER2-targeting than with either drug alone. Trastuzumab partially decreased PI3K activity, but not mTOR activity (Miller, 2009). Increased PI3K signaling is a validated mechanism of trastuzumab resistance, but its association with lapatinib resistance is yet to be determined due to conflicting data (Eichhorn, 2008; O'Brien, 2010). Patients with HER2-overexpressing breast cancer who have developed resistance to trastuzumab may be given the dual EGFR/HER2 tyrosine kinase inhibitor lapatinib. Response to single agent lapatinib is less than 25%, indicating cross-resistance between trastuzumab and lapatinib (Blackwell, 2010; Eichhorn, 2008). As with trastuzumab treatment, the small subset of patients who initially responded to lapatinib eventually developed resistance, at which point there is no standard therapeutic approach available. Phase I trials have indicated that in patients with trastuzumab-resistant, heavily pretreated breast cancer, combined everolimus plus trastuzumab could be a promising treatment (Jerusalem, 2011). It is thought that the inability of trastuzumab to completely inhibit PI3K/Akt/mTOR signaling may permit escape from growth inhibition; mTOR inhibitors would thus synergize with trastuzumab to prevent the continued growth of HER2-dependent cancer cells.

In contrast to PI3K, very little has been published regarding the role of MAPK signaling in trastuzumab resistance. Our data suggests that phosphorylation of Erk1/2, which is a marker of MAPK activity, is not increased in resistant cells (Fig. 2A). Inhibition of MEK (upstream of Erk1/2) using a small molecule MEK kinase inhibitor called PD0325901 reduces p-Erk1/2 levels in parental HER2-overexpressing breast cancer cells and in acquired trastuzumab-resistant and primary trastuzumab-resistant cells (Fig. 2B). However, trastuzumab-naïve and trastuzumab-resistant cells are relatively resistant to PD0325901, in that doses up to 10 uM do not block proliferation of HER2-overexpressing trastuzumab-naïve or resistant cells (Fig. 2C). Thus, our data indicate that MAPK signaling may not be a major mechanism of trastuzumab resistance.

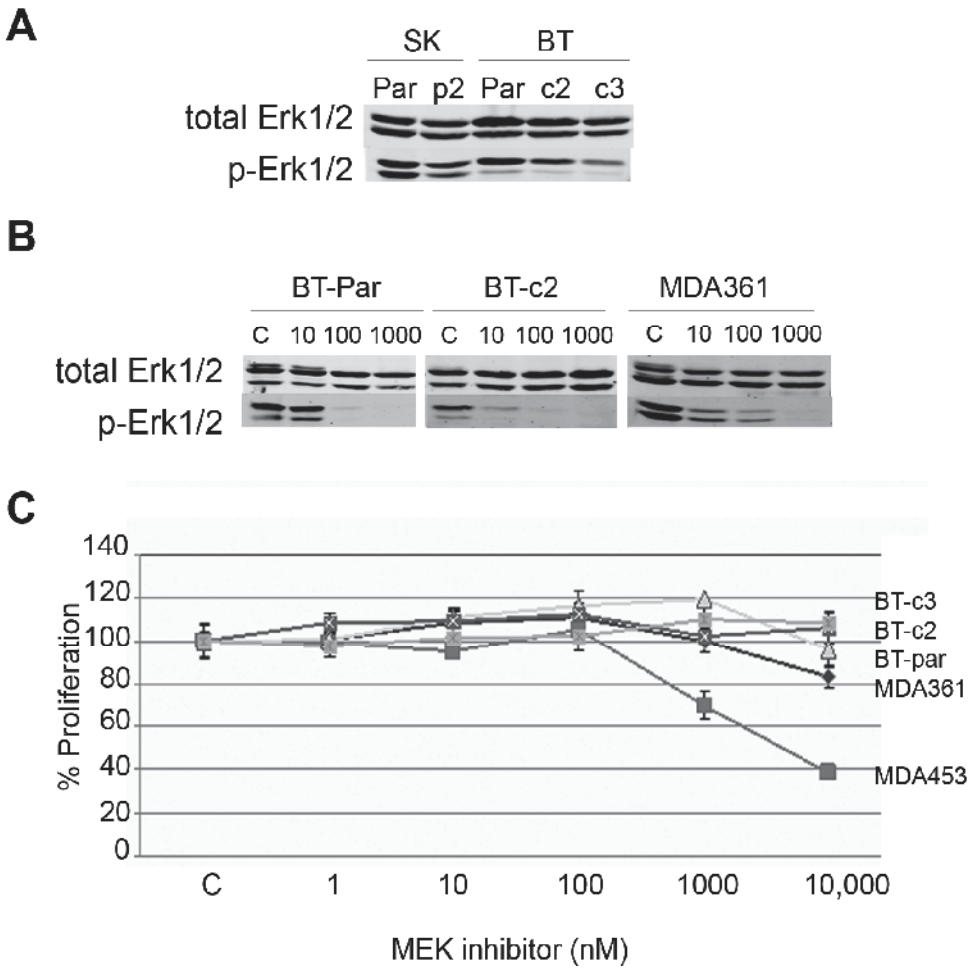


Fig. 2. Role of MAPK signaling in trastuzumab-resistant cells. (A) SKBR3 parental, trastuzumab-resistant pool 2, and BT474 parental, and trastuzumab-resistant clone 2 and clone 3 cells were Western blotted for phosphorylated and total Erk1/2. (B) BT-parental, BT-c2 (resistant clone 2), and MDA-MB-361 primary trastuzumab-resistant cells were treated with MEK inhibitor PD0325901 at 10, 100, or 1000nM for 6 hours or with DMSO control (*C*) corresponding to the volume found in the highest dose of PD0325901. Total protein lysates were Western blotted for phosphorylated and total Erk1/2. (C) BT-parental, resistant clone 2 and 3, MDA361, and MDA453 cells were treated with MEK inhibitor PD0325901 at 1, 10, 100, 1000, or 10, 000nM for 48 hours with six replicates per treatment group. Control cells were treated with DMSO corresponding to the volume found in the highest dose of PD0325901. Proliferation was assessed by MTS assay, and is shown as a percentage of control group per line.

4. Targeting IGF-IR signaling in HER2-overexpressing breast cancer

The insulin-like growth factor receptor I (IGF-IR) is a heterotrimeric transmembrane tyrosine kinase receptor that regulates cell metabolism and growth (Chaves, 2010), and has

been associated with increased risk and maintenance of multiple cancers including HER2-overexpressing breast cancer (Esparis-Ogando, 2008; Hankinson, 1998; Surmacz, 2000). Circulating ligands of the insulin-like growth factor (IGF) system include IGF-I and IGF-II, with IGF-I having the highest affinity for IGF-IR. Upon binding to IGF-IR, a receptor conformational change is induced that leads to tyrosine phosphorylation and activation of several downstream survival signaling pathways such as the Ras/Raf/mitogen activated protein kinase pathway (MAPK), and the PI3K/Akt/mTOR pathway. Activation of these pathways results in cell cycle progression and resistance to apoptosis (Chaves, 2011; Adams, 2000). The IGF binding proteins (IGFBPs) modulate IGF-IR activity by binding to the IGF ligands thereby sequestering them and preventing ligand-induced receptor activation (Adams, 2000). Higher levels of circulating IGF-I have been linked to trastuzumab resistance in HER2-overexpressing breast cancer, with the addition of IGFBP3 decreasing IGF-IR activity, and subsequently resulting in an increased response to trastuzumab (Lu, 2001; Jerome, 2006).

We found by gene microarray analysis that IGFBP3 and IGFBP5 were down-regulated in resistant versus sensitive cells (Table 2). However, ELISA of secreted IGFBP3 (Fig. 3A) or real-time PCR analysis of endogenous IGFBP3 or IGFBP5 transcript level (Fig. 3B) failed to show any differences in IGFBP3 or IGFBP5 level in resistant versus parental cells. Thus, our data do not support down-regulation of IGFBP3 or IGFBP5 as a mechanism of increased IGF-IR signaling in trastuzumab resistance.

Gene Name	Fold Change	ILMN_GENE	DEFINITION
IGFBP5	-20. 55848937	IGFBP5	Homo sapiens insulin-like growth factor binding protein 5 (IGFBP5), mRNA.
IGFBP5	-20. 0185274	IGFBP5	Homo sapiens insulin-like growth factor binding protein 5 (IGFBP5), mRNA.
IGFBP3	-7. 77282369	IGFBP3	Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 2, mRNA.
PKIA	-6. 484521044	PKIA	Homo sapiens protein kinase (cAMP-dependent, catalytic) inhibitor alpha (PKIA), transcript variant 7, mRNA.
IGFBP3	-6. 193624741	IGFBP3	Homo sapiens insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 1, mRNA.
PKIA	-5. 371909749	PKIA	Homo sapiens protein kinase (cAMP-dependent, catalytic) inhibitor alpha (PKIA), transcript variant 6, mRNA.
BASP1	-4. 444496135	BASP1	Homo sapiens brain abundant, membrane attached signal protein 1 (BASP1), mRNA.
HERC6	-4. 048474978	HERC6	Homo sapiens hect domain and RLD 6 (HERC6), mRNA.
FRAS1	-3. 988854857	FRAS1	Homo sapiens Fraser syndrome 1 (FRAS1), mRNA.
THBS1	-3. 966312615	THBS1	Homo sapiens thrombospondin 1 (THBS1), mRNA.

Table 2. Genes that are down-regulated in SKBR3- and BT474-derived acquired trastuzumab-resistant cells versus parental SKBR3 and BT474 cells by 4-fold or more.

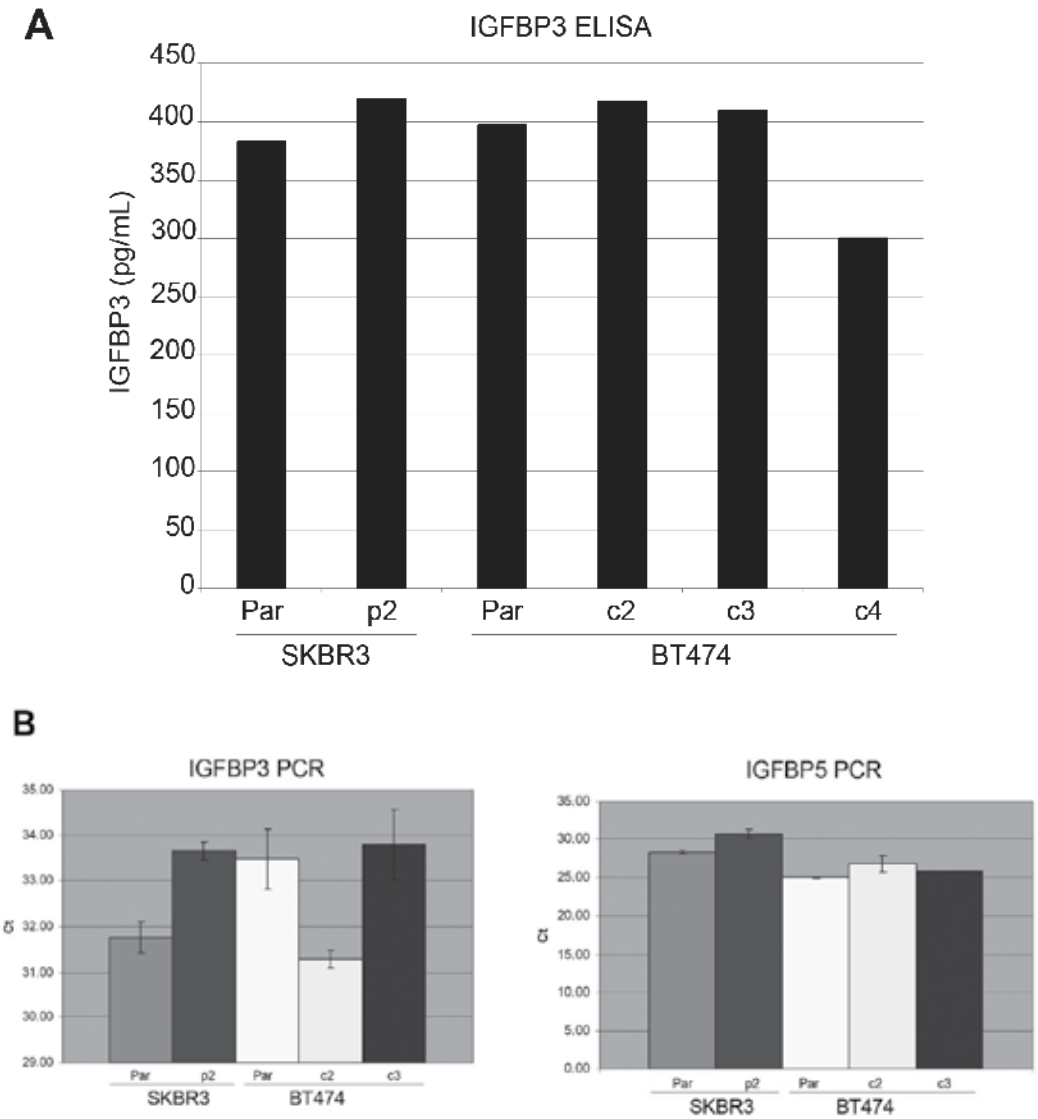


Fig. 3. IGFBP3 and IGFBP5 in resistant and sensitive cells. (A) Secreted IGFBP3 was assessed by ELISA in SKBR3 parental, resistant pool 2, BT474 parental, resistant clone 2 and clone 3 cells. IGFBP3 is shown in pg/mL and was measured in triplicate with reproducible results per line. (B) Real-time PCR analysis of IGFBP3 and IGFBP5 was examined in triplicate per line, with error bars representing standard deviation between replicates. Housekeeping gene RPLPO was measured as an internal control; IGFBP3 and IGFBP5 values are normalized to RPLPO.

A subset of HER2-/ IGF-IR-overexpressing cells were found to be less sensitive to the growth inhibitory effects of trastuzumab when compared to HER2-overexpressing cells that do not overexpress IGF-IR (Lu, 2001). Flow cytometry revealed that after trastuzumab

treatment, HER2 overexpressing cells were less likely to progress through the cell cycle and stopped at the G1 phase, while a greater number of HER2/IGF-IR overexpressing cells passed the restriction point and completed the cell cycle. These results demonstrate that IGF-IR interferes with the growth inhibitory actions of trastuzumab, supporting therapeutic strategies that co-target HER2 and IGF-IR. Further, we discovered that signaling interactions exist between IGF-IR and HER2 in trastuzumab-resistant cancers (Nahta, 2005; Jin, 2008). Immunoprecipitation and immunoblotting experiments revealed that IGF-I stimulation results in an increase in IGF-IR phosphorylation more rapidly in trastuzumab-resistant cells than in trastuzumab-sensitive cells. Furthermore, IGF-IR heterodimerization with HER2 results in HER2 activation in trastuzumab-resistant cells, but not in trastuzumab-sensitive cells, indicating crosstalk between the two receptors. Kinase inhibition or antibody blockade of IGF-IR restores trastuzumab sensitivity. Treatment of trastuzumab-resistant breast cancer cells with the highly specific IGF-IR antibody alpha IR3 disrupted the IGF-IR/HER2 heterodimer and increased trastuzumab sensitivity. These results suggest that IGF-IR-targeted treatments may be useful in combination with trastuzumab.

The association of increased IGF-IR activity with the development of trastuzumab resistance in HER2-overexpressing breast cancer makes IGF-IR an important target. Researchers have been working toward the goal of developing agents that target IGF-IR for the past several years with each generation of agents aimed at producing a greater benefit for the patient while decreasing adverse effects. IGF-IR and the insulin receptor (IR) are 60% homologous, with one of the adverse effects of IGF-IR antibody treatment being downregulation of the IR, leading to hyperglycemia (Sachdev, 2006). In an effort to remedy this problem, pharmacological agents like the small molecule tyrosine kinase inhibitor NVP-AEW541 (Novartis Pharma, Basel Switzerland) are specific for IGF-IR and less likely to interfere with glucose metabolism. Combination treatment with NVP-AEW541 and trastuzumab showed synergistic growth inhibitory effects, indicating that inhibiting IGF-IR plus HER2 could benefit patients whose tumors overexpress both receptors (Esparis-Ogando, 2008).

IGF-IR overexpression and crosstalk with HER2 suggests that IGF-IR plays a crucial role in conferring trastuzumab resistance. The molecular signaling pathways by which IGF-IR confers resistance to trastuzumab is not clear, although downstream focal adhesion kinase (FAK) and PI3K/Akt pathway signaling likely play a role (Yang, 2010). This data linking IGF-IR to the development of trastuzumab resistance, along with the increased sensitivity to trastuzumab upon IGF-IR inhibition provides a rational for the development of combinatorial HER2 and IGF-IR targeting.

5. Targeting Src in HER2-overexpressing breast cancer

Trastuzumab treatment of HER2-overexpressing breast cancer cells results in inhibition of Src non-receptor tyrosine kinase (Nagata, 2004). Src inhibition appears to be important to trastuzumab-mediated anti-cancer activity, as increased Src signaling is associated with trastuzumab resistance (Mitra, 2009; Liang, 2010; Zhang, 2011). One mechanism leading to increased Src activity appears to be a variant of HER2 called HER2 delta 16 (Mitra, 2009), which shows increased oncogenic activity. Local disease progression involved HER2Delta16 in 89% of breast cancer patients with HER2-positive tumors (Mitra, 2009). Transfection of MCF7 or NIH3T3 cells with HER2 delta 16 promoted receptor dimerization, invasion, and trastuzumab resistance (Mitra, 2009). The oncogenic properties of HER2Delta16 were mediated through direct interaction of HER2Delta16 with Src kinase. Activated Src kinase

was found in 44% of HER2Delta16-positive breast carcinomas (Mitra, 2009). Dual targeting of HER2Delta16 plus Src with dasatinib resulted in Src inactivation, destabilization of HER2Delta16, and decreased tumorigenicity (Mitra, 2009). In addition, Src activation via Jak2 has been shown to reduce trastuzumab activity (Liang, 2010). Recombinant human erythropoietin activated Jak2-Src signaling and inactivated PTEN in HER2-positive cells (Liang, 2010). Combined treatment with recombinant human erythropoietin plus trastuzumab reduced response to trastuzumab in cell culture and *in vivo* models. Further, shorter progression-free and overall survival was found in patients with HER2-positive breast cancer treated concurrently with erythropoietin and trastuzumab (Liang, 2010). Src was also shown to be activated in primary and acquired trastuzumab resistance as a consequence of PTEN loss (Zhang, 2011). Src-targeted therapy blocked growth of trastuzumab-resistant tumors *in vivo* (Zhang, 2011). Thus, Src activation may occur via multiple mechanisms, ultimately abrogating sensitivity to trastuzumab. Combining Src-targeted therapy with trastuzumab may offer benefit to patients with HER2-overexpressing breast cancer.

6. Role of p27 and cdk2 in HER2-overexpressing breast cancer

Trastuzumab induces G1 arrest by several mechanisms including increased expression of cyclin-dependent kinase inhibitor p27kip1, which inhibits cyclin E/cdk2 and cyclin A/cdk2 complexes and blocks cell cycle progression through S phase (Lane, 2001; Le, 2003). Trastuzumab induces p27kip1expression by suppressing expression of proteins that sequester p27kip1, which also results in increased interaction between p27kip1 and cdk2 leading to cdk2 inactivation (Lane, 2001). We previously reported (Nahta, 2004b) that cells with acquired trastuzumab resistance showed increased proliferation, reduced p27kip1 expression, reduced p27kip1-cdk2 interaction, and increased cdk2 activity relative to parental, trastuzumab-sensitive cells. Transfection of wild-type p27kip1 increased trastuzumab sensitivity in cells with acquired resistance (Nahta, 2004b). Yakes et al. (Yakes, 2002) showed that knockdown of p27kip1 reduced trastuzumab sensitivity in HER2-overexpressing breast cancer cell lines, further supporting a requirement of p27kip1 expression for optimal response to trastuzumab. Post-translational modification of p27kip1 occurs primarily by phosphorylation, with subsequent protein ubiquitination and degradation. Preliminary data supporting ubiquitin-proteasome degradation of p27kip1 as a mechanism of p27kip1 down-regulation in trastuzumab resistance includes our finding that proteasome inhibitor MG132 induced p27 expression and reduced viability of resistant cells (Nahta, 2004b). Further, Cardoso et al. (Cardoso, 2006) showed that proteasome inhibitor bortezomib induced p27kip1 and increased the efficacy of trastuzumab in HER2-overexpressing breast cancer cells. PI3K inhibition has been shown to induce p27kip1 expression, and is believed to contribute to p27kip1 down-regulation and acquired trastuzumab resistance. In addition to observing reduced p27kip1 levels in models of acquired resistance, our data indicates that p27kip1 expression is down-regulated post-transcriptionally in cells with primary trastuzumab resistance (Fig. 4). Cyclin E expression has been shown to be regulated by HER2 expression status, in that HER2 knockdown resulted in reduced cyclin E level and reduced cyclin E-associated kinase activity (Mittendorf, 2010). In addition, HER2-overexpressing breast cancers that also show increased cyclin E expression have lower 5 year disease-free survival versus those that have lower cyclin E levels (Mittendorf, 2010). Recently, cyclin E overexpression

in HER2-overexpressing breast cancer cells that have acquired trastuzumab resistance was shown to be due to amplification of the *cyclin E* gene (Scaltriti, 2011). Amongst 34 patients with HER2-overexpressing breast cancer, cyclin E amplification was associated with worse response to trastuzumab (Scaltriti, 2011). Knockdown of cyclin E or cdk2 inhibition reduced proliferation and induced apoptosis of trastuzumab-resistant tumors (Scaltriti, 2011). Thus, cdk2 inhibition is a potential pharmacologic strategy for treating trastuzumab-resistant HER2-overexpressing breast cancers that show reduced p27kip1 or increased cyclin E levels.

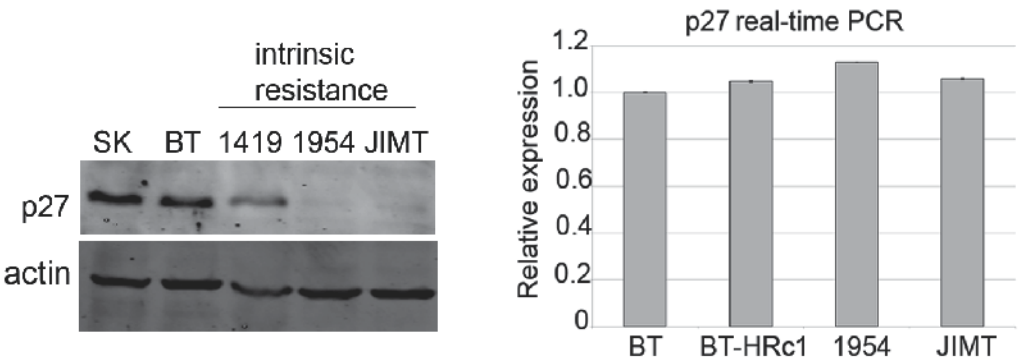


Fig. 4. p27 down-regulation in models of intrinsic (primary resistance). (A) SKBR3 and BT474 trastuzumab-sensitive cells and trastuzumab-resistant HCC1419, HCC1954, and JIMT-1 cells were examined by Western blotting for p27 and actin internal control. (B) BT474 and acquired resistant clone BT-HRc1 and primary resistant HCC1954 and JIMT-1 cells were examined by real-time PCR for p27 transcript which was normalized to RPLPO housekeeping gene.

7. Combining multiple HER2-targeted agents in HER2-overexpressing breast cancer

Two HER2-targeted agents are currently approved for use in the setting of metastatic HER2-positive breast cancer, trastuzumab and lapatinib. These agents target HER2 via distinct mechanisms (Fig. 5). Trastuzumab is a monoclonal antibody that specifically recognizes and binds to an extracellular part of HER2. Since antibodies are large, bulky molecules, trastuzumab is unable to cross the blood-brain barrier and thus cannot combat brain metastases. In contrast, lapatinib is a small molecule kinase inhibitor targeted against the EGFR and HER2 active sites. Since it is a small molecule, it is believed that lapatinib has the potential to enter the brain and target metastatic cells that overexpress HER2. A phase II trial of lapatinib in patients with trastuzumab-refractory disease and CNS metastases showed some volumetric changes in brain lesions and improved neurologic symptoms (Lin, 2008; Lin, 2009). Amongst 50 patients who were terated with lapatinib plus capecitabine, 20% showed a CNS objective response and 40% experienced 20% or greater volumetric reduction in their CNS lesions (Lin, 2009), suggesting that lapatinib may have some utility in limiting CNS metastases of primary HER2-overexpressing breast cancers.

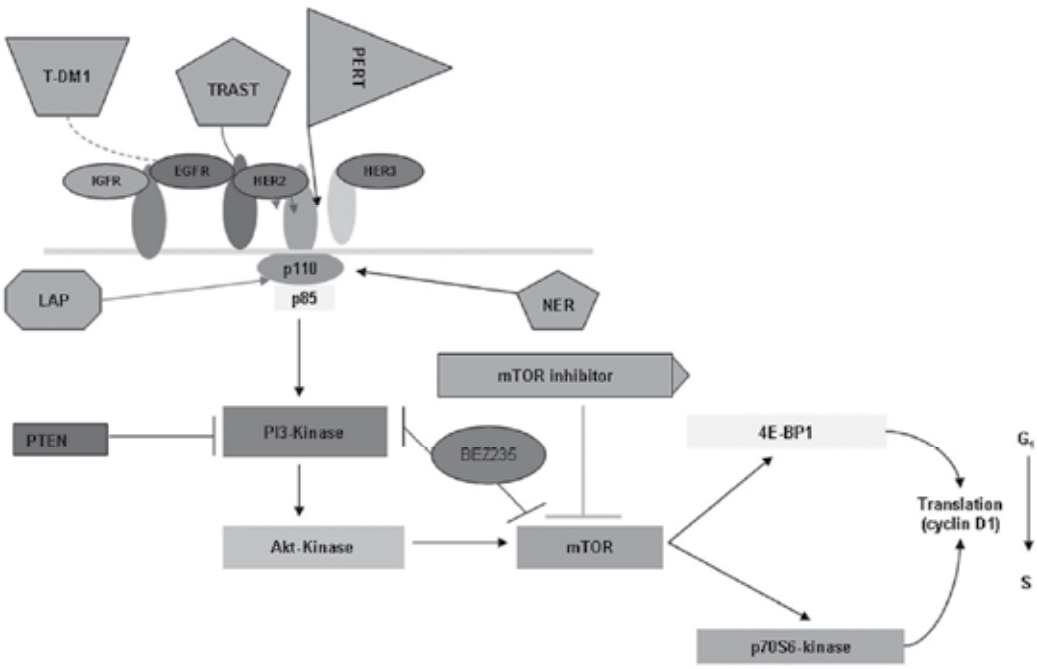


Fig. 5. Novel targeted agents in trastuzumab-resistant HER2-positive breast cancer. T-DM1, Trastuzumab-DM1; TRAST, Trastuzumab; PERT, Pertuzumab; IGFR, insulin growth factor receptor; EGFR, epidermal growth factor receptor; LAP, lapatinib; NER, neratinib.

7.1 Combining trastuzumab with lapatinib

Combination of trastuzumab plus lapatinib has been shown to induce apoptosis in part via down-regulation of survivin in cell culture and animal models (Xia, 2005). Initial phase I data suggested that the combination is well-tolerated and elicits partial or complete responses in a subset of patients who have progressed on prior trastuzumab therapy (Storniolo, 2008). The combination has been tested clinically in advanced phase trials in patients who have progressed on trastuzumab-based regimens. Progression-free survival and quality of life were improved in patients treated with the combination versus lapatinib alone (Wu, 2011). EGF104900 showed that the combination was superior to lapatinib alone in the trastuzumab-resistant setting, with a clonical benefit rate of 24. 7% versus 12. 4% (Blackwell, 2010). A potentially important mechanism of action of this drug combination is that lapatinib has been shown to induce accumulation of inactive HER2 dimers via reduced receptor ubiquitination, providing increased pharmacologic target for trastuzumab-mediated antibody-dependent cellular cytotoxicity (Scaltriti, 2009). Combining trastuzumab with lapatinib offers a chemotherapy-free option for treating HER2-positive trastuzumab-resistant disease.

7.2 Combining trastuzumab with pertuzumab

Pertuzumab is an anti-HER2 monoclonal antibody that targets an extracellular epitope distinct from what is targeted by trastuzumab. Pertuzumab binds to HER2 near the center of

domain II, sterically blocking a binding pocket necessary for receptor dimerization and signaling (Franklin, 2004). In contrast, trastuzumab does not significantly inhibit HER2 interaction with other erbB receptors. We were the first to show that combining pertuzumab with trastuzumab results in synergistic inhibition of proliferation of HER2-overexpressing breast cancer cells (Nahta, 2004a). Trastuzumab increased pertuzumab-mediated disruption of HER2 dimerization with EGFR and HER3, and further reduced pertuzumab-mediated inhibition of PI3K signaling (Nahta, 2004a). Phase II data shows that combining trastuzumab with pertuzumab in patients who have progressed on prior trastuzumab regimens achieves clinical benefit rate of 50%, objective response rates of 24%, and median progression-free survival of 5. 5 months (Baselga, 2010a). A potential mechanism of synergy is non-overlapping mechanisms by single agents, trastuzumab-mediated inhibition of p95HER2 cleavage and pertuzumab-mediated disruption of dimerization (Scheuer, 2009). Clinical evaluation of pertuzumab and trastuzumab (CLEOPATRA) is an international, randomized, double-blind, placebo-controlled phase III trial. Patients with HER2-positive breast cancer with locally recurrent or metastatic disease will be randomized to receive docetaxel, trastuzumab, and pertuzumab or docetaxel, trastuzumab, and placebo. Progresion-free survival will be assessed to determine efficacy of combination pertuzumab plus trastuzumab in the trastuzumab-refractory setting (Baselga, 2010b).

8. Novel HER2-targeted agents in clinical development

8.1 Trastuzumab-DM1

One novel preparation of trastuzumab is a drug conjugate called trastuzumab-DM1, which is trastuzumab conjugated to a microtubule-depolymerizing drug called maytansinoid (Lewis Phillips, 2008). Trastuzumab-DM1 blocks growth of trastuzumab-naive and trastuzumab-refractory HER2-overexpressing breast tumors *in vivo* (Lewis Phillips, 2008), and retains the mechanistic activity of unconjugated trastuzumab (Junttila, 2010). Antibody-dependent cellular cytotoxicity was induced by trastuzumab-DM1, and tumor growth of trastuzumab-resistant cells was blocked by trastuzumab-DM1 due to induction of apoptosis and mitotic catastrophe (Barok, 2011). A phase I dose-escalation study in patients who had progressed on trastuzumab showed clinical benefit of 73% in 15 of 24 patients, including objective responses in 5 patients (Krop, 2010). A phase II study of trastuzumab-DM1 in patients with trastuzumab-refractory HER2-positive breast cancer showed objective response of 25. 9% and median progression-free survival of 4. 6 months (Burris, 2011). Thus, trastuzumab-DM1 HER2 antibody-chemotherapy conjugate is a promising treatment for HER2-positive breast cancer that has progressed on prior HER2-directed therapies.

8.2 Irreversible pan-HER kinase inhibitors

In contrast to lapatinib, which is a reversible EGFR/HER2 kinase inhibitor, irreversible pan-HER inhibitors are being developed for use against HER2-dependent breast cancers (Ocana, 2009). Neratinib, an irreversible EGFR/HER2 inhibitor, achieved a response rate of 26% in trastuzumab-pretreated patients and 55% in trastuzumab-naïve patients (Burstein, 2009). Progression-free survival at 16 weeks was 60% and 77%, respectively, for trastuzumab-pretreated and naïve patients (Burstein, 2009). Finally, the median time to progression was 23 weeks and 40 weeks, respectively, for trastuzumab-pretreated and naïve patients (Burstein, 2009). Canertinib (CI-1033) is an irreversible inhibitor of all HER proteins. Response to canertinib was higher in patients with HER2-positive breast cancer, although toxicity at the most effective dose was limiting and unacceptable (Rixe, 2009).

9. Conclusion

In conclusion, several major mechanisms of trastuzumab resistance have been proposed, including increased signaling from PI3K/mTOR, Src, and IGF-IR, as well as reduced p27kip1 and increased cdk2 activity. These mechanisms have uncovered new therapeutic targets for which multiple pharmacologic agents have been developed. Some of the most promising include mTOR-targeted agents derived from rapamycin and trastuzumab-DM1. Combining multiple HER2-targeted agents appears to be beneficial due to different mechanisms of action. Future studies should more clearly address the role of IGF-IR in acquired versus primary resistance, and test IGF-IR-targeted agents in combination with trastuzumab and/or lapatinib in a trastuzumab-refractory setting. In addition, studies examining the role of estrogen receptor (ER) signaling in trastuzumab resistant HER2-positive ER-positive disease should be performed. Finally, biological predictors of response or resistance need to be developed to determine which patients are most likely to benefit from trastuzumab therapy, thus allowing for more specific individualization of targeted therapy in patients with HER2-overexpressing breast cancer.

10. References

Adams TE, Epa VC, Garrett TP, & Ward CW. (2000). Structure and function of the type 1 insulin-like growth factor receptor. *Cell Mol Life Sci*, Vol. 57, No. 7, (July 2000), pp. 1050-93

Barok M, Tanner M, Koninki K, & Isola J. (2011). Trastuzumab-DM1 causes tumor growth inhibition by mitotic catastrophe in trastuzumab-resistant breast cancer cells in vivo. Breast *Cancer Res* Vol. 13, No. 2, (2011 Apr 21), pp. R46

Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC, & Norton L. (1996). Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol*, Vol. 14, No. 3, (March 1996), pp. 737-44

Baselga J, Gelmon KA, Verma S, Wardley A, Conte P, Miles D, Bianchi G, Cortes J, McNally VA, Ross GA, Fumoleau P, & Gianni L. (2010a). Phase II trial of pertuzumab and trastuzumab in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer that progressed during prior trastuzumab therapy. *J Clin Oncol* Vol. 28, No. 7, (2010 Mar 1), pp. 1138-44

Baselga J & Swain SM. (2010b). CLEOPATRA: a phase III evaluation of pertuzumab and trastuzumab for HER2-positive metastatic breast cancer. *Clin Breast Cancer* Vol. 10, No. 6, (2010 Dec 1), pp. 489-91

Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, Linn SC, Gonzalez-Angulo AM, Stemke-Hale K, Hauptmann M, Beijersbergen RL, Mills GB, van de Vijver MJ, & Bernards R. (2007). A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell* Vol. 12, No. 4, (2007 Oct), pp. 395-402

Blackwell KL, Burstein HJ, Storniolo AM, Rugo H, Sledge G, Koehler M, Ellis C, Casey M, Vukelja S, Bischoff J, Baselga J, O'Shaughnessy J. (2010). Randomized study of Lapatinib alone or in combination with trastuzumab in women with ErbB2-

positive, trastuzumab-refractory metastatic breast cancer. *J Clin Oncol*, Vol. 28, No. 7, (March 2010), pp. 1124-30

Burris HA 3rd, Rugo HS, Vukelja SJ, Vogel CL, Borson RA, Limentani S, Tan-Chiu E, Krop IE, Michaelson RA, Girish S, Amler L, Zheng M, Chu YW, Klencke B, & O'Shaughnessy JA. (2011). Phase II study of the antibody drug conjugate trastuzumab-DM1 for the treatment of human epidermal growth factor receptor 2 (HER2)-positive breast cancer after prior HER2-directed therapy. *J Clin Oncol* Vol. 29, No. 4, (2011 Feb 1), pp. 398-405

Burstein HJ, Sun Y, Dirix LY, Jiang Z, Paridaens R, Tan AR, Awada A, Ranade A, Jiao S, Schwartz G, Abbas R, Powell C, Turnbull K, Vermette J, Zacharchuk C, & Badwe R. (2010). Neratinib, an irreversible ErbB receptor tyrosine kinase inhibitor, in patients with advanced ErbB2-positive breast cancer. *J Clin Oncol* Vol. 28, No. 8, (2010 Mar 10), pp. 1301-7

Cardoso F, Durbecq V, Laes JF, Badran B, Lagneaux L, Bex F, Desmedt C, Willard-Gallo K, Ross JS, Burny A, Piccart M, & Sotiriou C. (2006). Bortezomib (PS-341, Velcade) increases the efficacy of trastuzumab (Herceptin) in HER-2-positive breast cancer cells in a synergistic manner. *Mol Cancer Ther* Vol. 5, No. 12, (2006 Dec), pp. 3042-51

Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, & Shepard HM. (1992). Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci U S A* Vol. 89, No. 10, (1992 May 15), pp. 4285-9

Casalini P, Iorio MV, Berno V, Bergamaschi A, Børresen Dale AL, Gasparini P, Orlandi R, Casati B, Tagliabue E, & Ménard S. (2007). Relationship between p53 and p2 7 expression following HER2 signaling. *Breast*, Vol. 16, No. 6, (December 2007), pp. 597-605

Chaves J, Saif MW. IGF system in cancer: from bench to clinic. (2011). *Anticancer Drugs*, Vol. 22, No. 3, (March 2011), pp. 206-12

Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, Wolter JM, Paton V, Shak S, Lieberman G, & Slamon DJ. (1999). Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol*, Vol. 17, No. 9, pp. 2639-48

Cuello M, Ettenberg SA, Clark AS, Keane MM, Posner RH, Nau MM, Dennis PA, & Lipkowitz S. (2001). Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2. *Cancer Res*, Vol. 61, No. 12, (June 2001), pp. 4892-4900

Eccles SA. (2001). The role of c-erbB-2/HER2/neu in breast cancer progression and metastasis. *J Mammary Gland Biol Neoplasia* Vol. 6, No. 4, (2001 Oct), pp. 393-406

Eichhorn PJ, Gili M, Scaltriti M, Serra V, Guzman M, Nijkamp W, Beijersbergen RL, Valero V, Seoane J, Bernards R, Baselga J. Phosphatidylinositol 3-kinase hyperactivation results in lapatinib resistance that is reversed by the

mTOR/phosphatidylinositol 3-kinase inhibitor NVP-BEZ235. *Cancer Res* Vol. 68, No. 22, (2008 Nov 15), pp. 9221-30

Esparís-Ogando A, Ocaña A, Rodríguez-Barrueco R, Ferreira L, Borges J & Pandiella A. (2008). Synergic antitumoral effect of an IGF-IR inhibitor and trastuzumab on HER2-overexpressing breast cancer cells. *Ann Oncol*, Vol. 19, No. 11, (November 2008) pp. 1860-9

Esteva FJ, Valero V, Booser D, Guerra LT, Murray JL, Pusztai L, Cristofanilli M, Arun B, Esmaeli B, Fritsche HA, Sneige N, Smith TL, & Hortobagyi GN. (2002). Phase II study of weekly docetaxel and trastuzumab for patients with HER-2-overexpressing metastatic breast cancer. *J Clin Oncol*, Vol. 20, No. 7 (2002 Apr 1), pp. 1800-8

Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, & Sliwkowski MX. (2004). Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell* Vol. 5, No. 4, (2004 Apr), pp. 317-28

Gajria D, & Chandarlapaty S. (2011). HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies. *Expert Rev Anticancer Ther,* Vol. 11, No. 2, (February 2011), pp. 263-75

Geyer CE, Forster J, Lindquist D, Chan S, Romieu CG, Pienkowski T, Jagiello-Gruszfeld A, Crown J, Chan A, Kaufman B, Skarlos D, Campone M, Davidson N, Berger M, Oliva C, Rubin SD, Stein S, & Cameron D. (2006). Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med*, Vol. 355, No. 26 (2006 Dec 28), pp. 2733-43.

Graus-Porta D, Beerli RR, Daly JM, & Hynes NE. (1997). ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J*, Vol. 16, No. 7, (April 1997), pp. 1647-55

Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, Rosner B, Speizer FE, & Pollak M. Circulating concentrations of insulin-like growth factor-I and risk of breast cancer. *Lancet* Vol. 351, No. 9113, (1998 May 9), pp. 1393-6.

Huang X, Qian X, Cheng C, He S, Sun L, Ke Q, Zhang L, Pan X, He F, Wang Q, Meng J, Ni R, & Shen A. (2011). Expression of Pirh2, a p27(Kip1) ubiquitin ligase, in hepatocellular carcinoma: correlation with p27(Kip1) and cell proliferation. *Hum Pathol*, Vol. 42, No. 4, (April 2011), pp. 507-15

Jerome L, Alami N, Belanger S, Page V, Yu Q, Paterson J, Shiry L, Pegram M, & Leyland-Jones B. (2006). Recombinant human insulin-like growth factor binding protein 3 inhibits growth of human epidermal growth factor receptor-2-overexpressing breast tumors and potentiates herceptin activity in vivo. *Cancer Res* Vol. 66, No. 14, (2006 Jul 15), pp. 7245-52

Jerusalem G, Fasolo A, Dieras V, Cardoso F, Bergh J, Vittori L, Zhang Y, Massacesi C, Sahmoud T, & Gianni L. (2011). Phase I trial of oral mTOR inhibitor everolimus in combination with trastuzumab and vinorelbine in pre-treated patients with HER2-overexpressing metastatic breast cancer. *Breast Cancer Res Treat*, Vol. 125, No. 2, (January 2011), pp. 447-55

Jin Q & Esteva FJ. (2008). Cross-talk between the ErbB/HER family and the type I insulin-like growth factor receptor signaling pathway in breast cancer. *J Mammary Gland Biol Neoplasia*, Vol. 13, No. 4, (December 2008), pp. 485-98

Junttila TT, Akita RW, Parsons K, Fields C, Lewis Phillips GD, Friedman LS, Sampath D, & Sliwkowski MX. (2009). Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. *Cancer Cell,* Vol. 15, No. 5, (May 2009), pp. 429-40

Junttila TT, Li G, Parsons K, Phillips GL, & Sliwkowski MX. (2010). Trastuzumab-DM1 (T-DM1) retains all the mechanisms of action of trastuzumab and efficiently inhibits growth of lapatinib insensitive breast cancer. *Breast Cancer Res Treat* (2010 Aug 21. [Epub ahead of print])

Kalinsky K, Jacks LM, Heguy A, Patil S, Drobnjak M, Bhanot UK, Hedvat CV, Traina TA, Solit D, Gerald W, & Moynahan ME. (2009). PIK3CA mutation associates with improved outcome in breast cancer. *Clin Cancer Res*, Vol. 15, No. 16, (August 2009), pp. 5049-59

Kotoshiba S, Kamura T, Hara T, Ishida N, & Nakayama KI. (2005). Molecular dissection of the interaction between p27 and Kip1 ubiquitylation-promoting complex, the ubiquitin ligase that regulates proteolysis of p27 in G1 phase. *J Biol Chem,* Vol. 280, No. 18, (May 2005), pp. 17694-700

Krop IE, Beeram M, Modi S, Jones SF, Holden SN, Yu W, Girish S, Tibbitts J, Yi JH, Sliwkowski MX, Jacobson F, Lutzker SG, & Burris HA. (2010). Phase I study of trastuzumab-DM1, an HER2 antibody-drug conjugate, given every 3 weeks to patients with HER2-positive metastatic breast cancer. *J Clin Oncol* Vol. 28, No. 16, (2010 Jun 1), pp. 2698-704

Lane HA, Motoyama AB, Beuvink I, & Hynes NE. (2001). Modulation of p27/Cdk2 complex formation through 4D5-mediated inhibition of HER2 receptor signaling. *Ann Oncol* Vol. 12, Suppl 1, (2001), pp. S21-2.

Lang SA, Hackl C, Moser C, Fichtner-Feigl S, Koehl GE, Schlitt HJ, Geissler EK, & Stoeltzing O. (2010). Implication of RICTOR in the mTOR inhibitor-mediated induction of insulin-like growth factor-I receptor (IGF-IR) and human epidermal growth factor receptor-2 (Her2) expression in gastrointestinal cancer cells. *Biochim Biophys Acta*, Vol. 4, (April 2010), pp. 435-42

Le XF, Claret FX, Lammayot A, Tian L, Deshpande D, LaPushin R, Tari AM, & Bast RC Jr. (2003). The role of cyclin-dependent kinase inhibitor p27Kip1 in anti-HER2 antibody-induced G1 cell cycle arrest and tumor growth inhibition. *J Biol Chem*, Vol. 278, No. 26, (June 2003), pp. 23441-50

Lewis Phillips GD, Li G, Dugger DL, Crocker LM, Parsons KL, Mai E, Blättler WA, Lambert JM, Chari RV, Lutz RJ, Wong WL, Jacobson FS, Koeppen H, Schwall RH, Kenkare-Mitra SR, Spencer SD, & Sliwkowski MX. (2008). Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. Cancer Res Vol. 68, No. 22, (2008 Nov 15), pp. 9280-90

Liang K, Esteva FJ, Albarracin C, Stemke-Hale K, Lu Y, Bianchini G, Yang CY, Li Y, Li X, Chen CT, Mills GB, Hortobagyi GN, Mendelsohn J, Hung MC, & Fan Z. (2010). Recombinant human erythropoietin antagonizes trastuzumab treatment of breast

cancer cells via Jak2-mediated Src activation and PTEN inactivation. *Cancer Cell* Vol. 18, No. 5, (2010 Nov 16), pp. 423-35

Lin NU, Carey LA, Liu MC, Younger J, Come SE, Ewend M, Harris GJ, Bullitt E, Van den Abbeele AD, Henson JW, Li X, Gelman R, Burstein HJ, Kasparian E, Kirsch DG, Crawford A, Hochberg F, & Winer EP. (2008). Phase II trial of lapatinib for brain metastases in patients with human epidermal growth factor receptor 2-positive breast cancer. *J Clin Oncol* Vol. 26, No. 12, (2008 Apr 20), pp. 1993-9

Lin NU, Diéras V, Paul D, Lossignol D, Christodoulou C, Stemmler HJ, Roché H, Liu MC, Greil R, Ciruelos E, Loibl S, Gori S, Wardley A, Yardley D, Brufsky A, Blum JL, Rubin SD, Dharan B, Steplewski K, Zembryki D, Oliva C, Roychowdhury D, Paoletti P, & Winer EP. (2009). Multicenter phase II study of lapatinib in patients with brain metastases from HER2-positive breast cancer. *Clin Cancer Res* Vol. 15, No. 4, (2009 Feb 15), pp. 1452-9

Lu Y, Zi X, Zhao Y, Mascarenhas D, & Pollak M. (2001). Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J Natl Cancer Inst,* Vol. 93, No. 24, (December 2001), pp. 1852-7

Marches R, & Uhr JW. (2004). Enhancement of the p27Kip1-mediated antiproliferative effect of trastuzumab (Herceptin) on HER2-overexpressing tumor cells. *Int J Cancer*, Vol. 112, No. 3, (November 2004), pp. 492-501

Miller TW, Forbes JT, Shah C, Wyatt SK, Manning HC, Olivares MG, Sanchez V, Dugger TC, de Matos Granja N, Narasanna A, Cook RS, Kennedy JP, Lindsley CW, & Arteaga CL. (2009). Inhibition of mammalian target of rapamycin is required for optimal antitumor effect of HER2 inhibitors against HER2-overexpressing cancer cells. *Clin Cancer Res*, Vol. 15, No. 23, (December 2009), pp. 7266-76

Mitra D, Brumlik MJ, Okamgba SU, Zhu Y, Duplessis TT, Parvani JG, Lesko SM, Brogi E, & Jones FE. (2009). An oncogenic isoform of HER2 associated with locally disseminated breast cancer and trastuzumab resistance. *Mol Cancer Ther* Vol. 8, No. 8, (2009 Aug), pp. 2152-62

Mittendorf EA, Liu Y, Tucker SL, McKenzie T, Qiao N, Akli S, Biernacka A, Liu Y, Meijer L, Keyomarsi K, & Hunt KK. (2010). A novel interaction between HER2/neu and cyclin E in breast cancer. *Oncogene* Vol. 29, No. 27, (2010 Jul 8), pp. 3896-907

Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, & Baselga J. (2001). Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer Res*, Vol. 61, No. 12, (June 2001), pp. 4744-9

Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, Klos KS, Li P, Monia BP, Nguyen NT, Hortobagyi GN, Hung MC, & Yu D. (2004). PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell*, Vol. 6, No. 2, (2004 Aug), pp. 117-27

Nahta R, Hung MC, & Esteva FJ. (2004a). The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. *Cancer Res* Vol. 64, No. 7, (2004 Apr 1), pp. 2343-6

Nahta R, Takahashi T, Ueno NT, Hung MC, & Esteva FJ. (2004b). P27(kip1) down-regulation is associated with trastuzumab resistance in breast cancer cells. *Cancer Res,* Vol. 64, No. 11, (June 2004), pp. 3981-6

Nahta, R, Yuan, L X, Zhang, Kobayashi, R, & Esteva FJ. (2005). Insulin-like growth factor-i receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. *Cancer Res*, Vol. 65, No. 23, (Nov 2008), pp. 11118-28

Nielsen DL, Andersson M, & Kamby C. (2008). HER2-targeted therapy in breast cancer. Monoclonal antibodies and tyrosine kinase inhibitors. *Cancer Treat Rev*, Vol. 35, No. 2, (April 2009), pp. 121-36

O'Brien NA, Browne BC, Chow L, Wang Y, Ginther C, Arboleda J, Duffy MJ, Crown J, O'Donovan N, & Slamon DJ. (2010). Activated phosphoinositide 3-kinase/AKT signaling confers resistance to trastuzumab but not lapatinib. *Mol Cancer Ther* Vol. 9, No. 6, (June 2010), pp. 1489-502

Oakman C, Pestrin M, Zafarana E, Cantisani E, & Di Leo A. (2010). Role of lapatinib in the first-line treatment of patients with metastatic breast cancer. *Cancer Manag Res*, Vol. 2, (January 2010), pp. 13-25

Ocaña A & Amir E. (2009). Irreversible pan-ErbB tyrosine kinase inhibitors and breast cancer: current status and future directions. *Cancer Treat Rev* Vol. 35, No. 8, (2009 Dec), pp. 685-91

Rixe O, Franco SX, Yardley DA, Johnston SR, Martin M, Arun BK, Letrent SP, & Rugo HS. (2009). A randomized, phase II, dose-finding study of the pan-ErbB receptor tyrosine-kinase inhibitor CI-1033 in patients with pretreated metastatic breast cancer. *Cancer Chemother Pharmacol* Vol. 64, No. 6, (2009 Nov), pp. 1139-48

Sachdev D, Singh R, Fujita-Yamaguchi Y, & Yee D. (2006). Down-regulation of insulin receptor by antibodies against the type I insulin-like growth factor receptor: implications for anti-insulin-like growth factor therapy in breast cancer. *Cancer,* Vol. 66, No. 4, (February 2006), pp. 2391-402

Sakr RA, Barbashina V, Morrogh M, Chandarlapaty S, Andrade VP, Arroyo CD, Olvera N, & King TA. (2010). Protocol for PTEN expression by immunohistochemistry in formalin-fixed paraffin-embedded human breast carcinoma. *Appl Immunohistochem Mol Morphol* Vol. 18, No. 4, (July 2010), pp. 371-4

Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL, Pedersen K, Smith DJ, Landolfi S, Ramon y Cajal S, Arribas J, & Baselga J. (2009). Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. *Oncogene* Vol. 28, No. 6, (2009 Feb 12), pp. 803-14

Scaltriti M, Eichhorn PJ, Cortés J, Prudkin L, Aura C, Jiménez J, Chandarlapaty S, Serra V, Prat A, Ibrahim YH, Guzmán M, Gili M, Rodríguez O, Rodríguez S, Pérez J, Green SR, Mai S, Rosen N, Hudis C, & Baselga J. (2011). Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients. *Proc Natl Acad Sci U S A* Vol. 108, No. 9, (2011 Mar 1), pp. 3761-6

Scheuer W, Friess T, Burtscher H, Bossenmaier B, Endl J, & Hasmann M. (2009). Strongly enhanced antitumor activity of trastuzumab and pertuzumab combination treatment on HER2-positive human xenograft tumor models. *Cancer Res* Vol. 69, No. 24, (2009 Dec 15), pp. 9330-6

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, & McGuire WL. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* Vol. 235, No. 4785 (1987 Jan 9), pp. 177-82

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, & Norton L. (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* Vol. 15, No. 344, (March 2001), pp. 783-92

Sliwkowski MX, Lofgren JA, Lewis GD, Hotaling TE, Fendly BM, & Fox JA. (1999). Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). *Semin Oncol* Vol. 26, No. 4 Suppl 12, (1999 Aug), pp. 60-70

Spector NL & Blackwell KL. (2009). Understanding the mechanisms behind trastuzumab therapy for human epidermal growth factor receptor 2-positive breast cancer. *J Clin Oncol*, Vol. 27. No. 34 (December 2009), pp. 5838-47

Storniolo AM, Pegram MD, Overmoyer B, Silverman P, Peacock NW, Jones SF, Loftiss J, Arya N, Koch KM, Paul E, Pandite L, Fleming RA, Lebowitz PF, Ho PT, & Burris HA 3rd. (2008). Phase I dose escalation and pharmacokinetic study of lapatinib in combination with trastuzumab in patients with advanced ErbB2-positive breast cancer. *J Clin Oncol* Vol. 26, No. 20, (2008 Jul 10), pp. 3317-23

Surmacz E. (2000). Function of the IGF-I receptor in breast cancer. *J Mammary Gland Biol Neoplasia*, Vol. 5, No. 1 (January 2000), pp. 95-105

Van der Heijden, M S, & Bernards, R. (2010). Inhibition of the PI3K pathway: Hope we can believe in? *Clin Cancer Res*, Vol. 16, No. 12 (June 2010), pp. 3094-9

Vermeulen K, Van Bockstaele DR, & Berneman ZN. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif*. Vol. 36, No. 3, (June 2003), pp. 131-49

Wu Y, Amonkar MM, Sherrill BH, O'Shaughnessy J, Ellis C, Baselga J, Blackwell KL, & Burstein HJ. (2011). Impact of lapatinib plus trastuzumab versus single-agent lapatinib on quality of life of patients with trastuzumab-refractory HER2+ metastatic breast cancer. *Ann Oncol* (2011 Mar 15 [Epub ahead of print])

Wullschleger S, Loewith R, Oppliger W, & Hall MN. (2005). Molecular organization of target of rapamycin complex 2. *J Biol Chem*, Vol. 280, No. 235, (September 2005), pp. 30697-704

Xia W, Gerard CM, Liu L, Baudson NM, Ory TL, & Spector NL. (2005). Combining lapatinib (GW572016), a small molecule inhibitor of ErbB1 and ErbB2 tyrosine kinases, with therapeutic anti-ErbB2 antibodies enhances apoptosis of ErbB2-overexpressing breast cancer cells. *Oncogene* Vol. 24, No. 41, (2005 Sep 15), pp. 6213-21

Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, & Arteaga CL. (2002). Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. *Cancer Res* Vol. 62, No. 14, (2002 Jul 15), pp. 4132-41

Yang XH, Flores LM, Li Q, Zhou P, Xu F, Krop IE, & Hemler ME. (2010). Disruption of laminin-integrin-CD151-focal adhesion kinase axis sensitizes breast cancer cells to ErbB2 antagonists. *Cancer Res* Vol. 70, No. 6, (2010 Mar 15), pp. 2256-63

Zhang S, Huang WC, Li P, Guo H, Poh SB, Brady SW, Xiong Y, Tseng LM, Li SH, Ding Z, Sahin AA, Esteva FJ, Hortobagyi GN, & Yu D. (2011). Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. *Nat Med* Vol. 17, No. 4, (2011 Apr), pp. 461-9

2

Therapeutic Targeting of Osteopontin in Breast Cancer Cells

Gopal C. Kundu et al.*
National Centre for Cell Science, NCCS Complex, Pune, India

1. Introduction

Osteopontin (OPN), a cytokine like ECM associated member of Small Integrin Binding LIgand N-linked Glycoprotein (SIBLING) family of protein plays an important role in determining the metastatic potential of many cancers. The function of OPN in various pathophysiological conditions, especially in cancer indicated that the variation in post-translational modification generate different functional forms that might alter its normal physiological functions. Recent data indicated that OPN regulates tumor growth through induction of pro-angiogenic and metastatic genes like COX-2, and VEGF expressions and activation of matrix metalloproteinase (MMP) in cancer cells. The exact role of stroma- and tumor-derived OPN in regulation of tumor growth and angiogenesis in various cancers is not well understood. Therefore, it is important to delineate the mechanism by which both tumor and stroma-derived OPN control the cell migration and tumor growth. p70S6 kinase, STAT3 and VEGF are directly involved in regulation of breast tumor growth and angiogenesis. But, the mechanism by which OPN regulates p70S6 kinase and STAT3 activation and VEGF expression leading to breast cancer cell migration, tumor growth and angiogenesis are not well defined. We have recently shown that OPN induces p70S6 kinase phosphorylation in a site specific manner. Interestingly, OPN has no effect on mTOR phosphorylation, but overexpression of mTOR does not regulate OPN-induced phosphorylation of p70S6 kinase. Overexpression of mTOR/p70S6 kinase suppresses OPN-induced ICAM-1 expression, while treatment with rapamycin enhances OPN-induced ICAM-1 expression. Our recent data also indicated that OPN upregulates JAK2 dependent STAT3 activation in breast cancer cells. Wild type STAT3 enhanced whereas mutant STAT3 suppressed OPN-induced breast tumor cell migration. Cells overexpressing STAT3 upregulate whereas mutant STAT3 downregulate OPN-induced tumor growth leading to

* Supriya Saraswati, Megha Sanyal, Anuradha Bulbule, Anuja Ramdasi, Dhiraj Kumar, Reeti Behera[1], Mansoor Ahmed[2], Goutam Chakraborty[3], Vinit Kumar[4], Shalini Jain[5], Gowrishankar S. and Pompom Ghosh
[1]*Present Address: H. Lee Moffitt Cancer Center and Research Institute, FL*
[2]*Present Address: University of Virginia, VA*
[3]*Present Address: Memorial Sloan-Kettering Cancer Center, NY*
[4]*Present Address: H. Lee Moffitt Cancer Center and Research Institute, FL*
[5]*PresentAddress: The University of Texas MD Anderson Cancer Center, TX USA*

Bcl2 and cyclin D1 expressions. Our data also revealed that OPN augments breast cancer cell migration, angiogenesis and tumor growth through induction of VEGF expression. Thus, targeting OPN and its regulated signalling cascade may develop an effective therapeutic approach for the management of breast cancer.

2. General features of breast cancer

The critical features that define cancer encompass the six core hallmarks of the disease as described recently (Hanahan and Weinberg, 2011). These hallmarks are sustained proliferative signalling, evading growth suppressors, activating invasion and metastasis, overcoming replicative senescence, inducing angiogenesis and resisting cell death (Hanahan and Weinberg, 2000). Breast cancer represents malignant transformation of the epithelial cells lining the ducts or lobules of the breast, occurring as a result of unrestricted cellular proliferation possibly owing to accumulation of a series of somatic or germ line mutations. Majority of the breast cancer is a result of somatic or acquired mutations and it is the most common form of cancer affecting women worldwide. Benign breast tumors are treatable and hence not a grave threat in contrast to malignant breast cancer where many complex processes are involved that are difficult to target. Invasion, angiogenesis and metastasis are the defining attributes of malignancy and occur as early events in cancer progression.

Mutations in certain genes lead to sporadic cases of breast cancer. Tumor suppressor genes like p53 control unrestricted proliferation of cells. It is noteworthy to mention about two related genes such as p63 and p73 which are yet to assume importance as candidates for alternative regimens for the treatment of cancer. These are reported to be involved in embryonic development and their roles in attenuating cancer progression are under study. Boominathan has provided mechanistic insights into how p53, p63, and p73 regulate the components of the miRNA processing and how p53, TA-p63, and TA-p73 regulated miRNAs inhibit tumorigenesis, EMT, metastasis, and cancer stem cell proliferation (Boominathan, 2010). The first clinical trials of attempting to use p73 to combat a hard-to-treat-type of breast cancer have been initiated (Leslie, 2011).

The other two breast cancer specific tumor suppressor genes, BRCA1 and BRCA2 protect the cells from dysregulation leading to unrestrained cellular proliferation (Stefansson et al, 2009). A member of EGF receptor superfamily called erbB2 or HER2/neu is a receptor for human epidermal growth factor that is present on the breast cancer cells and stimulates the cells to grow and divide. Overexpression of HER2/neu due to gene amplification is associated with transformation of human breast epithelium. Apart from mutations in tumor suppressor genes and oncogenes, breast cancer is also associated with the presence of ER and PR. Breast cancers are sub-divided into four groups based on IHC profile of ER/PR and Her2/neu expression: luminal A (ER and/or PR +ve, HER2 –ve), luminal B (ER and/or PR +ve, HER2 +ve), HER2 positive (ER and PR -ve, HER2 +ve) and triple negative (all –ve).

These classifications of breast cancers are based on which hormone fuels their growth and helps decide the course of hormone targeted therapy. Triple negative breast cancer is marked by the absence of hormone receptors and HER2/neu and forms belligerent tumors that are unresponsive to hormonal therapies (tamoxifene, aromatase inhibitors) or HER2 directed therapies (herceptin, lapatinib) (Chen and Russo, 2009). Staging of breast cancer is performed by employing the widely accepted TNM classification which describes the individual stages of the tumor, node and metastases (TNM) of the cancer. The tumor grade of invasive carcinomas is classified according to the Scarff–Bloom–Richardson (SBR) system.

Clinical studies have revealed that higher expression of OPN is found in tumor tissue and serum of breast cancers (Shevde et al, 2006). Enhanced expression of OPN can be correlated with increase in tumor growth and metastasis, suggesting that OPN can be used as a diagnostic and prognostic biomarker for breast cancer. Earlier micro array analysis data revealed that expression of OPN is upregulated in metastatic breast cancers (Cook, 2005). OPN is an extracellular matrix (ECM)-associated, SIBLING family of cytokine-like, noncollagenous, sialic acid rich phosphoglycoprotein (Rangaswami, et al 2006). OPN controls normal physiological and various pathophysiological processes such as myocardial necrosis, restenosis, atherosclerosis and autoimmune diseases (Panda et al, 1997). OPN acts as an important oncogenic molecule which is involved in all the stages of cancer progression including tumor invasion, angiogenesis and metastasis. Previous reports have indicated that OPN is also overexpressed in tumor-educated stromal cells suggesting its involvement in the crosstalk between tumor and stromal compartment that ultimately leads to cancer progression (Osterreicher, 2011). Earlier results indicate that OPN could regulate the expression of several oncogenic and angiogenic molecules through activation of various signalling mechanism (Chakraborty et al, 2006).

3. Structure, functions and mediators of osteopontin

Osteopontin was initially characterized in 1979 as a phosphoprotein secreted by transformed malignant epithelial cells and has since been under extensive study. The human OPN gene sprawls across 8 kilobases and is localized at chromosome 4q13 in human as a single copy gene with seven exons and six introns (Wai and Kuo, 2004). Alternative splicing yields three distinct splice variants- OPN-A, the full-length transcript, OPN-B, lacking exon 5 and OPN-C lacking exon 4 (He et al, 2006). Two isoforms of OPN, a full-length secreted OPN (Opn-s) and an intracellular OPN (Opn-i) are generated from alternative translation of a non-AUG site downstream of the canonical AUG sequence (Shinohara et al, 2008). These two isoforms occupy characteristic intracellular sites and mediate distinct functions in dendritic and T cells (Shinohara et al, 2008).

A full length human OPN consists of about 314 amino acid residues with a molecular weight in the range of 44-75 kDa, resulting from the varying degree of posttranslational modifications. Within the functional domains of OPN, there are specific motifs essential for the binding of OPN to its cell surface receptors, integrins and CD44 for mediating its biological activities (Figure 1). Whereas the N-terminal fragment contains the RGD motif, the SVVYGLR motif, a thrombin cleavage site and an aspartic acid rich site, the C-terminal fragment contains a calcium-binding site and CD44 binding site. The RGD motif necessary for the attachment of integrins such as $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 1$ and $\alpha 5\beta 1$ is embedded within exon 6. A central thrombin cleavage site distal to the RGD motif divides OPN into two similar-sized fragments. The SVVYGLR motif binds to integrins, $\alpha 9\beta 1$ and $\alpha 4\beta 1$ and the aspartic acid rich site binds hydroxyapatite in bones. The CD44 interacts through the C-terminal of OPN. OPN is involved in maintaining calcium homeostasis via its calcium binding site. OPN upon binding with integrins or CD44 regulates breast cancer cell proliferation, migration, invasion and chemotaxis. OPN plays an important role in regulation of tumor progression, angiogenesis and metastasis in breast cancer. OPN is detected in many biological fluids like plasma of metastatic breast cancer patients, urine, milk and seminal fluids. The ligation of OPN to its receptors stimulates a cascade of signalling pathways which cross talk and foster neoplastic growth in breast cancer (Rangaswami et al, 2006).

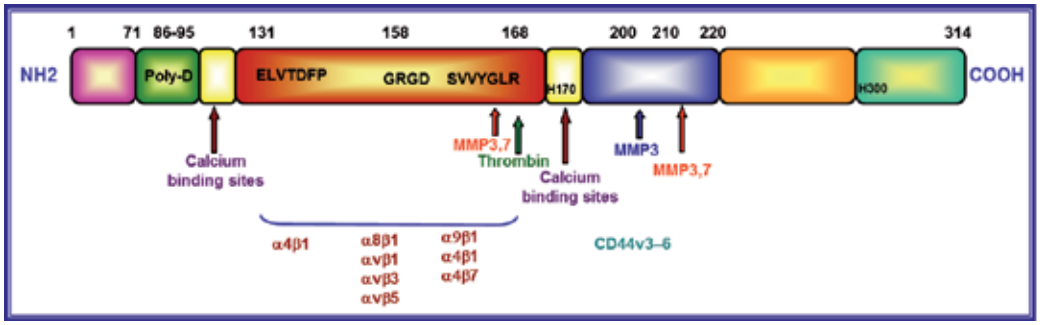


Fig. 1. Schematic representation of the domain structure of OPN. The N-terminal fragment contains a poly D rich region, calcium binding site, RGD motif and SVVYGLR. Various integrins interact with the N-terminal domain of OPN while C-terminal domain of it interacts with CD44, v3-6.

4. Pleiotropic function of OPN in breast cancer

Breast cancer progression depends on an accumulation of metastasis supporting cell signaling molecules that target various signal transduction pathways. These complex signaling mechanisms can result in changes in gene expression, which ultimately lead to alterations in cellular properties involved in malignancy such as adhesion, migration, invasion, enhanced tumor cell survival, angiogenesis and metastasis (Figure 2). Increased expressions of OPN and its receptors, integrins and CD44 correlate with enhanced breast tumor epithelial cell migration, tumor progression and metastasis. Among all splice variants of OPN, OPN-C is a highly specific marker for transformed breast cancer cells (He et al, 2005). Rittling et al have reported that OPN associated with tumors is primarily soluble, and that OPN can neither support endothelial cell proliferation nor prevent apoptosis of these cells in the absence of adhesion (Rittling et al, 2002).

OPN activates αvβ3 integrin-mediated PI 3'-kinase/IKK-dependent NF-κB activation and uPA secretion leading to breast cancer cell migration (Das et al, 2003). Previous reports have shown that OPN induces αvβ3 integrin-mediated AP-1 activation and uPA secretion through c-Src/EGFR/ERK signaling pathways and all of these ultimately control breast cancer cell migration (Das et al, 2004). Recent studies suggest that mutant OPN lacking thrombin cleavable domain decreases cell adhesion and primary tumor latency time, and increases uPA expression, primary tumor growth and lymph node metastatic burden in MDA-MB-468 breast cancer cells (Beausoleil et al, 2011). Cook et al have shown that hyaluronan synthase 2 (HAS2) is found to be upregulated by OPN in breast cancer cells (Cook et al, 2006). It is reported that OPN induces NF-κB activation and NF-κB dependent AP1-mediated ICAM-1 expression through mTOR/p70S6 kinase pathways in breast cancer cells. The study suggests that inhibition of mTOR by rapamycin induces whereas overexpression of mTOR/p70S6 kinase suppresses OPN-induced ICAM-1 expression. Thus OPN stimulates p70S6 kinase phosphorylation at Thr-421/Ser-424, but not at Thr-389 or Ser-371 and mTOR phosphorylation at Ser-2448. Overexpression of mTOR has no effect in regulation of OPN-induced phosphorylation of p70S6 kinase at Thr-421/Ser-424 (Ahmed and Kundu, 2010). Recent reports also suggested that OPN induces αvβ3 integrin-mediated JAK2 dependent STAT3 activation in breast cancer cells. OPN protects the cells from staurosporine (STS)-induced apoptosis through JAK2/STAT3 pathway. Wt STAT3 in

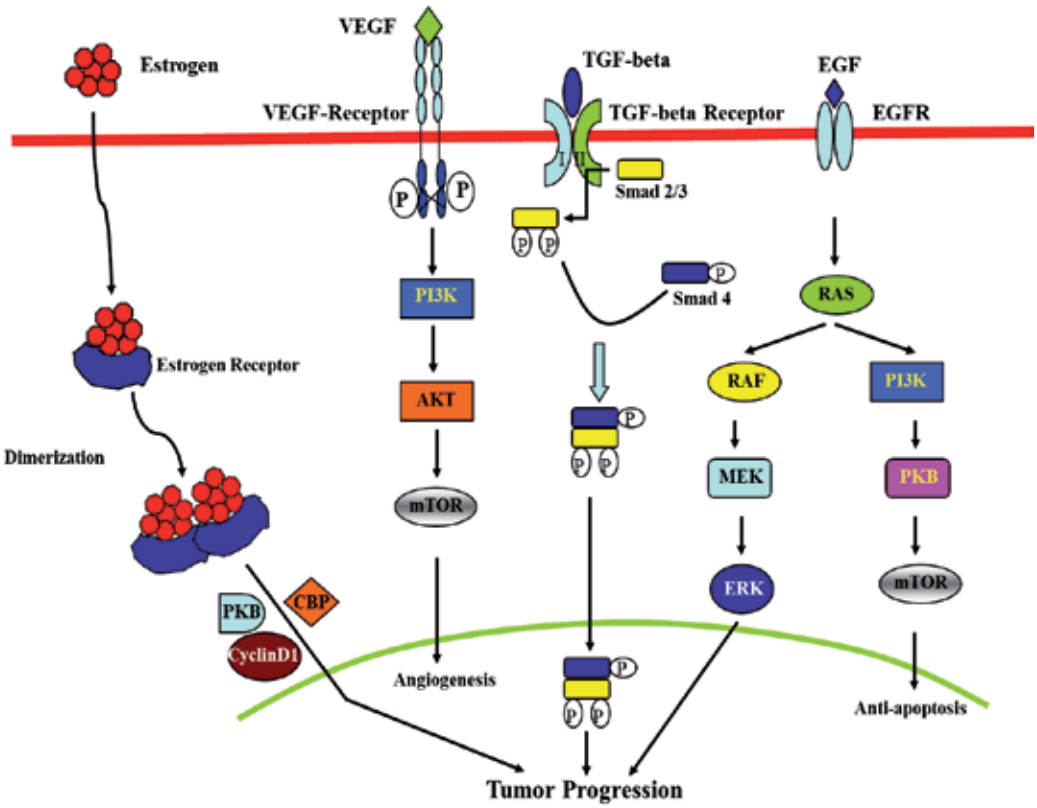


Fig. 2. Model depicting various signalling pathways involved in breast cancer cells. These pathways include estradiol, VEGF, TGF beta and EGF-induced signalling that promote cell growth, angiogenesis and prevention of cell death.

presence of OPN induces breast tumor progression through up regulation of Bcl2 and cyclin D1 expression in breast cancer cells (Behera et al, 2010). It has been also reported that both exogenous and tumor-derived OPN triggers vascular endothelial growth factor (VEGF)–dependent tumor progression and angiogenesis by activating breast tumor kinase (Brk)/NF-κB)/ATF-4 signaling cascades through autocrine and paracrine mechanisms in breast cancer models (Chakraborty et al, 2008). Curcumin inhibits OPN-induced VEGF expression leading to suppression of tumor angiogenesis in breast cancer (Chakraborty et al, 2008). Mi et al have demonstrated that OPN promotes CCL5-mesenchymal stromal cell (MSC) mediated breast cancer metastasis. They have shown that tumor derived OPN induces MSC expression of CCL5 through integrin mediated AP1 transactivation and further demonstrated that concomitant inoculation of MSC with MDA-MB-231 induces tumor growth and metastasis. These results suggested that tumor derived OPN promotes tumor progression through transformation of MSC into Cancer associated fibroblast (CAF) (Mi et al, 2011).

5. OPN as a chemoattractant cytokine and pro-angiogenic factor

OPN mediates RGD dependent chemotaxis, attachment and migration in many epithelial cell types (Celetti et al, 2005). It aids preferential metastasis of breast cancer cells to bone

(Kang et al, 2003). OPN functions in cell adhesion, chemotaxis, macrophage-directed interleukin-10 (IL-10) suppression, stress-dependent angiogenesis, prevention of apoptosis, and anchorage-independent growth of tumor cells by regulating cell-matrix interactions and cellular signaling through binding with integrin and CD44 receptors (Wai et al, 2004). Correlative evidence has shown that the αvβ3 integrin receptor appears to be preferentially used by more malignant breast epithelial cell lines in binding and migrating toward OPN (Tuck et al, 2000). Cancer metastasis involves invasion by the cancer cells, angiogenesis, circulation of cancer cells, colonization at a distant site and finally evasion of the host immune response. Motility of the cancer cells and degradation of extracellular matrix are essential for invasion. Cells cross the basement membrane and move to secondary organ sites. This phenomenon occurs due to the secretion of chemokines. Extracellular matrix degradation, by both tumor and host cells occurs by the secretion of proteases (Wong et al, 1998). On the molecular level, the metastatic phenotype is generated by the deregulation of cell surface receptors, their ligands, their downstream signaling molecules and extracellular matrix proteases. Unlike oncogenes, the genes involved in metastasis are not mutated but their expression is deregulated. OPN overexpression or exogenous addition in breast cancer cell lines increases the invasiveness of the cells and uPA expression through cell surface interactions between integrin and uPA/uPAR. Constitutive activation of NF-κB has been detected in lymphomas, melanomas and breast cancers and has been shown to correlate with oncogenesis.

A large number of proangiogenic factors and their cognate receptors have been identified including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), angiopoietin-1, transforming growth factor beta-1 (TGF-β1), transforming growth factor alpha (TGF-α), and epidermal growth factor (EGF) (Liotta et al, 2001). VEGF is one of the best characterized pro-angiogenic factors among other growth factors in terms of its specificity for the vascular endothelium (Mcmahon, 2000). OPN is involved in angiogenesis through αvβ3 integrin-mediated upregulation of VEGF expression. It can stimulate adhesion and migration of endothelial cells. Therefore, OPN and αvβ3 integrin play significant roles in vascular repair and regeneration. It has been reported that OPN protects the endothelial cells from apoptosis. This interaction is mediated by αvβ3 integrin and NF-κB dependent pathway.

6. Osteopontin regulates various signaling pathways in breast cancer

6.1 OPN controls tumor angiogenesis through VEGF/VEGFR signaling pathway

The molecular mechanism of OPN-induced VEGF expression and its potential role in regulating in vitro cell motility which ultimately controls in vivo tumor growth and angiogenesis in breast cancer model was described earlier (Chakraborty et al, 2008). The study highlighted the role of OPN in induction of neovascularization by enhancing VEGF expression through activation of breast tumor kinase (Brk)/NF-κB/ATF-4 pathways (Figure 3). OPN was shown to trigger VEGF-dependent tumor progression and angiogenesis by activating Brk/NF-κB/ATF-4 signaling cascades through autocrine and paracrine mechanisms in breast cancer cells. VEGF promoter activity and its expression in human breast carcinoma cell lines was found to be regulated by OPN. OPN induces Brk/NIK-dependent NF-κB-mediated ATF-4 activation that leads to VEGF expression. The study revealed that OPN-induced VEGF binds with neuropilin-1 (NRP-1) and enhances VEGF–NRP-1-dependent tumor cell migration through autocrine pathway. Moreover, OPN

induces VEGF dependent KDR phosphorylation leading to increased endothelial cell migration and angiogenesis in a paracrine manner. Tumor-endothelial cell interaction through binding with NRP-1 and KDR in endothelial cells was observed to be regulated by tumor derived VEGF in response to OPN in a juxtacrine manner. Blocking tumor-derived VEGF or silencing tumor-derived OPN and NRP-1 significantly suppressed breast tumor progression and angiogenesis in nude mice model. Clinical specimen analysis of solid human breast tumors exhibited strong correlation between the OPN and VEGF expression with different pathologic grades of tumors. Previous reports have also shown that VEGF induces mRNA encoding OPN in endothelial cells (Sengar et al, 1996). OPN plays a crucial role in determining spontaneous metastatic performance of orthotopic human breast cancer xenografts. Changes in levels of OPN induced by silencing with its shRNA or upregulation by cDNA altered the ability of breast cancer cells to colonize to distant organs. It has been shown that silencing of OPN resulted in reduction of *in vivo* tumorigenicity through down regulation of molecules like uPA, MMP-2 and -9. OPN knocked out mice showed slower progression of tumor growth in breast cancer model as compared to wild type mice (Chakraborty et al, 2008).

6.2 OPN inhibits staurosporine (STS)-induced apoptosis through JAK2/STAT3 signaling pathway

Earlier reports have indicated that enhanced expression of STAT3 correlates with increased tumor growth and poor survival in breast cancer (Garcia et al, 1997). Behera et al have recently demonstrated that OPN induces αvβ3 integrin-mediated JAK2 dependent STAT3 activation in breast cancer cells (Behera et al, 2010). The mechanism by which OPN controls JAK2/STAT3 signaling pathway and regulates apoptosis and breast tumor growth was studied. OPN was found to activate STAT3 by inducing its phosphorylation through αvβ3 integrin mediated pathway. OPN has been observed to regulate STAT3 nuclear translocation through αvβ3 integrin mediated and JAK2 dependent pathway. It was further established that OPN, through promoting STAT3-DNA binding ultimately regulates the expression of downstream molecules such as cyclin D1 and Bcl2 and thus influences survival and cell migration in breast cancer (Figure 3). Cells transfected with wt STAT3 showed enhanced cell migration as well as anti-apoptotic function in response to OPN, as opposed to cells transfected with the mutant forms of STAT3. The study revealed that OPN protects the cells from staurosporine (STS)-induced apoptosis through JAK2/STAT3 pathway. Cells stably transfected with wt STAT3 and not with mutant STAT3 were observed to enhance tumor growth in response to OPN in mice models. Enhanced expressions of Bcl2 and cyclin D1 in STAT3- overexpressed tumors in response to OPN were indicative of the significance of STAT3 in OPN-induced Bcl2 and cyclin D1 expression and tumor progression. Clinical specimen analysis revealed an enhanced expression of OPN and phosphorylated STAT3 and their correlation with higher grades of breast cancer as compared to the peripheral normal and lower grades.

6.3 OPN regulates breast cancer cell motility through mTOR/p70S6 kinase pathway

mTOR, a serine threonine kinase regulates both cell growth and cell cycle progression (Ahmed and Kundu, 2010). mTOR initiates translation by activating the p70S6 kinase. Inhibition of mTOR by rapamycin attenuates its ability to control cell cycle progression, cell growth and proliferation in normal and malignant cells. They have recently reported

that OPN regulates p70S6 kinase and mTOR phosphorylation in breast cancer cells (Ahmed and Kundu, 2010). The results revealed that OPN controls NF-κB mediated ICAM-1 expression in these cells. The data also showed that OPN induced NF-κB controls AP-1 transactivation indicating a cross talk between NF-κB and AP-1 which in turn regulates ICAM-1 expression in these cells (Figure 3). The study suggested that inhibition of mTOR by rapamycin enhanced whereas overexpression of mTOR/p70S6 kinase inhibited OPN-induced ICAM-1 expression. OPN-induced NF-κB and AP-1-DNA binding and transcriptional activity was inhibited by mTOR overexpression whereas rapamycin was noted to enhance these OPN-induced effects. In the same study, OPN was shown to selectively phosphorylate p70S6 kinase at Thr-421/Ser-424 through MEK/ERK pathway but it did not phosphorylate p70S6 kinase at Thr-389 and Ser-371 sites which further suggested that mTOR inhibitor, rapamycin suppresses p70S6 kinase phosphorylation at Ser-371 and does not affect p70S6 kinase phosphorylation at Thr-421/Ser-424 and Thr-389 sites indicating that Ser-371 phosphorylation is primarily responsible for p70S6 kinase activation in these cells (Figure 3).

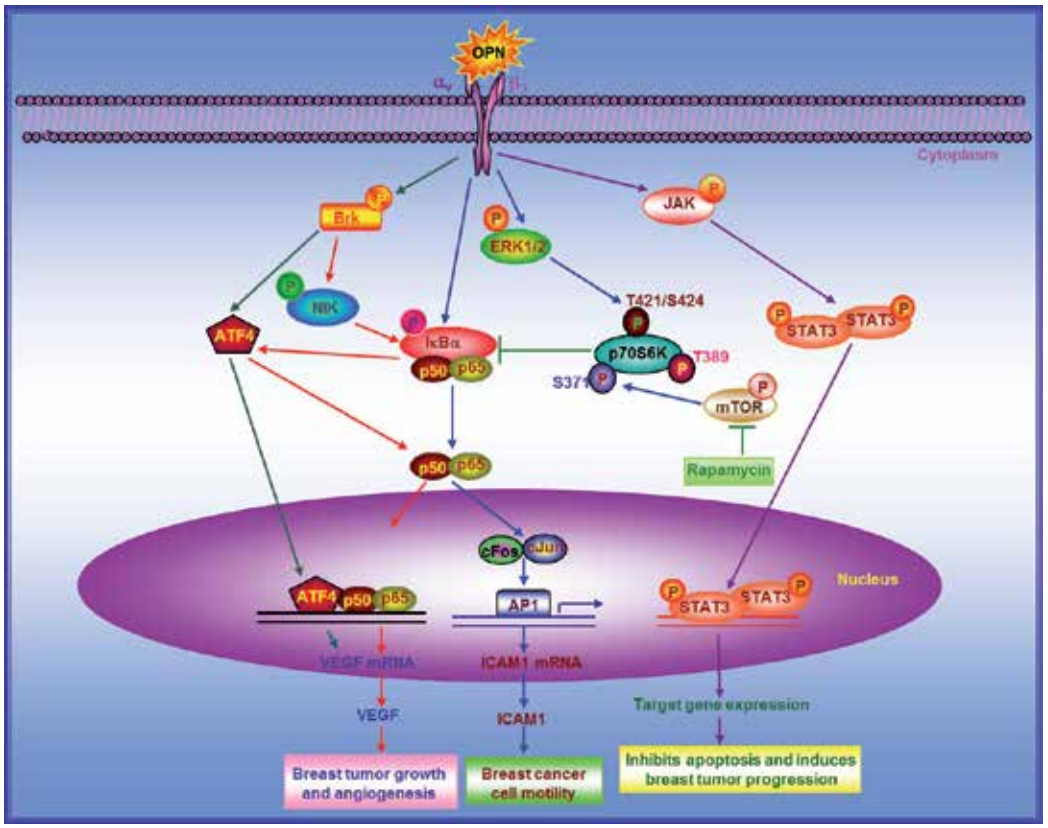


Fig. 3. Diagramatic representation of OPN-induced signaling cascades mediated by its cell surface receptor, integrin. These signaling pathways lead to upregulation of various oncogenic and angiogenic molecules that augment breast cancer cell migration, tumor growth, angiogenesis and inhibition of apoptosis, (Adapted from Chakraborty et al., 2008; Ahmed and Kundu, 2010; Behera et al., 2010 with modification).

7. Clinicopathological significance of osteopontin in breast cancer

Effective management of breast cancer is possible by surgical removal of the tumor. Metastasis of tumor cells to secondary sites like bone, lung, liver and brain leads to poor survival. Although the detection system is not well established owing to the multifactorial nature and heterogeneity of cancer, early diagnosis can be made possible by identifying cancer biomarkers. Many earlier publications suggested that OPN may be considered as one of the potential candidate biomarkers in breast cancer. OPN is overexpressed in human breast cancer cells and tissues as well as in stromal compartment including CAFs. OPN plays a critical role in generation of calcification which is allied with breast cancer. Enhanced expression of OPN has been found in plasma and tumors of metastatic breast cancer suggesting that OPN may be considered as a prognostic marker (Bramwell et al, 2006). The plasma OPN level in women with known metastatic breast carcinoma is significantly higher than that of normal healthy individuals. The plasma OPN level in patients with metastatic breast cancer is higher than 138 ng/ml versus control groups which have 123 ng/ml. Gene profiles compared between lobular versus ductal breast carcinomas using microarray analysis reveal 11 genes including OPN, and a specific change in gene expression (Korkola et al, 2003). An mRNA transcript analysis of OPN in normal, non-invasive, invasive and metastatic human breast cancer specimens shows that it's level increases with enhanced malignancy. Moreover, a splice variant of OPN, namely OPN-C has been shown to be an important marker of breast cancer. It has been shown that OPN-C is selectively expressed in invasive, but not in non-invasive breast tumor cell lines. When the significance of OPN-C was studied in various tumor grades of breast cancer, the level of OPN-C increased from grade 1 to 3. Conclusively, these reports suggested that OPN-C is a selective marker of breast cancer (He et al, 2005).

8. Therapeutic potential of OPN and its receptors

Many OPN specific monoclonal and polyclonal antibodies have been generated. It has been observed that humanized anti-OPN antibody inhibits cell migration, adhesion, invasion, colony formation, tumor growth and lung metastasis in breast cancer (Dai et al, 2010). Thus for effective cancer management, targeting OPN by its specific blocking antibody may provide a novel therapeutic approach (Figure 4). The binding of OPN and its receptor controls the expression of various oncogenic molecules leading to tumor progression through various signalling pathways. Therefore, disruption of OPN and its receptor ligation may attenuate tumor growth and metastasis. αvβ3 integrin blocking antibody inhibits OPN-induced tumor growth and angiogenesis through attenuating various signaling cascades (Rangaswami et al, 2006). Decreased expression of OPN, integrin linked kinase (ILK), uPA and MMP-2 in murine mammary epithelial cancer cells was observed by blocking αvβ3 integrin (Mi et al, 2006). OPN can interact with various integrins and the specific blocking antibodies against these receptors can significantly suppress tumor-stromal interaction and reduce OPN-induced tumor progression (Figure 4). It has been recently documented that αvβ3 integrin blocking antibody inhibits AP1 activation in response to OPN in breast cancer cells (Ahmed and Kundu, 2010). Inhibition of OPN and αvβ3 integrin binding by LM609 and RGD peptide attenuates STAT3 DNA-binding and suppresses cell migration and breast tumor growth by down regulating the expression of cyclinD1 and Bcl2 (Behera et al, 2010). Previous results have demonstrated that non-RGD-based integrin binding peptide (ATN-161) suppresses breast tumor growth and metastasis (Khalili et al, 2006).

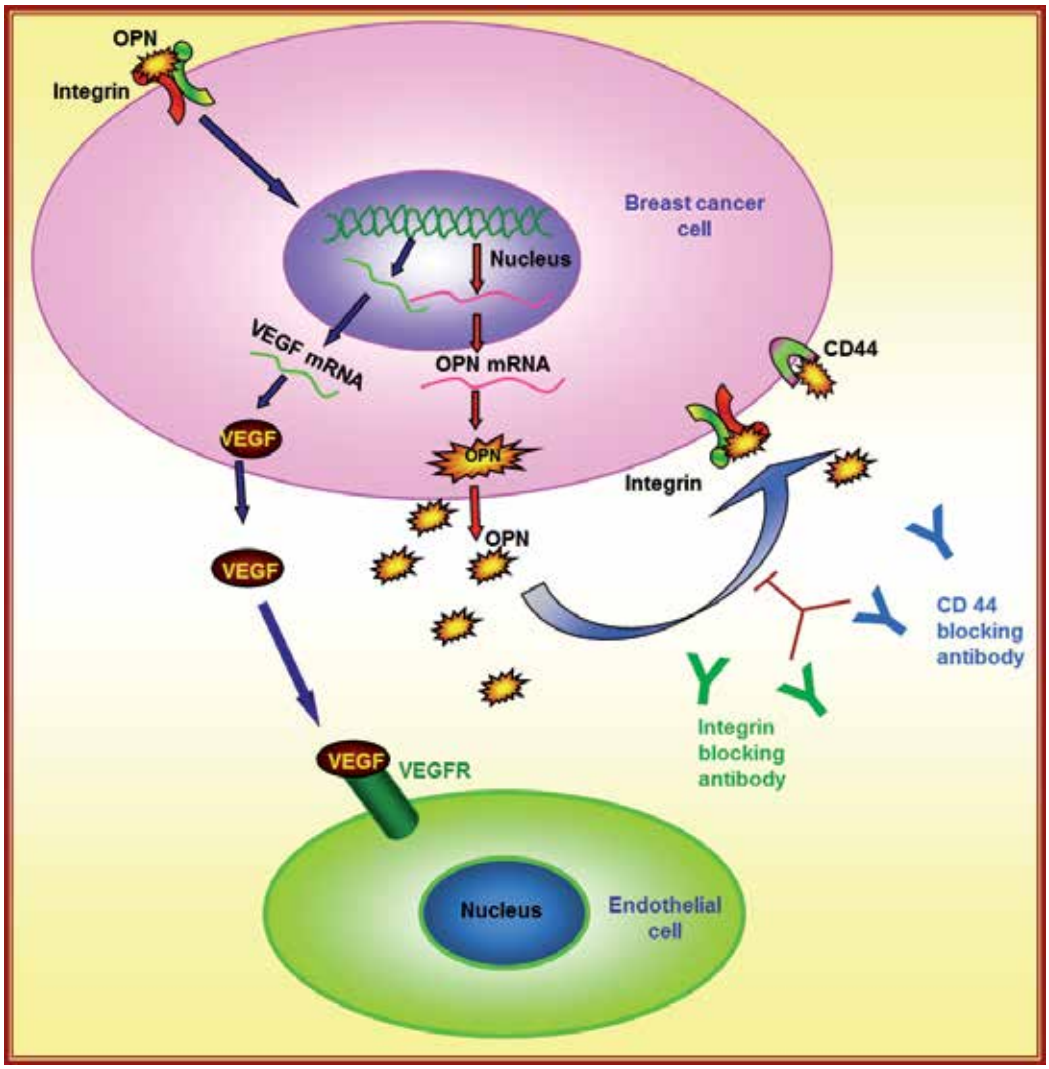


Fig. 4. Therapeutic targeting of OPN in breast cancer. The blocking antibody against OPN or its receptors such as integrin and CD44 impedes OPN regulated cancer signalling pathways leading to inhibition of breast tumor growth and angiogenesis through disruption of tumor-endothelial cell interaction.

9. Conclusion

Breast cancer accounts for major cancer related death in women around the world. Tremendous efforts are being made everyday in reducing the occurrence of breast cancer. Because of the complexity of the diseases, precise detection system is not available till date to diagnose the cancer at the early stages. Therefore, identification of novel biomarkers is the need of the hour. According to numerous publications, OPN may be considered as a potential biomarker in breast cancer because of its involvement in all the stages of tumor progression. Hence targeting OPN would be a rational approach for the treatment of cancer. In addition to tumor derived OPN, stromal OPN also plays a crucial role in regulation of tumor progression and angiogenesis. In conclusion, we have demonstrated that OPN regulates breast cancer cell migration through mTOR/p70S6 kinase dependent ICAM-1 expression. Moreover, OPN also induces breast tumor growth and inhibits apoptosis through induction of JAK2/STAT3 dependent expression of Bcl2 and cyclin D1. Furthermore, OPN controls VEGF dependent breast cancer growth and angiogenesis through tumor-endothelial cell interaction via Brk/NIK dependent NF-κB activation pathway. Thus in depth knowledge of OPN regulated signalling mechanism may be useful in developing novel molecular diagnostics and targeted therapy for the management of breast cancer.

10. Acknowledgements

The author's research is aided in part by Department of Biotechnology, Department of Science and Technology and Council of Scientific and Industrial Research, Government of India (to GCK). We apologize to the colleagues whose contributions we could not site due to lack of space.

11. References

Ahmed, M. & Kundu, GC. (2010). Osteopontin selectively regulates p70S6K/mTOR phosphorylation leading to NF-κB dependent AP-1-mediated ICAM-1 expression in breast cancer cells. *Molecular Cancer*, Vol. 9, No. 101, (May 2010), pp 101-13.

Beausoleil, MS., et al. (2011). Deletion of the thrombin cleavage domain of osteopontin mediates breast cancer cell adhesion, proteolytic activity, tumorgenicity, and metastasis. *BMC Cancer*, Vol. 11, No. 25, (January 2011), pp 1-12.

Behera,R., et al. (2010). Activation of JAK2/STAT3 signaling by osteopontin promotes tumor growth in human breast cancer cells. *Carcinogenesis*, Vol. 31, No. 2, (February 2010), pp 192–200.

Boominathan, L. (2010). The tumor suppressors p53, p63, and p73 are regulators of microRNA processing complex. *PLoS One*, Vol. 5, No. 5, (May 2010), pp. 1-13.

Bramwell, V.H.C., et al (2006). Serial plasma osteopontin levels have prognostic value in metastatic breast cancer. *Clinical Cancer Research.* Vol.12, (June 2006), pp. 3337-43.

Celetti et al. (2005). Overexpression of the cytokine osteopontin identifies aggressive laryngeal squamous cell carcinomas and enhances carcinoma cell proliferation and invasiveness, *Clinical Cancer Research,* Vol.11, (November 2005), pp. 8019-27.

Chakraborty, G., et al. (2006). The multifaceted roles of osteopontin in cell signaling, tumor progression and angiogenesis. *Current Molecular Medicine*. Vol. 6, No. 8, (December 2006), pp. 819-30.

Chakraborty, G., et al. (2008). Osteopontin promotes vascular endothelial growth factor-dependent breast tumor growth and angiogenesis via autocrine and paracrine mechanisms. *Cancer Research*, Vol. 68, No. 1, (January 2008), pp 152-61.

Chakraborty, G., et al (2008). Curcumin suppresses breast tumor angiogenesis by abrogating osteopontin-induced VEGF expression. *Molecular Medicine Reports*, Vol. 1, No. 5, (June 2008) pp. 641-46.

Chen, JQ. & Russo, J. (2009). ER alpha negative and triple negative breast cancer: molecular features and potential therapeutic approaches. *Biochimica et Biophysica Acta*, Vol. 1796, No. 2, (December 2009), pp. 162-75.

Cook, A.C., et al (2005). Osteopontin induces multiple changes in gene expression that reflect the six ''Hallmarks of Cancer'' in a model of breast cancer progression. *Molecular Carcinogenesis*, Vol. 43, No. 4, (August 2005), pp- 225-36.

Cook, A.C., et al (2006). Osteopontin induction of hyaluronan synthase 2 expression promotes breast cancer malignancy. *Journal of Biological Chemistry*, Vol. 281, No. 34, (August 2006), pp. 24381-89.

Dai, J., et al (2010). A humanized anti-osteopontin antibody inhibits breast cancer growth and metastasis in vivo. *Cancer Immunology and Immunotherapeutics*, Vol.59, No.3, (March 2010), pp. 355-66.

Das, R., et al. (2003). Osteopontin stimulates cell motility and nuclear factor kappaB-mediated secretion of urokinase type plasminogen activator through phosphatidylinositol 3-kinase/Akt signaling pathways in breast cancer cells. *Journal of Biological Chemistry*, Vol. 278, No. 31, (August 2003), pp 28593-606.

Das, R., et al. (2004). Osteopontin induces AP-1-mediated secretion of urokinase-type plasminogen activator through c-Src-dependent epidermal growth factor receptor transactivation in breast cancer cells. *Journal of Biological Chemistry*, Vol. 279, No.12, (March 2004), pp 11051-64.

Garcia, R., et al (1997). Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. *Cell Growth and Differentiation*. Vol.8, No.12 (December 1997), pp. 1267-76.

Hanahan, D. & Weinberg, RA. (2000). The hallmarks of cancer. *Cell*, Vol. 100, No.1, (January 2000), pp. 57-70.

Hanahan, D. & Weinberg, RA. (2011). Hallmarks of cancer: The next generation. *Cell,* Vol. 144, No. 5, (March 2011), pp. 646-74.

He, B., et al. (2005). An osteopontin splice variant induces anchorage independence in human breast cancer cells. *Oncogene,* Vol. 25, No. 15, (April 2006), pp. 2192-202.

Kang, Y. et al., (2003). A multigenic program mediating breast cancer metastasis to bone, *Cancer Cell,* Volume 3, No. 6 (June 2003) pp. 537-49.

Khalili, P., et al (2006). A non-RGD-based integrin binding peptide (ATN-161) blocks breast cancer growth and metastasis in vivo. *Molecular Cancer Therapeutics*, Vol.5, No.9, (September 2006), pp. 2271-80.

Korkola, J.E., et al (2003). Differentiation of lobular versus ductal breast carcinomas by expression microarray analysis. *Cancer Research,* Vol.63, No.21, (November 2003), pp. 7167-75.

Leslie, M. (2011). Brothers in arms against cancer. *Science*, Vol. 331, No. 6024, (March 2011), pp. 1551 - 52.

Liotta, L. A., & Kohn, EC. (2001). The microenvironment of the tumor-host interface, *Nature*, Vol. 411, No. 6835 (May 2001), pp. 375 –79.

Mcmahon (2000). VEGF receptor signaling in tumor angiogenesis, *The Oncologist,* Vol.5, (April 2000), pp. 3-10.

Mi, Z., et al (2006). Integrin-linked kinase regulates osteopontin-dependent MMP-2 and uPA expression to convey metastatic function in murine mammary epithelial cancer cells. *Carcinogenesis,* Vol.27, No.6, (June 2006), pp. 1134-45.

Mi, Z., et al (2011). Osteopontin promotes CCL5-mesenchymal stromal cell-mediated breast cancer metastasis, *Carcinogenesis*, Vol.32, No. 4, (April 2011), pp. 477-87.

Osterreicher, C.H., et al. (2011). Fibroblast-specific protein 1 identifies an inflammatory subpopulation of macrophages in the liver *Proceedings of the National Academy of Sciences,* Vol. 108 No. 1, (January 2011) pp 308-13.

Panda, D., et al. (1997). Potential roles of osteopontin and alphavbeta3 integrin in the development of coronary artery restenosis after angioplasty, *Proceedings of the National Academy of Sciences,* Vol. 94, No. 17, (August 1997), pp. 9308–13.

Rangaswami, H., et al. (2006). Osteopontin: role in cell signaling and cancer progression. *Trends in Cell Biology*, Vol. 16, No 2, (February 2006), pp. 79-87.

Rittling, S.R., et al. (2002). Tumor-derived osteopontin is soluble, not matrix associated. *Journal of Biological Chemistry*, Vol. 277, No. 11, (March 2002), pp. 9175-82.

Senger, D.R., et al. (1996). Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin. *American Journal of Pathology,* Vol.149, No.1 (July 1996), pp. 293-305.

Shevde, L.A., et al (2006). Osteopontin knockdown suppresses tumorigenicity of human metastatic breast carcinoma, MDA-MB-435. *Clinical Experimental Metastasis*. Vol. 23, No. 2, (July 2006), pp. 123-33.

Shinohara, M.L., et al. (2008). Alternative translation of osteopontin generates intracellular and secreted isoforms that mediate distinct biological activities in dendritic cells. *Proceedings of the National Academy of Sciences*, Vol. 105, No 20, (May 2008), pp. 7235-39.

Stefansson, O.A., et al. (2009). Genomic profiling of breast tumours in relation to BRCA abnormalities and phenotypes. *Breast Cancer Research*, Vol. 11, No. 4, (July 2009), pp. 1-14.

Tuck et al. (2000). Osteopontin-induced, integrin-dependent migration of human mammary epithelial cells involves activation of the hepatocyte growth factor receptor (Met). *Journal of Cellular Biochemistry*, Vol.78, No. 3, (June 2000), pp. 465–75.

Wai, PY. & Kuo, PC. (2004). The role of Osteopontin in tumor metastasis. *Journal of Surgical Research*. Vol. 121, No. 2, (October 2004), pp 228-41.

Wong et al. (1998). Alphav integrins mediate adhesion and migration of breast carcinoma cell lines. *Clinical and Experimental Metastasis*, Vol.16, No. 1, (January 1998) pp. 50 – 61.

3

Targeting Cas Family Proteins as a Novel Treatment for Breast Cancer

Joerg Kumbrink and Kathrin H. Kirsch
Department of Biochemistry, Boston University School of Medicine, Boston, MA USA

1. Introduction

Frequently, breast cancer is treated before and after surgery with chemotherapy, hormone, and radiation therapies. However, breast cancers can evolve and stop responding to chemotherapeutic drugs, including adriamycin (doxorubicin), and hormone therapy with tamoxifen. A new generation of targeted biological agents demonstrates a high effectiveness at lower toxicity. Treatment with these specific drugs is limited to subsets of breast cancers that depend on their targets, and eventually patients develop resistance to these drugs as well. This strongly indicates the need to develop novel approaches to fight breast tumor cells and to prevent or reduce drug-resistance. The Cas family of proteins play significant roles in development, proliferation, cell cycle control, cell survival, migration, and invasion. Some of its members, in particular p130^{Cas}/*BCAR1*, has been implicated with tamoxifen as well as adriamycin resistance in mammary tumors. Here we review the role of the Cas family of proteins in breast cancer and summarize the potential development of anti-cancer therapeutics targeting this important family of adapter-type proteins.

1.1 Cas family

Proteins of the Cas (Crk-associated substrate) family function as scaffolds for large multi-protein complexes that integrate the response to numerous stimuli including growth factors, integrin engagement, and hormone release (Tikhmyanova *et al.* 2010; Bouton *et al.* 2001). Cas family members comprise p130^{Cas}/*BCAR1* (Sakai *et al.* 1994a; Brinkman *et al.* 2000), HEF1 (also known as *NEDD9*, CASS2, Cas-L) (Law *et al.* 1996; Minegishi *et al.* 1996), Efs/Sin (CASS3) (Alexandropoulos & Baltimore 1996; Ishino *et al.* 1995), and CASS4 (HEPL) (Singh *et al.* 2008). p130^{Cas} is the founding member and was first identified as a major tyrosine-phosphorylated 130 kDa protein in cells transformed by the v-crk and v-src oncogenes (Sakai *et al.* 1994a). HEF1 (human enhancer of filamentation)/*NEDD9* (neural precursor cell expressed, developmentally downregulated 9) was isolated in a screen for human proteins that confer morphoregulatory changes leading to filamentous budding in yeast *Saccharomyces cerevisiae* (Law *et al.* 1996). In addition, HEF1 was independently isolated based on its homology to p130^{Cas} (Minegishi *et al.* 1996).
Efs/Sin (embryonal Fyn-associated substrate/Src-interacting protein) was identified as a protein binding to the Src-homology (SH) 3 domain of Fyn (Alexandropoulos & Baltimore 1996; Ishino *et al.* 1995). Most recently, the fourth member CASS4 (Cas scaffolding protein family member 4)/HEPL (HEF1-Efs-p130^{Cas}-Like) was identified using reiterative BLAST

analysis for protein and mRNA sequences of Cas family members (Singh *et al.* 2008). The expression patterns of Cas family members are distinct. $p130^{Cas}$ is ubiquitously expressed in adult tissues, suggesting that it plays an essential role in normal cell physiology (Defilippi *et al.* 2006; Sakai *et al.* 1994a). HEF1 is found primarily in lymphocytes and in lung and breast epithelium (Law *et al.* 1996; Law *et al.* 1998; Minegishi *et al.* 1996). Highest levels of Efs/Sin and CASS4 expression are found in the placenta and brain (Ishino *et al.* 1995), and lung and spleen (Singh *et al.* 2008), respectively.

By facilitating the interaction of Src family kinases (SFKs), focal adhesion kinase (FAK), and recruiting the adaptor proteins Crk and Nck, members of the Cas family play significant roles in signaling networks involved in cell survival (Cabodi *et al.* 2006; Kim *et al.* 2004), cell cycle regulation (Law *et al.* 1998; Ma *et al.* 2007; Yamakita *et al.* 1999), proliferation, and invasion (Huang *et al.* 2002; Klemke *et al.* 1998) (as depicted in Figure 1). These cellular programs are frequently deregulated in different cancer types (Cabodi *et al.* 2010a; Henderson & Feigelson 2000; Marcotte & Muller 2008; Hanahan & Weinberg 2011) and concordantly members of the Cas family have been extensively associated with the development and progression of different tumors in particular mammary carcinomas as reviewed in Section 2.

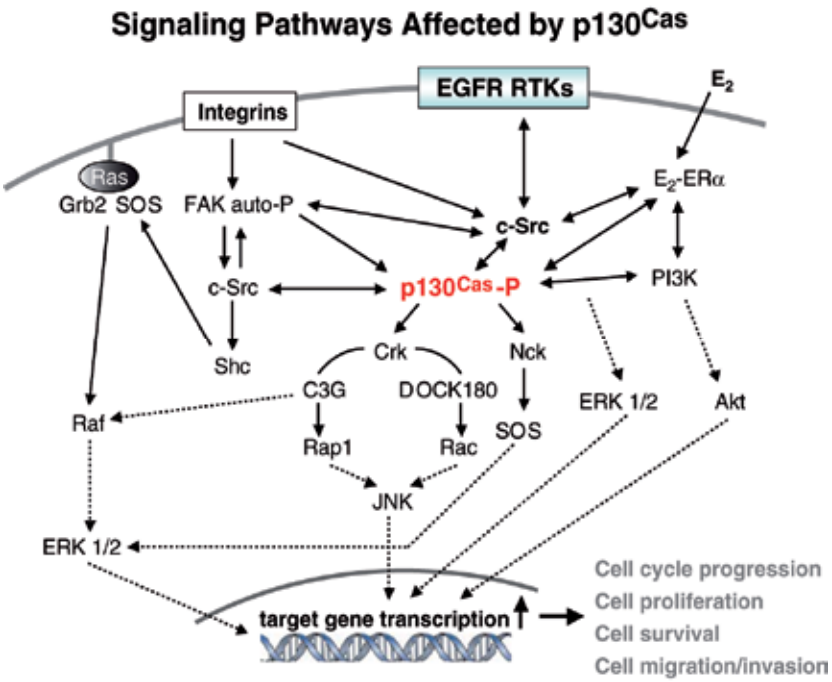


Fig. 1. Major signaling pathways affected by $p130^{Cas}$. Integrin engagement, growth factor-mediated activation of receptor tyrosine kinases (RTKs), and estrogen (E_2)-induced non-genomic estrogen receptor (ER) alpha signaling results in tyrosine phosphorylation of $p130^{Cas}$. The phosphorylated/activated $p130^{Cas}$ recruits various effector proteins, thereby generating a signaling node, that activates downstream pathways leading to the induction of transcriptional programs promoting cell cycle progression, proliferation, survival, and migration/invasion. Solid lines depict direct interactions; dashed lines show pathways that have additional steps in between.

1.2 The domain structure of Cas family proteins

Cas proteins exhibit a highly conserved modular domain structure and vary from 561 to 870 amino acids (Tikhmyanova *et al.* 2010). To date no evidence has been found for intrinsic enzymatic activity of the Cas family members. The members are characterized by multiple protein-protein interaction domains including an amino-terminal SH3 domain, a central substrate domain (SD) containing multiple tyrosine phosphorylation sites, a serine-rich region (SER), and a carboxy-terminal domain (CTD) containing a bi-partite Src-binding motif (SBM) (Figure 2). Cas proteins have numerous binding partners for each domain which are summarized in Table 1.

Domain	Interacting partners	Reference
SH3 domain	C3G	(Kirsch *et al.* 1998)
	CMS/CD2AP	(Kirsch *et al.* 1999)
	CIZ	(Nakamoto *et al.* 2000)
	FAK	(Polte & Hanks 1995; Law *et al.* 1996; Singh *et al.* 2008)
	FRANK	(Harte *et al.* 1996)
	PR-39	(Chan & Gallo 1998)
	PTP-1B	(Liu *et al.* 1996)
	PTP-PEST	(Garton *et al.* 1997)
	Pyk2	(Astier *et al.* 1997; Lakkakorpi *et al.* 1999)
Substrate Domain	Crk family: CrkI, CrkII, CrkL	(Burnham *et al.* 1996; Ishino *et al.* 1995; Petruzzelli *et al.* 1996; Sakai *et al.* 1994a; Salgia *et al.* 1996)
	Nck	(Schlaepfer *et al.* 1997)
	SHP-2	(Prasad *et al.* 2001; Yo *et al.* 2009)
	c-Src	(Shin *et al.* 2004)
Serine-rich Domain	14-3-3	(Briknarova *et al.* 2005)
Carboxy-terminal domain	AIP4	(Feng *et al.* 2004)
	APC/C, CDH1	(Nourry *et al.* 2004)
	Bmx/Etk	(Abassi *et al.* 2003)
	NSP family: BCAR3/NSP2, NSP1, CHAT	(Gotoh *et al.* 2000; Lu *et al.* 1999; Sakakibara & Hattori 2000)
	Nephrocystin	(Donaldson *et al.* 2000)
	$p130^{Cas}$, HEF1, Id2	(Law *et al.* 1999)
	p140Cap	(Di Stefano *et al.* 2004)
	PI3K	(Li *et al.* 2000)
	Src family: c-SRC, LCK, LYN, HCK, FYN, YES	(Alexandropoulos & Baltimore 1996; Ishino *et al.* 1995; Kanda *et al.* 1999; Nakamoto *et al.* 1996; Nasertorabi *et al.* 2006; Nishio & Suzuki 2002; Pellicena & Miller 2001; Singh *et al.* 2008)
Not mapped/ indirect	DDR	(Shintani *et al.* 2008)
	ER alpha	(Cabodi *et al.* 2004)
	Grb2	(Wang *et al.* 2000)
	Smad3	(Liu *et al.* 2000)

Table 1. Interacting partners of Cas family proteins.

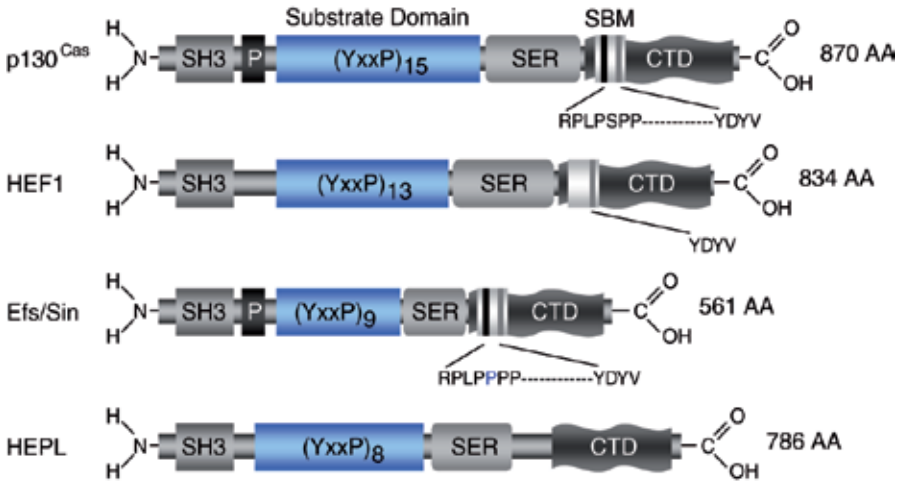


Fig. 2. Domain structure of the Cas family members. SH3, Src homology 3 domain. P, proline-rich region. SER, serine-rich domain. SBM, bi-partite Src-binding motif. CTD, carboxy-terminal domain. The number of YxxP motifs, representing SH2 domain binding sites when phosphorylated, are indicated.

1.2.1 The SH3 domain

The amino-terminal SH3 domain is an interaction module that associates with proteins containing proline-rich motifs with the core consensus sequence PxxP (Ren *et al.* 1993). The SH3 domain of p130^{Cas} selects binding sites sharing the consensus motif *XX*Pp+Pp*X* (where + and *X* represent positively charges and non-conserved residues, respectively; lower case positions contain residues that tend to be proline) (Kirsch *et al.* 1998). The specificity of the p130^{Cas} SH3 domain is strongly dependent on the positively charged amino acid in the position P_2 (see nomenclature in Yu *et al.* 1994). The functional relevance of the interaction of Cas family members with FAK via the Cas SH3 domain has been extensively studied (Polte & Hanks 1995; Parsons *et al.* 2000; Law *et al.* 1996; Singh *et al.* 2008; Provenzano & Keely 2009; Tikhmyanova *et al.* 2010). Utilizing p130^{Cas} SH3 domain deletion mutants, studies indicated that this domain and its interaction with FAK are necessary for phosphorylation and the localization of p130^{Cas} to focal adhesions (FAs) (Nakamoto *et al.* 1997). More recent studies, further explored the temporal and spatial involvement of p130^{Cas} in FA dynamics and showed the influence of p130^{Cas} in FA turnover and controlling the migratory response (Donato *et al.* 2010; Meenderink *et al.* 2010). The p130^{Cas} SH3 domain was shown to be necessary for tyrosine phosphorylation of the SD and the promotion of cell migration (Donato *et al.* 2010), in conditional FAK-deficient mammary tumor cells a reduction in the phosphorylation of Y249 within the p130^{Cas} SD was observed (Provenzano *et al.* 2008). These studies uncovered the significance of the Cas SH3 domain in the spatial regulation of Cas protein function and in particular its role for the phosphorylation of the SD of the Cas proteins (as described in the next section).

1.2.2 The substrate domain (SD)

The SD region of the Cas family members is situated adjacent to the SH3 domain and contains clusters of YxxP tyrosine phosphorylation sites (Figure 2). The SD of p130^{Cas} and

HEF1 contain 15 and 13 YxxP motifs, whereas only eight and nine of these motifs are present in Efs/Sin and HEPL, respectively (Alexandropoulos & Baltimore 1996; Ishino *et al.* 1995; Law *et al.* 1996; Sakai *et al.* 1994a), which upon phosphorylation by Src family members recruit small adaptor proteins such as Crk, CRKII, CrkL, and Nck via their respective SH2 domains (Burnham *et al.* 1996; Harte *et al.* 1996; Minegishi *et al.* 1996).

The SFKs, in particular c-Src, play significant roles in the activation of Cas proteins by phosphorylation of tyrosine residues within the SD, resulting in coupling to downstream effector molecules (Sakai *et al.* 1997; Schlaepfer *et al.* 1997). Correspondingly, c-Src-deficient cells show reduced Cas phosphorylation levels. Furthermore, *in vitro* kinase assays show that c-Src, in comparison to FAK, has a stronger ability to phosphorylate the SD of $p130^{Cas}$ (Ruest *et al.* 2001).

In order to prevent permanent activation of Cas-related pathways, the protein tyrosine phosphatases (PTPs) PTP-1B (Liu *et al.* 1996), PTP-PEST (Garton *et al.* 1997; Cote *et al.* 1998), and leukocyte antigen related (LAR)-PTP (Hoon *et al.* 2003) can be recruited to Cas family members to dephosphorylate Cas proteins and other associated molecules. This results, for instance, in the subsequent cleavage and degradation of the $p130^{Cas}$ protein (Hoon *et al.* 2003; Weng *et al.* 1999) and/or the disassembly of signaling complexes in FAs (Angers-Loustau *et al.* 1999).

The tyrosine phosphorylation/activation status of Cas family members is altered by diverse stimuli, including environmental influences such as growth factors, integrin, and estrogen signaling as well as intrinsic signals, thereby modulating multiple signal transduction networks as depicted in Figure 1 (reviewed in Bouton *et al.* 2001; Cabodi *et al.* 2010a; Tikhmyanova *et al.* 2010). SD phosphorylation correlates with transformation and the effector signaling pathways relevant in breast cancer development and progression involving the Cas family proteins are reviewed in Section 2.

1.2.3 The serine-rich domain (SER)

This domain is situated between the SD and the CTD and is enriched in serines and threonines (Alexandropoulos & Baltimore 1996; Law *et al.* 1996; Sakai *et al.* 1994a; Sakai *et al.* 1994b; Singh *et al.* 2008). Although the SER was initially thought to separate and orient the SD and SBM, several studies identified important properties. During mitosis an increase in the phosphorylation of serine and threonine residues of $p130^{Cas}$ has been observed (Yamakita *et al.* 1999). Notably, adhesion-dependent serine phosphorylation of $p130^{Cas}$ is associated with an invasive phenotype in breast cancer cells (Makkinje *et al.* 2009). The solution structure of the SER of $p130^{Cas}$ was determined by nuclear magnetic resonance spectroscopy revealing that it folds as a four-helix bundle. Site-directed mutagenesis and binding assays characterized this domain as an interaction site for 14-3-3 proteins (Briknarova *et al.* 2005). Proteins of the 14-3-3 family act as chaperones or scaffolds and are involved in signaling, cell cycle control, and apoptosis (reviewed in Bridges & Moorhead 2004).

1.2.4 The carboxy-terminal domain (CTD)

Although the CTD, which folds as a helix-loop-helix (HLH) structure, is the most conserved region among Cas family members, one of the striking differences regarding this domain is the presence or absence of the bi-partite SBM. The SBM consists of a proline-rich motif (RPLP S/P PP) that interacts with the SH3 domain of SFKs and a YDYVHL motif that, when

phosphorylated, interacts with the SH2 domain of SFKs. This region is present in p130Cas and Efs/Sin but absent in CASS4 (Alexandropoulos & Baltimore 1996; Nakamoto *et al.* 1996; Singh *et al.* 2008; Nasertorabi *et al.* 2006). HEF1 contains the SH2 binding motif but lacks the SH3 binding motif (Law *et al.* 1996). This may influence the ability of HEF1 to bind SFKs and to become phosphorylated, since amino acid substitutions in the RPLP S/P PP sequence reduces/abolishes binding to Src (Burnham *et al.* 1999; Burnham *et al.* 2000; Nakamoto *et al.* 1996). However, TGF-β-mediated tyrosine phosphorylation of HEF1 (Zheng & McKeown-Longo 2002) and attachment-induced tyrosine phosphorylation of CASS4 (Singh *et al.* 2008), are both dependent on Src kinase activity. It has been suggested that additonal mechanisms may regulate this modification such as FAK-dependent recruitment of c-Src to p130Cas (Ruest *et al.* 2001). The differences in the SBM of Cas proteins likely indicate distinct functions among the Cas proteins, which are highly dependent on the phosphorylation of the SD by SFKs and represents an important event in different cellular programs (see 1.2.2).
The CTD along with the SH3 domain target p130Cas to FAs, as deletion of the CTD prevents the localization of p130Cas to FAs (Harte *et al.* 2000). In addition, the CTD mediates the homo- and heterodimerization of p130Cas and HEF1 (Law *et al.* 1999) thereby potentially generating additional regulatory mechanisms.

2. Involvement of Cas family members in mammary carcinomas

Over the last decade *in vivo* studies in different organisms and *in vitro* studies in cells in culture accumulated evidence that individual Cas family members play central roles in the development and progression of mammary carcinomas. The p130Cas and HEF1 proteins are the best studied in this context. Primary breast tumors contain elevated p130Cas levels, which correlate with increased rate of relapse and with poor response to tamoxifen treatment (van der Flier *et al.* 2000). Increased p130Cas expression was also found in tumor cells isolated from pleural effusions of breast cancer patients in comparison to primary tumors (Konstantinovsky *et al.* 2010). In feline and canine breast cancers the levels of p130Cas positively correlate with advanced breast disease as well (Scibelli *et al.* 2003). Furthermore, our *in vitro* studies have shown increased p130Cas levels in the tamoxifen resistant breast cancer cells TAM-R (Soni *et al.* 2009), which were derived from tamoxifen sensitive MCF-7 cells (Knowlden *et al.* 2003).
In 2000, Brinkman and colleagues identified the gene of p130Cas, in a retroviral insertion screen as a factor that mediates resistance to tamoxifen in breast cancer cell lines (Brinkman *et al.* 2000; Dorssers *et al.* 1993). Subsequently, the gene located on chromosome 16q23.1 was named *BCAR1* (breast cancer anti-estrogen resistance 1). Although HEF1 has a similar domain structure it was unable to support long-term anti-estrogen resistant cell proliferation (Brinkman *et al.* 2009). Chimeric p130Cas/HEF1 proteins generated by exchange of defined domains, identified the SD of p130Cas as the region contributing to anti-estrogen resistance in breast cancer cells (Brinkman *et al.* 2009). Accordingly, disruption of the p130Cas signaling node by ectopic expression of an isolated constitutively tyrosine phosphorylated SD of p130Cas in the cytoplasm (as described in detail in Section 3) led to reduced proliferation and re-sensitization of tamoxifen resistant breast cancer cells to tamoxifen (Kirsch *et al.* 2002; Soni *et al.* 2009).
The mechanisms by which Cas proteins may promote mammary carcinomas and acquired tamoxifen resistance are manifold and under extensive investigation. It has been shown that estrogen treatment triggers the rapid and transient association of p130Cas with the estrogen

receptor (ER) alpha in the cytoplasm, thus mediating non-genomic ER signaling in human breast cancer cells (Cabodi *et al.* 2004). This is dependent on c-Src activation and results in the formation of a multi-molecular complex containing p130^{Cas}, c-Src, and the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and subsequent activation of extracellular-signal regulated kinase (ERK) 1/2. Importantly, overexpression of p130^{Cas} as well as short-interfering (si) RNA-mediated reduction of p130^{Cas} experiments in T47D breast cancer cells indicated that p130^{Cas} enhances the estrogen-dependent Src and Erk1/2 activities (and accelerates the kinetics in response to stimulation) (Cabodi *et al.* 2004). Long-term treatment of estrogen-dependent mammary carcinoma cells with the estrogen antagonist tamoxifen led to increased phosphorylation levels of p130^{Cas} (Cowell *et al.* 2006; Soni *et al.* 2009), suggesting that anti-estrogens modulate intrinsic mechanisms to deregulate Cas protein function.

Resistance to the anti-estrogens tamoxifen and fulvestrant, is associated with enhanced growth factor signaling involving the upregulation of epidermal growth factor receptor (EGFR) family and alteration of the AKT signaling pathway (Knowlden *et al.* 2003; Soni *et al.* 2009; Zhang *et al.* 2009; Frogne *et al.* 2009). Consistently, interference with p130^{Cas} signaling results in the attenuation of the ERK and PI3K/Akt survival pathways in breast cancer cells (Soni *et al.* 2009). Moreover, overexpression of p130^{Cas} mediates resistance to the chemotherapeutic drug adriamycin in mammary tumor cells by activating c-Src, Akt, and ERK1/2 growth and survival pathways (Ta *et al.* 2008).

More recent *in vivo* studies in transgenic mice overexpressing p130^{Cas} in mammary epithelial cells, showed substantial mammary epithelial cell hyperplasia during development and pregnancy, and delayed involution (Cabodi *et al.* 2006). Activation of Src, ERK1/2, mitogen-activated protein kinase (MAPK), and Akt pathways contribute to these phenotypes by inducing proliferation and inhibiting apoptosis.

Importantly, accelerated mammary tumor formation has been observed in double transgenic mice that overexpress both p130^{Cas} and the activated form of HER2/neu (human epidermal growth factor receptor 2) compared to the HER2/neu single transgenic mice without p130^{Cas} (Cabodi *et al.* 2006). Delivery of p130^{Cas}/*BCAR1*-specific siRNAs into the mammary gland of transgenic BALB–HER2/neu mice carrying the activated *HER2/neu* oncogene was sufficient to inhibit HER2/neu signaling and decreased the growth of spontaneous tumors *in vivo* (Cabodi *et al.* 2010b).

The balance between canonical and noncanonical transforming growth factor (TGF)-β signaling in mammary carcinomas is also regulated by p130^{Cas} (Wendt *et al.* 2009). Maintaining this balance is critical as TGF-β acts as both a tumor-suppressor or tumor-promoter depending on the tumor microenvironment and tumor stage (as reviewed in Ikushima & Miyazono 2010; Meulmeester & Ten Dijke 2011). Forced expression of either full length p130^{Cas} or the CTD of p130^{Cas} in mammary epithelial cells (MECs) shifted TGF-β signaling from Smad2/3 to p38 MAPK activation resulting in resistance of TGF-β-induced growth arrest and increased invasion and metastasis of MECs *in vivo* utilizing an orthotopic mouse model (Wendt *et al.* 2009).

In addition to p130^{Cas}, HEF1, also mediates TGF-β tumor promoting activities. TGF-β signaling upregulates HEF1 thereby enhancing mammary carcinoma cell scattering and the transition from collective cell motility to single cell motility (Giampieri *et al.* 2009). Similar to the TGF-β study, HEF1 overexpression in MCF-7 breast cancer cells increases the migration and invasion *in vitro* (Fashena *et al.* 2002). In MMTV-polyoma virus middle T antigen

(PyMT) mice crossed with the HEF1-deficient (HEF$^{-/-}$) mice delayed mammary tumor formation and reduced tumor incidence was observed (Izumchenko *et al.* 2009). Most of the mammary tumors excised from PyMT/HEF1$^{-/-}$ mice showed reduced activation of AKT, FAK, Src, and ERK1/2 compared to HEF1 wildtype animals. In contrast, an siRNA screening approach to identify genes that regulate migration in non-transformed mammary epithelial MCF-10A cells demonstrated an inhibitory function of HEF1 on migration (Simpson *et al.* 2008). Furthermore, HEF1 is part of a lung metastasis signature for primary breast cancers (Minn *et al.* 2005). In this study, an orthotopic MDA-MB-231 breast cancer mouse model was used and HEF1 was found to be down-regulated in highly lung metastatic mammary cancer cells. These results may suggest, that high levels of HEF1 contribute to early stages of breast cancer and a loss of expression during tumor progression may promote later stages leading to metastases formation of tumor cells.
In summary, the studies reviewed here indicate the extensive involvement of Cas family members, specifically p130^{Cas} and HEF1, in the transformation of mammary epithelium as well as acquired resistance of breast cancers to several therapeutic agents. The effects of the Cas proteins might be further amplified by simultaneous and synergistic activation of multiple signaling effector pathways, in particular down-stream of the SD of p130^{Cas} and HEF1.

2.1 Role of Cas SD effector protein signaling in mammary carcinomas

As described above, phosphorylaion of the Cas SD and subsequent coupling to effector molecules has been implicated in the transformation of cells, breast cancer progression, and acquired tamoxifen resistance (reviewed in Tikhmyanova *et al.* 2010). To better understand how the SD of the Cas proteins may contribute to these malignant processes the involvement of SD-interacting proteins in breast cancer is reviewed in this section.

2.1.1 The Crk family

Members of the Crk (chicken tumor virus no. 10 regulator of kinase) family, consisting of CRKI, CRKII, generated by alternative splicing of transcripts of the *CRK* gene, and CRKL (CRK-like protein) are SH2 and SH3 domain containing adaptor proteins (Matsuda *et al.* 1992; ten Hoeve *et al.* 1993; Feller & Lewitzky 2006). The Crk family SH2 domains bind to p130^{Cas} upon phosphorylation of the SD and recruit additional downstream effectors via their SH3 domains. The amino-terminal SH3 domain of Crk binds to guanine nucleotide exchange factors (GEFs), including C3G, Sos (Son of sevenless) (Feller *et al.* 1995; Okada & Pessin 1996), and DOCK1 (dedicator of cytokinesis 1, also known as DOCK180) (Hasegawa *et al.* 1996). This complex leads to the activation of the small GTPases Rap1 (Gotoh *et al.* 1995) and Rac (Dolfi *et al.* 1998), and subsequently JNK (c-Jun N-terminal kinase) signaling (shown in part in Figure 1) (Dolfi *et al.* 1998).
Crk proteins have been associated with several different tumor types and especially with the promotion of an invasive phenotype and cell migration (Cabodi *et al.* 2010a; Tikhmyanova *et al.* 2010). Interestingly, Klemke's group was the first to reveal that p130^{Cas}-crk coupling is involved in HER2/neu -mediated cell migration (Spencer *et al.* 2000). Subsequently, Park's group found elevated CrkI and CrkII protein levels in human mammary tumors and showed that siRNA-mediated CrkI/II knockdown results in a significant decrease in migration and invasion of breast cancer cells (Rodrigues *et al.* 2005). More recently, the same group observed in post-pubertal MMTV-CrkII transgenic mice premature ductal branching

that was associated with increased proliferation (Fathers *et al.* 2010). The mammary tumor incidence in MMTV-CrkII mice was 17.6% compared to 4% in female control mice with a similar latency of approximately 15 months. While Crk has been shown to induce cell migration, no metastatic lesions were found in any of the MMTV-CrkII animals. This suggests that CrkII plays a more important role at the early stages of breast carcinomas *in vivo*.

2.1.2 The Src-family tyrosine kinases (SFKs)

The SFKs are comprised of ten members of which c-Src, Lck, Lyn, Hck, Fyn, and Yes phosphorylate the SD of the Cas proteins (Alexandropoulos & Baltimore 1996; Ishino *et al.* 1995; Kanda *et al.* 1999; Nakamoto *et al.* 1996; Nasertorabi *et al.* 2006; Nishio & Suzuki 2002; Pellicena & Miller 2001; Singh *et al.* 2008). In addition to phosphorylating the $p130^{Cas}$ SD, c-Src binds to phosphorylated tyrosine residues within SD *in vitro* (Shin *et al.* 2004).

SFKs have a conserved structure containing SH1 (kinase), SH2, SH3, and SH4 (membrane targeting) domains and transactivate interacting partners by phosphorylation resulting in the activation of multiple signaling pathways as presented in part in Figure 1 (reviewed in Mayer & Krop 2010; Wheeler *et al.* 2009). The kinase activity is tightly regulated by tyrosine-phosphorylation on the carboxyl terminus by CSK (c-src tyrosine kinase) resulting in intramolecular binding and an inactive closed conformation (Superti-Furga *et al.* 1993). The precise cellular regulation of c-Src is of major relevance and $p130^{Cas}$ has been postulated to activate c-Src by disrupting the intramolecular inactive conformation (Nasertorabi *et al.* 2006; Burnham *et al.* 2000).

SFKs are central players in multiple cellular programs that are often dysregulated during tumorigenesis and progression and several of the members have been associated with malignant transformation. The founding member c-Src was the first proto-oncogene to be sequenced in the early 1980s (Czernilofsky *et al.* 1980; Schwartz *et al.* 1983; Takeya & Hanafusa 1982) and over the past 30 years several therapeutic agents to inhibit Src kinase activity have been developed and clinical trials are ongoing (summarized in Aleshin & Finn 2010; Mayer & Krop 2010).

Several studies have demonstrated the relevance of Src in breast cancer as enhanced expression and activity of c-Src was observed in human mammary carcinoma cell lines and tumor tissues (Biscardi *et al.* 1998; Jacobs & Rubsamen 1983; Ottenhoff-Kalff *et al.* 1992; Verbeek *et al.* 1996). Studies by Muller's group unequivocally showed that expression of an activated c-Src protein in the mammary gland of mice induces tumor formation, though with long latency (Webster *et al.* 1995). Importantly, tumor formation in transgenic mice expressing the PyMT oncogene under the control of the MMTV promoter is c-Src dependent as PyMT-Src-deficient mice rarely developed mammary tumors, whereas a more rapid tumor progression was found in the MMTV-PyMT control mice (Guy *et al.* 1994). Furthermore, aberrated c-Src expression and activity has been associated with Her2/neu transformation *in vivo* (Muthuswamy & Muller 1995). Several SFK specific inhibitors have been developed which suppress migration and invasion of human breast cancer cells (Vultur *et al.* 2008). These compounds also reduce the incidence of metastasis formation after intracardiac injection of human breast cancer cells in nude mice (Rucci *et al.* 2006).

2.1.3 SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase)

Through its interactions with numerous proteins via its two SH2 domains, the protein tyrosine phosphatase SHP-2 regulates oncogenic transformation (as reviewed in Matozaki *et*

al. 2009). Conflicting results have been reported for a role of SHP-2 in mammary adenocarcinomas in regard to the effect on migration. Upon tyrosine phosphorylation of HEF1, SHP-2 associates with the HEF1 SD and dephosphorylates it thereby inhibiting HEF1-mediated cell migration (Yo *et al.* 2009). Conversely, SHP-2 acts as a positive regulator of migration of MCF7 breast cancer cells *in vitro* and promotes metastasis of MCF7 cells, when injected into the abdominal cavity of nude mice *in vivo* (Wang *et al.* 2005).

2.1.4 The Nck family

Similar to the Crk proteins, the two Nck family members are SH2/SH3 domain-containing adapters which regulate tyrosine kinase signaling (reviewed in Buday *et al.* 2002). Nck was first identified in a screen of a human melanoma cDNA library using antibodies against the melanoma cell adhesion molecule (MCAM) (Lehmann *et al.* 1990). In MCF-7 breast cancer cells, Nck is required for fibroblast growth factor (FGF)-2-induced DNA synthesis (Liu et al. 1999). Furthermore, Nck facilitates invadopodia formation and extracellular matrix (ECM) degradation in various tumor cell lines including breast cancer cells (Stylli et al. 2009).

These studies summarized here emphasize the importance of Cas effector proteins in the promotion of breast cancer *in vitro* and *in vivo*. They highlight the potential importance of targeting the $p130^{Cas}$ signaling node in human breast cancers, as Cas family members might contribute to this malignancy through their association with these SD interacting molecules.

3. Novel strategies to develop therapeutic agents for targeting Cas signaling in mammary carcinomas

Though many studies have suggested that $p130^{Cas}$ and HEF1 are critical for phenotypic changes that drive breast cancer progression and metastasis (see Section 2), no therapeutic agents (drugs) have been developed that target these important proteins. It might be feasible to inactivate cancer promoting adaptor proteins by several mechanisms among them (a) downregulating their expression or (b) by interfering with specific protein-protein interaction modules. Defillipi's group used an RNAi-based approach to mediate downregulation of $p130^{Cas}$ by intranipple injection of siRNA resulting in a reduction of tumor growth in BALB-HER2/neu mice (Cabodi *et al.* 2010b). These results are very promising and may warrant further exploration.

Current research has now revealed a clearer picture regarding the functions of the different domains in $p130^{Cas}$ signaling and tumor formation (see Sections 1 and 2). Therefore, inhibitors targeting individual domains, such as the SD, SH3, and/or CTD domain, might confer additional specificity and maintain the functional properties of the other domains thereby reducing potential adverse effects. As an alternative approach, the Src*/CasSD decoy molecule (Kirsch et al., 2002 and summarized in Section 3.1) could be used as a starting point to develop inhibitors that target only certain Cas functions.

3.1 Blocking $p130^{Cas}$ SD signaling by utilization of a phosphorylated $p130^{Cas}$ SD

As discussed above, the important role for $p130^{Cas}$ in breast cancer has been demonstrated by several groups, with a critical function for the SD emerging in cell transformation. Kirsch and colleagues previously investigated the role of the Cas SD in transformation by functionally separating Cas from upstream signals (Kirsch *et al.* 2002). The $p130^{Cas}$ SD was fused to the Src kinase domain with attenuated activity [activating tyrosine 416 replaced

with phenylalanine (Y416F); designated as Src*/CasSD] (Figure 3) (Kmiecik & Shalloway 1987; Piwnica-Worms *et al.* 1987). As controls, a Src kinase inactive mutant (K295M) (Jove *et al.* 1987) fused to the p130^{Cas} SD (Src^{KM}/CasSD) and the isolated Src* were employed (Kirsch *et al.* 2002). The initial hypothesis, that this constitutively phosphorylated chimera would act as dominant active molecule by circumventing upstream signaling had to be revised as the results obtained in transient and stably expressing cell systems revealed a dominant negative effect on downstream signaling. It was subsequently found that the Src*/CasSD chimera attenuated cellular transformation. Expression of Src*/CasSD resulted in a significant reduction of colony formation of v-crk transformed NIH3T3 cells in soft agar assays. Further experiments, suggested that the isolated tyrosine phosphorylated CasSD acts as a decoy for v- and c-Crk thereby blocking the ability of v-crk to transform these cells involving a reduction in JNK activation (Kirsch *et al.* 2002).

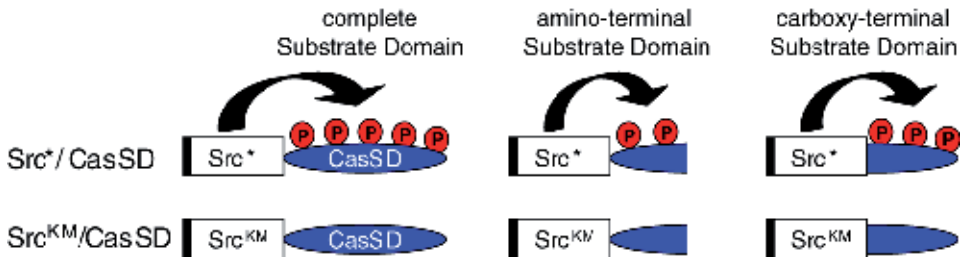


Fig. 3. Representation of the dominant negative p130^{Cas} (Src*/CasSD) and control (Src^{KM}/CasSD) fusion constructs generated by Kirsch and colleagues, 2002. Src*, attenuated Src kinase domain. Src^{KM}, inactive Src kinase domain. CasSD, substrate domain of p130^{Cas}. P, phosphorylation.

Additional studies utilizing this approach in different cellular contexts revealed proof of principle of the Src*/CasSD decoy approach. In BxPC3 pancreatic adenocarcinoma cells the Src*/CasSD expression prevented collagen I-mediated upregulation of N-cadherin and cell scattering (Shintani *et al.* 2008). In tamoxifen resistant breast cancer cells TAM-R, expression of the chimeric molecule attenuated several signaling pathways involved in breast carcinoma progression and acquired tamoxifen resistance (see below) (Soni *et al.* 2009).
TAM-R cells were established by long-term exposure of estrogen-dependent MCF-7 cells to tamoxifen (Hiscox *et al.* 2004). Importantly, similar to anti-estrogen resistant breast cancers, endogenous p130^{Cas} levels are increased and highly phosphorylated in these cells (Soni *et al.* 2009), a further indication that p130^{Cas} contributes to tamoxifen resistance. A major finding of our study investigating the effects of the inhibition of p130^{Cas} by the Src*/CasSD chimera on breast cancer cells was the re-sensitization of TAM-R cells to tamoxifen resulting in increased apoptosis (Soni *et al.* 2009). Of note, the Src*/CasSD induced apoptosis was specific for TAM-R cells and not detected in the parental MCF-7 cells expressing lower p130^{Cas} levels. Moreover, expression of Src*/CasSD resulted in reduced cell numbers of MCF-7 and TAM-R cells and in a reversion to a more epithelial-like phenotype in TAM-R cells. In TAM-R cells, these observations were accompanied by elevated levels of ERα, E-cadherin stabilization at cell-cell boundaries, and reduced migration and consistently, enhanced cell clustering. Furthermore, employment of the Src*/CasSD approach in TAM-R cells led to the reduction of growth factor signaling as seen by an attenuated PI3K/AKT pro-survival pathway, and a reduced activation of the MAPK/ERK pathway (Soni *et al.* 2009).

Taken together, these studies suggest, that blocking endogenous p130^{Cas} function by ectopic expression of a constitutively phosphorylated p130^{Cas} SD may represent an important tool not only for further elucidating the mechanisms by which Cas proteins contribute to breast cancer and other malignancies but also to develop potential therapeutic agents targeting this domain as discussed below.

3.2 The Src*/CasSD decoy approach - a starting point to develop potential therapeutics for breast cancer

The Src*/CasSD approach implies at least two different strategies to inhibit downstream signaling of Cas family proteins: **1.** Develop therapeutic agents reflecting the structure of important parts of the phosphorylated SD (mimetic), which act as a decoy for SD interacting molecules, thereby competing with endogenous Cas proteins for binding partners; **2.** Design compounds that bind to functionally relevant tyrosine motifs in the SD of the endogenous Cas proteins to block the recruitment of SD binding adapter proteins. **3.** Design drugs that bind to the SH2 domains of the interacting proteins as it has been elegantly demonstrated for Grb2 (Growth factor receptor-bound protein 2) inhibitors (Atabey *et al.* 2001; Dharmawardana *et al.* 2006). The advantage of the first two approaches lies in the fact that several molecules may be targeted simultaneously. On the other hand, this can be a disadvantage as well, due to the possibility of a greater degree of unwanted effects.

One requirement for specific inhibition of protein-protein interaction (PPI) and to limit potential adverse effects of drugs is to narrow down the targeting region. This will also increase alternative options for the choice of an application such as the utilization of peptide inhibitors or small-inhibitory molecules (discussed below).

Not all of the YxxP motifs in the Cas SD are phosphorylated by Src, or important for Crk, Src, and/or Nck binding (Kirsch *et al.* 2002; Shin *et al.* 2004) (summarized in Fig. 4), potentially suggesting that smaller fragments of the SD might be sufficient to mediate the inhibition of breast carcinoma progression. Importantly, the initial study describing the Src*/CasSD approach showed that the carboxy-terminal YxxP motifs six to fifteen of the p130^{Cas} SD were necessary for decoying/interacting with Crk (Kirsch *et al.* 2002). This correlated with the identified Crk-SH2 consensus sequence of YDxP (Birge *et al.* 1993; Songyang *et al.* 1993). In addition, the carboxy-terminal part of the p130^{Cas} SD is essential for p130^{Cas}-mediated cell migration (Shin *et al.* 2004). Furthermore, the majority of the 15 and 13 tyrosine motifs

within the p130^{Cas} and HEF1 SD, respectively, belong to two groups of sequences: YQxP and YDxP (Fig. 4B). PhosphoSitePlus™ (Hornbeck *et al.* 2004), a comprehensive resource of known phosphorylation sites, indicated that in both family members the amino-terminal motifs (primarily of the YQxP sequence) in the SD are rarely phosporylated, whereas the carboxy-terminal sites (primarily of the YDxP sequence) are significantly more often phosphorylated as measured by high-throughput mass spectometry screenings (Fig. 4B). In addition, these studies support the hypothesis that a drug based on the Src*/CasSD approach may block signaling mediated by both p130^{Cas} and HEF1 proteins. However, to date no studies addressing the influence of the Src*/CasSD chimera on HEF1 activity, and the importance of the carboxy-terminal part of the Cas SD for mediating the inhibition have been performed.

To test this hypothesis, experiments are currently ongoing in our laboratory using the Src*/CasSD approach to identify and define the smallest region of the SD that retains its inhibitory function in breast cancer *in vitro* and *in vivo*. Subsequently, the structure of this

constitutively phosphorylated peptide could be resolved by crystallization and *in silico* protein structure modeling, which may provide a starting point to design therapeutic agents for future testing.

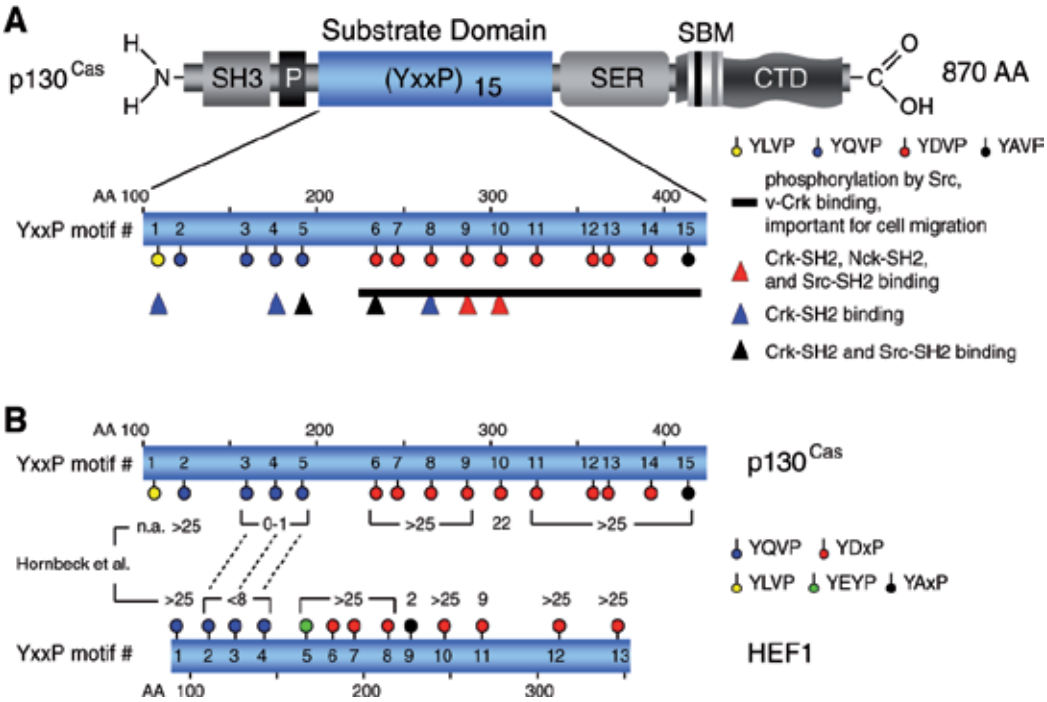


Fig. 4. Representation of the YxxP motifs in the p130Cas and HEF1 substrate domains. A, Interaction partners for p130Cas and a region important for cell migration are indicated. SH3, Src homology 3 domain. P, proline-rich region. SER, serine-rich domain. SBM, bi-partite Src-binding motif. CTD, carboxy-terminal domain. B, Comparison of the YxxP motifs in the SD of p130Cas and HEF1. The number of studies showing phosphorylation of certain motifs by high-throughput mass spectometry screening as curated at PhosphoSitePlus™ (Hornbeck *et al.* 2004) are indicated. n.a., not available.

4. Overview of potential approaches for targeting cytoplasmic adapter proteins

Over the past 15 years progress has been made in drug development and different novel approaches have become avaible to diversify the options of drug design and functional screening. For instance, humanized therapeutic antibodies, intrabodies, peptide inhibitors, peptidomimetics, or small-molecule inhibitors have been employed in cancer therapies and are undergoing clinical trials (Buchwald 2010; Leader *et al.* 2008; Lo *et al.* 2008).

Although therapeutic antibodies usually show a high specificity, they are in general not cell-permeable, thus excluding them as potential drug for targeting the intracellular Cas proteins. Intrabodies, antibodies designed to be expressed intracellularly, circumvent these obstacles and could possibly be directed to distinct subcellular locations (reviewed in Lo *et al.* 2008). It might be feasible to target a specific intracellular antigen e.g. in the nucleus, the endoplasmic

reticulum, mitochondria, or at the plasma membrane. This may represent an interesting approach to limit/focus the action of a drug to interfere with certain Cas protein functions.

Small molecules such as peptide inhibitors, peptidomimetics, or small-molecule inhibitors have recently become a focus for researchers to develop novel therapeutics. Great advances in the development of small molecules that modulate PPIs have been achieved (Arkin 2005; Arkin & Wells 2004; Wells & McClendon 2007). In the context of the Cas proteins that mediate their function as adapters by providing docking sites for multiple PPI, progress particularly in developing small-molecule inhibitors to block PPI is of major importance.

Peptide inhibitors represent potent therapeutic agents and over the past decade more than 50 peptides have been approved for the treatment of various diseases and several hundred are in preclinical development and clinical testing (Buchwald 2010). Advantages of peptide inhibitors are the high specificity, low toxicity, and low accumulation in tissues. However, peptide inhibitors exhibit a limited half-life in circulation, frequently possess restricted cell permeability, and are more expensive to produce than traditional small-molecule drugs. Often peptide inhibitors have become the starting point to subsequently develop PPI inhibitors (PPIIs) such as peptidomimetics (small protein-like chain based on a peptide with altered chemical structure designed to adjust the molecular properties to acheive increased stability or biological activity) or small-molecule inhibitors to circumvent these disadvantages.

Advances have been made in targeting PPI utilizing mimetic β-peptides (Kritzer *et al.* 2005), which consist of β amino acids that generally not appear in nature, thus increasing the resistance to proteolysis. Succesfully employed examples are β-peptides that target hDM2 (human double minutes-2, the human homologue of the murine p53 negative regulator MDM2) to prevent its interaction with the tumor suppressor p53 resulting in an upregulation of p53-dependent genes (Harker & Schepartz 2009; Kritzer *et al.* 2004). Importantly, subsequent structural modifications of these β-peptides enhanced their uptake in human coloncarcinoma cells.

In later stages of drug development, the goal would be to develop organic non-peptidic small-molecule inhibitors that are generally less expensive, cell-permeable and can be orally administered. Especially relevant to the CasSD approach, and proof of principle, is the progress made in the development of a small-inhibitory compound targeting the Grb2-HGFR (hepatocye growth factor receptor) interaction. The Grb2 antagonist C90, designed and validated to bind to the Grb2 SH2 domain thereby preventing specifically the association of Grb2 with phosphorylated tyrosine motifs within the HGFR (Atabey *et al.* 2001; Dharmawardana *et al.* 2006). The blockade of the Grb2 and HGFR interaction by the C90 small molecule resulted in the reduction of tumor metastases in two different mouse models (Giubellino *et al.* 2007) and inhibition of angiogenesis *in vivo* (Soriano *et al.* 2004). These studies elegantly demonstrate that small-molecule inhibitors possess the potential to block SH2 domain-mediated PPI with high specificity. Additional examples of small-inhibitory compounds with *in vivo* activity in cancer models include Nutlins (Vassilev 2007; Dickens *et al.* 2010), an inhibitor of MDM2, and the Bcl-2 blockers ABT-737 and ABT-263 (Vogler *et al.* 2009).

To summarize, progress has been made in developing different novel approaches in drug design for targeting cytoplasmic proteins. The successful application of small inhibitory compounds in preclinical and clinical trials shows their potential. Approaches employing small-molecule inhibitors may represent promising strategies to target specific regions and thus particular functions of Cas proteins.

5. Perspectives/Outlook on novel combinatorial adjuvant therapies for breast cancer

Increasingly, women are receiving adjuvant therapies in the form of chemotherapy, hormone- and/or radiation therapy (Yamashita 2008; Nicolini *et al.* 2006). Evidence is mounting that these therapies improve breast cancer survival. Adriamycin is one of the most frequently used agents for effective chemotherapeutic treatment of breast cancer (Gianni *et al.* 2008). Hormonal adjuvant therapy is a more targeted therapy with tamoxifen being a commonly used anti-estrogen for treatment of ERα positive breast cancers. Unfortunately, the use of these agents is limited by toxicity and the evolving resistance as seen for other chemotherapeutic drugs as well. This strongly indicates the need for the development of novel approaches to fight tumor cells and to prevent or reduce drug-resistance in breast cancer.

$p130^{Cas}$ was shown to be associated with tamoxifen (Dorssers *et al.* 2001) and adriamycin (Ta *et al.* 2008) resistance in human breast carcinomas *in vivo* and *in vitro*, respectively. Expression of Src*/CasSD in TAM-R cells re-sensitized TAM-R cells to tamoxifen (Soni *et al.* 2009) and RNAi-mediated depletion of $p130^{Cas}$ in breast cancer cells increased the efficiency of adriamycin treatment (Ta *et al.* 2008). Interestingly, expression of the Src*/CasSD in TAM-R cells attenuated signaling pathways which are also involved in $p130^{Cas}$-mediated adriamycin resistance, suggesting that the Src*/CasSD approach might increase the susceptibility to adriamycin as well. Implementation of the Src*/CasSD approach may potentially enhance the efficiency of other anti-estrogens, such as fulvestrant, or the dual tyrosine kinase inhibitor lapatinib (Tykerb), targeting EGFR and HER2/neu (Medina & Goodin 2008; Azim & Azim, Jr. 2008), as it results in an upregulation of ER alpha and HER2/neu in TAMR-R cells (Soni *et al.* 2009).

Data from these initial studies suggest that a therapeutic agent based on the Src*/CasSD approach may have the potential to open avenues for novel strategies for combinatorial adjuvant treatment of breast cancer in the future.

6. Conclusions/Summary

Although many studies indicate the critical involvement of the Cas family members in breast cancer progression, no therapeutic agents have been developed that target these proteins. Here we reviewed different approaches addressing this issue and presented in detail our SD decoy approach that represents an important novel tool to block endogenous Cas protein functions in mammary cancer. Ongoing studies with the aim to specify the region that is mediating these inhibitory effects may guide in the design of therapeutic agents in the future. As we discussed different future drug designs, small-molecule inhibitor approaches might be favourably suited for clinical applications to target Cas proteins, that may be extended to other PPI domains such as the SH3 and/or CTD, or SER domain.

7. Acknowledgments

The authors regret that there are many other important studies that they were unable to include owing to space limitations. This work was supported by the Public Health Service grants CA106468 and CA143108 from the National Cancer Institute and the Susan G. Komen for the Cure® Breast Cancer Foundation grant KG101208. We thank Dr. Matthew D. Layne for critical reading of the manuscript and for comments.

8. References

Abassi YA, Rehn M, Ekman N, Alitalo K & Vuori K 2003 p130Cas Couples the tyrosine kinase Bmx/Etk with regulation of the actin cytoskeleton and cell migration. *J Biol.Chem.* 278 35636-35643.

Aleshin A & Finn RS 2010 SRC: a century of science brought to the clinic. *Neoplasia.* 12 599-607.

Alexandropoulos K & Baltimore D 1996 Coordinate activation of c-Src by SH3- and SH2-binding sites on a novel p130Cas-related protein, Sin. *Genes Dev.* 10 1341-1355.

Angers-Loustau A, Cote JF, Charest A, Dowbenko D, Spencer S, Lasky LA & Tremblay ML 1999 Protein tyrosine phosphatase-PEST regulates focal adhesion disassembly, migration, and cytokinesis in fibroblasts. *J Cell Biol.* 144 1019-1031.

Arkin M 2005 Protein-protein interactions and cancer: small molecules going in for the kill. *Curr.Opin.Chem.Biol.* 9 317-324.

Arkin MR & Wells JA 2004 Small-molecule inhibitors of protein-protein interactions: progressing towards the dream. *Nat.Rev.Drug Discov.* 3 301-317.

Astier A, Manie SN, Law SF, Canty T, Haghayghi N, Druker BJ, Salgia R, Golemis EA & Freedman AS 1997 Association of the Cas-like molecule HEF1 with CrkL following integrin and antigen receptor signaling in human B-cells: potential relevance to neoplastic lymphohematopoietic cells. *Leuk.Lymphoma* 28 65-72.

Atabey N, Gao Y, Yao ZJ, Breckenridge D, Soon L, Soriano JV, Burke TR, Jr. & Bottaro DP 2001 Potent blockade of hepatocyte growth factor-stimulated cell motility, matrix invasion and branching morphogenesis by antagonists of Grb2 Src homology 2 domain interactions. *J.Biol.Chem.* 276 14308-14314.

Azim H & Azim HA, Jr. 2008 Targeting Her-2/neu in breast cancer: as easy as this! *Oncology* 74 150-157.

Birge RB, Fajardo JE, Reichman C, Shoelson SE, Songyang Z, Cantley LC & Hanafusa H 1993 Identification and characterization of a high-affinity interaction between v-Crk and tyrosine-phosphorylated paxillin in CT10-transformed fibroblasts. *Mol.Cell Biol.* 13 4648-4656.

Biscardi JS, Belsches AP & Parsons SJ 1998 Characterization of human epidermal growth factor receptor and c-Src interactions in human breast tumor cells. *Mol.Carcinog.* 21 261-272.

Bouton AH, Riggins RB & Bruce-Staskal PJ 2001 Functions of the adapter protein Cas: signal convergence and the determination of cellular responses. *Oncogene* 20 6448-6458.

Bridges D & Moorhead GB 2004 14-3-3 proteins: a number of functions for a numbered protein. *Sci.STKE.* 2004 re10.

Briknarova K, Nasertorabi F, Havert ML, Eggleston E, Hoyt DW, Li C, Olson AJ, Vuori K & Ely KR 2005 The serine-rich domain from Crk-associated substrate (p130cas) is a four-helix bundle. *J Biol.Chem.* 280 21908-21914.

Brinkman A, de Jong D, Tuinman S, Azaouagh N, van Agthoven T & Dorssers LC 2009 The substrate domain of BCAR1 is essential for anti-estrogen-resistant proliferation of human breast cancer cells. *Breast Cancer Res.Treat.*

Brinkman A, van der Flier S, Kok EM & Dorssers LC 2000 BCAR1, a human homologue of the adapter protein p130Cas, and antiestrogen resistance in breast cancer cells. *J.Natl.Cancer Inst.* 92 112-120.

Buchwald P 2010 Small-molecule protein-protein interaction inhibitors: therapeutic potential in light of molecular size, chemical space, and ligand binding efficiency considerations. *IUBMB.Life* 62 724-731.

Buday L, Wunderlich L & Tamas P 2002 The Nck family of adapter proteins: regulators of actin cytoskeleton. *Cell Signal.* 14 723-731.

Burnham MR, Bruce-Staskal PJ, Harte MT, Weidow CL, Ma A, Weed SA & Bouton AH 2000 Regulation of c-SRC activity and function by the adapter protein CAS. *Mol.Cell Biol.* 20 5865-5878.

Burnham MR, Harte MT & Bouton AH 1999 The role of SRC-CAS interactions in cellular transformation: ectopic expression of the carboxy terminus of CAS inhibits SRC-CAS interaction but has no effect on cellular transformation. *Mol.Carcinog.* 26 20-31.

Burnham MR, Harte MT, Richardson A, Parsons JT & Bouton AH 1996 The identification of p130cas-binding proteins and their role in cellular transformation. *Oncogene* 12 2467-2472.

Cabodi S, Moro L, Baj G, Smeriglio M, Di Stefano P, Gippone S, Surico N, Silengo L, Turco E, Tarone G & Defilippi P 2004 p130Cas interacts with estrogen receptor alpha and modulates non-genomic estrogen signaling in breast cancer cells. *J Cell Sci.* 117 1603-1611.

Cabodi S, Pilar Camacho-Leal M, Di Stefano P & Defilippi P 2010a Integrin signalling adaptors: not only figurants in the cancer story. *Nat.Rev.Cancer* 10 858-870.

Cabodi S, Tinnirello A, Bisaro B, Tornillo G, Pilar Camacho-Leal M, Forni G, Cojoca R, Iezzi M, Amici A, Montani M, Eva A, Di Stefano P, Muthuswamy SK, Tarone G, Turco E & Defilippi P 2010b p130Cas is an essential transducer element in ErbB2 transformation. *FASEB J* 24 3796-3808.

Cabodi S, Tinnirello A, Di Stefano P, Bisaro B, Ambrosino E, Castellano I, Sapino A, Arisio R, Cavallo F, Forni G, Glukhova M, Silengo L, Altruda F, Turco E, Tarone G & Defilippi P 2006 p130Cas as a new regulator of mammary epithelial cell proliferation, survival, and HER2-neu oncogene-dependent breast tumorigenesis. *Cancer Res.* 66 4672-4680.

Chan YR & Gallo RL 1998 PR-39, a syndecan-inducing antimicrobial peptide, binds and affects p130(Cas). *J Biol.Chem.* 273 28978-28985.

Cote JF, Charest A, Wagner J & Tremblay ML 1998 Combination of gene targeting and substrate trapping to identify substrates of protein tyrosine phosphatases using PTP-PEST as a model. *Biochemistry* 37 13128-13137.

Cowell LN, Graham JD, Bouton AH, Clarke CL & O'Neill GM 2006 Tamoxifen treatment promotes phosphorylation of the adhesion molecules, p130Cas/BCAR1, FAK and Src, via an adhesion-dependent pathway. *Oncogene* 25 7597-7607.

Czernilofsky AP, Levinson AD, Varmus HE, Bishop JM, Tischer E & Goodman HM 1980 Nucleotide sequence of an avian sarcoma virus oncogene (src) and proposed amino acid sequence for gene product. *Nature* 287 198-203.

Defilippi P, Di Stefano P & Cabodi S 2006 p130Cas: a versatile scaffold in signaling networks. *Trends Cell Biol.* 16 257-263.

Dharmawardana PG, Peruzzi B, Giubellino A, Burke TR, Jr. & Bottaro DP 2006 Molecular targeting of growth factor receptor-bound 2 (Grb2) as an anti-cancer strategy. *Anticancer Drugs* 17 13-20.

Di Stefano P, Cabodi S, Boeri EE, Margaria V, Bergatto E, Giuffrida MG, Silengo L, Tarone G, Turco E & Defilippi P 2004 P130Cas-associated protein (p140Cap) as a new tyrosine-phosphorylated protein involved in cell spreading. *Mol.Biol.Cell* 15 787-800.

Dickens MP, Fitzgerald R & Fischer PM 2010 Small-molecule inhibitors of MDM2 as new anticancer therapeutics. *Semin.Cancer Biol.* 20 10-18.

Dolfi F, Garcia-Guzman M, Ojaniemi M, Nakamura H, Matsuda M & Vuori K 1998 The adaptor protein Crk connects multiple cellular stimuli to the JNK signaling pathway. *Proc.Natl.Acad.Sci.U.S.A* 95 15394-15399.

Donaldson JC, Dempsey PJ, Reddy S, Bouton AH, Coffey RJ & Hanks SK 2000 Crk-associated substrate p130(Cas) interacts with nephrocystin and both proteins localize to cell-cell contacts of polarized epithelial cells. *Exp.Cell Res.* 256 168-178.

Donato DM, Ryzhova LM, Meenderink LM, Kaverina I & Hanks SK 2010 Dynamics and mechanism of p130Cas localization to focal adhesions. *J Biol.Chem.* 285 20769-20779.

Dorssers LC, van Agthoven T, Dekker A, van Agthoven TL & Kok EM 1993 Induction of antiestrogen resistance in human breast cancer cells by random insertional mutagenesis using defective retroviruses: identification of bcar-1, a common integration site. *Mol.Endocrinol.* 7 870-878.

Dorssers LC, van der FS, Brinkman A, van Agthoven T, Veldscholte J, Berns EM, Klijn JG, Beex LV & Foekens JA 2001 Tamoxifen resistance in breast cancer: elucidating mechanisms. *Drugs* 61 1721-1733.

Fashena SJ, Einarson MB, O'Neill GM, Patriotis C & Golemis EA 2002 Dissection of HEF1-dependent functions in motility and transcriptional regulation. *J Cell Sci.* 115 99-111.

Fathers KE, Rodrigues S, Zuo D, Murthy IV, Hallett M, Cardiff R & Park M 2010 CrkII transgene induces atypical mammary gland development and tumorigenesis. *Am.J Pathol.* 176 446-460.

Feller SM, Knudsen B & Hanafusa H 1995 Cellular proteins binding to the first Src homology 3 (SH3) domain of the proto-oncogene product c-Crk indicate Crk-specific signaling pathways. *Oncogene* 10 1465-1473.

Feller SM & Lewitzky M 2006 Potential disease targets for drugs that disrupt protein--protein interactions of Grb2 and Crk family adaptors. *Curr.Pharm.Des* 12 529-548.

Feng L, Guedes S & Wang T 2004 Atrophin-1-interacting protein 4/human Itch is a ubiquitin E3 ligase for human enhancer of filamentation 1 in transforming growth factor-beta signaling pathways. *J Biol.Chem.* 279 29681-29690.

Frogne T, Benjaminsen RV, Sonne-Hansen K, Sorensen BS, Nexo E, Laenkholm AV, Rasmussen LM, Riese DJ, de Cremoux P, Stenvang J & Lykkesfeldt AE 2009 Activation of ErbB3, EGFR and Erk is essential for growth of human breast cancer cell lines with acquired resistance to fulvestrant. *Breast Cancer Res.Treat.* 114 263-275.

Garton AJ, Burnham MR, Bouton AH & Tonks NK 1997 Association of PTP-PEST with the SH3 domain of p130cas; a novel mechanism of protein tyrosine phosphatase substrate recognition. *Oncogene* 15 877-885.

Giampieri S, Manning C, Hooper S, Jones L, Hill CS & Sahai E 2009 Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility. *Nat.Cell Biol.* 11 1287-1296.

Gianni L, Herman EH, Lipshultz SE, Minotti G, Sarvazyan N & Sawyer DB 2008 Anthracycline cardiotoxicity: from bench to bedside. *J.Clin.Oncol.* 26 3777-3784.

Giubellino A, Gao Y, Lee S, Lee MJ, Vasselli JR, Medepalli S, Trepel JB, Burke TR, Jr. & Bottaro DP 2007 Inhibition of tumor metastasis by a growth factor receptor bound protein 2 Src homology 2 domain-binding antagonist. *Cancer Res.* 67 6012-6016.

Gotoh T, Cai D, Tian X, Feig LA & Lerner A 2000 p130Cas regulates the activity of AND-34, a novel Ral, Rap1, and R-Ras guanine nucleotide exchange factor. *J Biol.Chem.* 275 30118-30123.

Gotoh T, Hattori S, Nakamura S, Kitayama H, Noda M, Takai Y, Kaibuchi K, Matsui H, Hatase O, Takahashi H &. 1995 Identification of Rap1 as a target for the Crk SH3 domain-binding guanine nucleotide-releasing factor C3G. *Mol.Cell Biol.* 15 6746-6753.

Guy CT, Muthuswamy SK, Cardiff RD, Soriano P & Muller WJ 1994 Activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in transgenic mice. *Genes Dev.* 8 23-32.

Hanahan D & Weinberg RA 2011 Hallmarks of cancer: the next generation. *Cell* 144 646-674.

Harker EA & Schepartz A 2009 Cell-permeable beta-peptide inhibitors of p53/hDM2 complexation. *Chembiochem.* 10 990-993.

Harte MT, Hildebrand JD, Burnham MR, Bouton AH & Parsons JT 1996 p130Cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. *J Biol.Chem.* 271 13649-13655.

Harte MT, Macklem M, Weidow CL, Parsons JT & Bouton AH 2000 Identification of two focal adhesion targeting sequences in the adapter molecule p130(Cas). *Biochim.Biophys.Acta* 1499 34-48.

Hasegawa H, Kiyokawa E, Tanaka S, Nagashima K, Gotoh N, Shibuya M, Kurata T & Matsuda M 1996 DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane. *Mol.Cell Biol.* 16 1770-1776.

Henderson BE & Feigelson HS 2000 Hormonal carcinogenesis. *Carcinogenesis* 21 427-433.

Hiscox S, Morgan L, Barrow D, Dutkowskil C, Wakeling A & Nicholson RI 2004 Tamoxifen resistance in breast cancer cells is accompanied by an enhanced motile and invasive phenotype: inhibition by gefitinib ('Iressa', ZD1839). *Clin.Exp.Metastasis* 21 201-212.

Hoon KD, Jeon CS, Kook S, Kim W & Keun SW 2003 Phosphorylation-dependent cleavage of p130cas in apoptotic rat-1 cells. *Biochem Biophys.Res.Commun.* 300 141-148.

Hornbeck PV, Chabra I, Kornhauser JM, Skrzypek E & Zhang B 2004 PhosphoSite: A bioinformatics resource dedicated to physiological protein phosphorylation. *Proteomics.* 4 1551-1561.

Huang J, Hamasaki H, Nakamoto T, Honda H, Hirai H, Saito M, Takato T & Sakai R 2002 Differential regulation of cell migration, actin stress fiber organization, and cell transformation by functional domains of Crk-associated substrate. *J.Biol.Chem.* 277 27265-27272.

Ikushima H & Miyazono K 2010 TGFbeta signalling: a complex web in cancer progression. *Nat.Rev.Cancer* 10 415-424.

Ishino M, Ohba T, Sasaki H & Sasaki T 1995 Molecular cloning of a cDNA encoding a phosphoprotein, Efs, which contains a Src homology 3 domain and associates with Fyn. *Oncogene* 11 2331-2338.

Izumchenko E, Singh MK, Plotnikova OV, Tikhmyanova N, Little JL, Serebriiskii IG, Seo S, Kurokawa M, Egleston BL, Klein-Szanto A, Pugacheva EN, Hardy RR, Wolfson M, Connolly DC & Golemis EA 2009 NEDD9 promotes oncogenic signaling in mammary tumor development. *Cancer Res.* 69 7198-7206.

Jacobs C & Rubsamen H 1983 Expression of pp60c-src protein kinase in adult and fetal human tissue: high activities in some sarcomas and mammary carcinomas. *Cancer Res.* 43 1696-1702.

Jove R, Kornbluth S & Hanafusa H 1987 Enzymatically inactive p60c-src mutant with altered ATP-binding site is fully phosphorylated in its carboxy-terminal regulatory region. *Cell* 50 937-943.

Kanda H, Mimura T, Hamasaki K, Yamamoto K, Yazaki Y, Hirai H & Nojima Y 1999 Fyn and Lck tyrosine kinases regulate tyrosine phosphorylation of p105CasL, a member of the p130Cas docking protein family, in T-cell receptor-mediated signalling. *Immunology* 97 56-61.

Kim W, Kook S, Kim DJ, Teodorof C & Song WK 2004 The 31-kDa caspase-generated cleavage product of p130cas functions as a transcriptional repressor of E2A in apoptotic cells. *J Biol.Chem.* 279 8333-8342.

Kirsch KH, Kensinger M, Hanafusa H & August A 2002 A p130Cas tyrosine phosphorylated substrate domain decoy disrupts v-crk signaling. *BMC.Cell Biol.* 3 18.

Kirsch KH, Georgescu MM & Hanafusa H 1998 Direct binding of p130(Cas) to the guanine nucleotide exchange factor C3G. *J Biol.Chem.* 273 25673-25679.

Kirsch KH, Georgescu MM, Ishimaru S & Hanafusa H 1999 CMS: an adapter molecule involved in cytoskeletal rearrangements. *Proc.Natl.Acad.Sci.U.S.A* 96 6211-6216.

Klemke RL, Leng J, Molander R, Brooks PC, Vuori K & Cheresh DA 1998 CAS/Crk coupling serves as a "molecular switch" for induction of cell migration. *J.Cell Biol.* 140 961-972.

Kmiecik TE & Shalloway D 1987 Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation. *Cell* 49 65-73.

Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, Harper ME, Barrow D, Wakeling AE & Nicholson RI 2003 Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology* 144 1032-1044.

Konstantinovsky S, Smith Y, Zilber S, Tuft SH, Becker AM, Nesland JM, Reich R & Davidson B 2010 Breast carcinoma cells in primary tumors and effusions have different gene array profiles. *J Oncol.* 2010 969084.

Kritzer JA, Lear JD, Hodsdon ME & Schepartz A 2004 Helical beta-peptide inhibitors of the p53-hDM2 interaction. *J Am.Chem.Soc.* 126 9468-9469.

Kritzer JA, Stephens OM, Guarracino DA, Reznik SK & Schepartz A 2005 beta-Peptides as inhibitors of protein-protein interactions. *Bioorg.Med.Chem.* 13 11-16.

Lakkakorpi PT, Nakamura I, Nagy RM, Parsons JT, Rodan GA & Duong LT 1999 Stable association of PYK2 and p130(Cas) in osteoclasts and their co-localization in the sealing zone. *J Biol.Chem.* 274 4900-4907.

Law SF, Estojak J, Wang B, Mysliwiec T, Kruh G & Golemis EA 1996 Human enhancer of filamentation 1, a novel p130cas-like docking protein, associates with focal adhesion kinase and induces pseudohyphal growth in Saccharomyces cerevisiae. *Mol.Cell Biol.* 16 3327-3337.

Law SF, Zhang YZ, Fashena SJ, Toby G, Estojak J & Golemis EA 1999 Dimerization of the docking/adaptor protein HEF1 via a carboxy-terminal helix-loop-helix domain. *Exp.Cell Res.* 252 224-235.

Law SF, Zhang YZ, Klein-Szanto AJ & Golemis EA 1998 Cell cycle-regulated processing of HEF1 to multiple protein forms differentially targeted to multiple subcellular compartments. *Mol.Cell Biol.* 18 3540-3551.

Leader B, Baca QJ & Golan DE 2008 Protein therapeutics: a summary and pharmacological classification. *Nat.Rev.Drug Discov.* 7 21-39.

Lehmann JM, Riethmuller G & Johnson JP 1990 Nck, a melanoma cDNA encoding a cytoplasmic protein consisting of the src homology units SH2 and SH3. *Nucleic Acids Res.* 18 1048.

Li E, Stupack DG, Brown SL, Klemke R, Schlaepfer DD & Nemerow GR 2000 Association of p130CAS with phosphatidylinositol-3-OH kinase mediates adenovirus cell entry. *J Biol.Chem.* 275 14729-14735.

Liu F, Hill DE & Chernoff J 1996 Direct binding of the proline-rich region of protein tyrosine phosphatase 1B to the Src homology 3 domain of p130(Cas). *J Biol.Chem.* 271 31290-31295.

Liu JF, Chevet E, Kebache S, Lemaitre G, Barritault D, Larose L & Crepin M 1999 Functional Rac-1 and Nck signaling networks are required for FGF-2-induced DNA synthesis in MCF-7 cells. *Oncogene* 18 6425-6433.

Liu X, Elia AE, Law SF, Golemis EA, Farley J & Wang T 2000 A novel ability of Smad3 to regulate proteasomal degradation of a Cas family member HEF1. *EMBO J* 19 6759-6769.

Lo AS, Zhu Q & Marasco WA 2008 Intracellular antibodies (intrabodies) and their therapeutic potential. *Handb.Exp.Pharmacol.* 343-373.

Lu Y, Brush J & Stewart TA 1999 NSP1 defines a novel family of adaptor proteins linking integrin and tyrosine kinase receptors to the c-Jun N-terminal kinase/stress-activated protein kinase signaling pathway. *J Biol.Chem.* 274 10047-10052.

Ma L, Teruya-Feldstein J & Weinberg RA 2007 Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* 449 682-688.

Makkinje A, Near RI, Infusini G, Vanden Borre P, Bloom A, Cai D, Costello CE & Lerner A 2009 AND-34/BCAR3 regulates adhesion-dependent p130Cas serine phosphorylation and breast cancer cell growth pattern. *Cell Signal.* 21 1423-1435.

Marcotte R & Muller WJ 2008 Signal transduction in transgenic mouse models of human breast cancer--implications for human breast cancer. *J.Mammary.Gland.Biol.Neoplasia.* 13 323-335.

Matozaki T, Murata Y, Saito Y, Okazawa H & Ohnishi H 2009 Protein tyrosine phosphatase SHP-2: a proto-oncogene product that promotes Ras activation. *Cancer Sci.* 100 1786-1793.

Matsuda M, Tanaka S, Nagata S, Kojima A, Kurata T & Shibuya M 1992 Two species of human CRK cDNA encode proteins with distinct biological activities. *Mol.Cell Biol.* 12 3482-3489.

Mayer EL & Krop IE 2010 Advances in targeting SRC in the treatment of breast cancer and other solid malignancies. *Clin.Cancer Res.* 16 3526-3532.

Medina PJ & Goodin S 2008 Lapatinib: a dual inhibitor of human epidermal growth factor receptor tyrosine kinases. *Clin.Ther.* 30 1426-1447.

Meenderink LM, Ryzhova LM, Donato DM, Gochberg DF, Kaverina I & Hanks SK 2010 P130Cas Src-binding and substrate domains have distinct roles in sustaining focal adhesion disassembly and promoting cell migration. *PLoS.One.* 5 e13412.

Meulmeester E & Ten Dijke P 2011 The dynamic roles of TGF-beta in cancer. *J Pathol.* 223 205-218.

Minegishi M, Tachibana K, Sato T, Iwata S, Nojima Y & Morimoto C 1996 Structure and function of Cas-L, a 105-kD Crk-associated substrate-related protein that is involved in beta 1 integrin-mediated signaling in lymphocytes. *J Exp.Med.* 184 1365-1375.

Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, Viale A, Olshen AB, Gerald WL & Massague J 2005 Genes that mediate breast cancer metastasis to lung. *Nature* 436 518-524.

Muthuswamy SK & Muller WJ 1995 Activation of Src family kinases in Neu-induced mammary tumors correlates with their association with distinct sets of tyrosine phosphorylated proteins in vivo. *Oncogene* 11 1801-1810.

Nakamoto T, Sakai R, Honda H, Ogawa S, Ueno H, Suzuki T, Aizawa S, Yazaki Y & Hirai H 1997 Requirements for localization of p130cas to focal adhesions. *Mol.Cell Biol.* 17 3884-3897.

Nakamoto T, Sakai R, Ozawa K, Yazaki Y & Hirai H 1996 Direct binding of C-terminal region of p130Cas to SH2 and SH3 domains of Src kinase. *J Biol.Chem.* 271 8959-8965.

Nakamoto T, Yamagata T, Sakai R, Ogawa S, Honda H, Ueno H, Hirano N, Yazaki Y & Hirai H 2000 CIZ, a zinc finger protein that interacts with p130(cas) and activates the expression of matrix metalloproteinases. *Mol.Cell Biol.* 20 1649-1658.

Nasertorabi F, Tars K, Becherer K, Kodandapani R, Liljas L, Vuori K & Ely KR 2006 Molecular basis for regulation of Src by the docking protein p130Cas. *J Mol.Recognit.* 19 30-38.

Nicolini A, Giardino R, Carpi A, Ferrari P, Anselmi L, Colosimo S, Conte M, Fini M, Giavaresi G, Berti P & Miccoli P 2006 Metastatic breast cancer: an updating. *Biomed.Pharmacother.* 60 548-556.

Nishio H & Suzuki K 2002 Ethanol-induced Cas tyrosine phosphorylation and Fyn kinase activation in rat brain. *Alcohol Clin.Exp.Res.* 26 38S-43S.

Nourry C, Maksumova L, Pang M, Liu X & Wang T 2004 Direct interaction between Smad3, APC10, CDH1 and HEF1 in proteasomal degradation of HEF1. *BMC.Cell Biol.* 5 20.

Okada S & Pessin JE 1996 Interactions between Src homology (SH) 2/SH3 adapter proteins and the guanylnucleotide exchange factor SOS are differentially regulated by insulin and epidermal growth factor. *J Biol.Chem.* 271 25533-25538.

Ottenhoff-Kalff AE, Rijksen G, van Beurden EA, Hennipman A, Michels AA & Staal GE 1992 Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-src oncogene product. *Cancer Res.* 52 4773-4778.

Parsons JT, Martin KH, Slack JK, Taylor JM & Weed SA 2000 Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene* 19 5606-5613.

Pellicena P & Miller WT 2001 Processive phosphorylation of p130Cas by Src depends on SH3-polyproline interactions. *J Biol.Chem.* 276 28190-28196.

Petruzzelli L, Takami M & Herrera R 1996 Adhesion through the interaction of lymphocyte function-associated antigen-1 with intracellular adhesion molecule-1 induces tyrosine phosphorylation of p130cas and its association with c-CrkII. *J Biol.Chem.* 271 7796-7801.

Piwnica-Worms H, Saunders KB, Roberts TM, Smith AE & Cheng SH 1987 Tyrosine phosphorylation regulates the biochemical and biological properties of pp60c-src. *Cell* 49 75-82.

Polte TR & Hanks SK 1995 Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. *Proc.Natl.Acad.Sci.U.S.A* 92 10678-10682.

Prasad N, Topping RS & Decker SJ 2001 SH2-containing inositol 5'-phosphatase SHIP2 associates with the p130(Cas) adapter protein and regulates cellular adhesion and spreading. *Mol.Cell Biol.* 21 1416-1428.

Provenzano PP, Inman DR, Eliceiri KW, Beggs HE & Keely PJ 2008 Mammary epithelial-specific disruption of focal adhesion kinase retards tumor formation and metastasis in a transgenic mouse model of human breast cancer. *Am.J Pathol.* 173 1551-1565.

Provenzano PP & Keely PJ 2009 The role of focal adhesion kinase in tumor initiation and progression. *Cell Adh.Migr.* 3 347-350.

Ren R, Mayer BJ, Cicchetti P & Baltimore D 1993 Identification of a ten-amino acid proline-rich SH3 binding site. *Science* 259 1157-1161.

Rodrigues SP, Fathers KE, Chan G, Zuo D, Halwani F, Meterissian S & Park M 2005 CrkI and CrkII function as key signaling integrators for migration and invasion of cancer cells. *Mol.Cancer Res.* 3 183-194.

Rucci N, Recchia I, Angelucci A, Alamanou M, Del Fattore A, Fortunati D, Susa M, Fabbro D, Bologna M & Teti A 2006 Inhibition of protein kinase c-Src reduces the incidence of breast cancer metastases and increases survival in mice: implications for therapy. *J Pharmacol.Exp.Ther.* 318 161-172.

Ruest PJ, Shin NY, Polte TR, Zhang X & Hanks SK 2001 Mechanisms of CAS substrate domain tyrosine phosphorylation by FAK and Src. *Mol.Cell Biol.* 21 7641-7652.

Sakai R, Iwamatsu A, Hirano N, Ogawa S, Tanaka T, Mano H, Yazaki Y & Hirai H 1994a A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. *EMBO J* 13 3748-3756.

Sakai R, Iwamatsu A, Hirano N, Ogawa S, Tanaka T, Nishida J, Yazaki Y & Hirai H 1994b Characterization, partial purification, and peptide sequencing of p130,the main phosphoprotein associated with v-Crk oncoprotein. *J Biol.Chem.* 269 32740-32746.

Sakai R, Nakamoto T, Ozawa K, Aizawa S & Hirai H 1997 Characterization of the kinase activity essential for tyrosine phosphorylation of p130Cas in fibroblasts. *Oncogene* 14 1419-1426.

Sakakibara A & Hattori S 2000 Chat, a Cas/HEF1-associated adaptor protein that integrates multiple signaling pathways. *J Biol.Chem.* 275 6404-6410.

Salgia R, Pisick E, Sattler M, Li JL, Uemura N, Wong WK, Burky SA, Hirai H, Chen LB & Griffin JD 1996 p130CAS forms a signaling complex with the adapter protein CRKL in hematopoietic cells transformed by the BCR/ABL oncogene. *J Biol.Chem.* 271 25198-25203.

Schlaepfer DD, Broome MA & Hunter T 1997 Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. *Mol.Cell Biol.* 17 1702-1713.

Schwartz DE, Tizard R & Gilbert W 1983 Nucleotide sequence of Rous sarcoma virus. *Cell* 32 853-869.

Scibelli A, d'Angelo D, Pelagalli A, Tafuri S, Avallone L, Della MR & Staiano N 2003 Expression levels of the focal adhesion-associated proteins paxillin and p130CAS in canine and feline mammary tumors. *Vet.Res.* 34 193-202.

Shin NY, Dise RS, Schneider-Mergener J, Ritchie MD, Kilkenny DM & Hanks SK 2004 Subsets of the major tyrosine phosphorylation sites in Crk-associated substrate (CAS) are sufficient to promote cell migration. *J.Biol.Chem.* 279 38331-38337.

Shintani Y, Fukumoto Y, Chaika N, Svoboda R, Wheelock MJ & Johnson KR 2008 Collagen I-mediated up-regulation of N-cadherin requires cooperative signals from integrins and discoidin domain receptor 1. *J Cell Biol.* 180 1277-1289.

Simpson KJ, Selfors LM, Bui J, Reynolds A, Leake D, Khvorova A & Brugge JS 2008 Identification of genes that regulate epithelial cell migration using an siRNA screening approach. *Nat.Cell Biol.* 10 1027-1038.

Singh MK, Dadke D, Nicolas E, Serebriiskii IG, Apostolou S, Canutescu A, Egleston BL & Golemis EA 2008 A novel Cas family member, HEPL, regulates FAK and cell spreading. *Mol.Biol.Cell* 19 1627-1636.

Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, Lechleider RJ &. 1993 SH2 domains recognize specific phosphopeptide sequences. *Cell* 72 767-778.

Soni S, Lin BT, August A, Nicholson RI & Kirsch KH 2009 Expression of a phosphorylated p130(Cas) substrate domain attenuates the phosphatidylinositol 3-kinase/Akt survival pathway in tamoxifen resistant breast cancer cells. *J Cell Biochem* 107 364-375.

Soriano JV, Liu N, Gao Y, Yao ZJ, Ishibashi T, Underhill C, Burke TR, Jr. & Bottaro DP 2004 Inhibition of angiogenesis by growth factor receptor bound protein 2-Src homology 2 domain bound antagonists. *Mol.Cancer Ther.* 3 1289-1299.

Spencer KS, Graus-Porta D, Leng J, Hynes NE & Klemke RL 2000 ErbB2 is necessary for induction of carcinoma cell invasion by ErbB family receptor tyrosine kinases. *J Cell Biol.* 148 385-397.

Stylli SS, Stacey TT, Verhagen AM, Xu SS, Pass I, Courtneidge SA & Lock P 2009 Nck adaptor proteins link Tks5 to invadopodia actin regulation and ECM degradation. *J Cell Sci.* 122 2727-2740.

Superti-Furga G, Fumagalli S, Koegl M, Courtneidge SA & Draetta G 1993 Csk inhibition of c-Src activity requires both the SH2 and SH3 domains of Src. *EMBO J* 12 2625-2634.

Ta HQ, Thomas KS, Schrecengost RS & Bouton AH 2008 A novel association between p130Cas and resistance to the chemotherapeutic drug adriamycin in human breast cancer cells. *Cancer Res.* 68 8796-8804.

Takeya T & Hanafusa H 1982 DNA sequence of the viral and cellular src gene of chickens. II. Comparison of the src genes of two strains of avian sarcoma virus and of the cellular homolog. *J Virol.* 44 12-18.

ten Hoeve J, Morris C, Heisterkamp N & Groffen J 1993 Isolation and chromosomal localization of CRKL, a human crk-like gene. *Oncogene* 8 2469-2474.

Tikhmyanova N, Little JL & Golemis EA 2010 CAS proteins in normal and pathological cell growth control. *Cell Mol.Life Sci.* 67 1025-1048.

van der Flier S, Brinkman A, Look MP, Kok EM, Meijer-van Gelder ME, Klijn JG, Dorssers LC & Foekens JA 2000 Bcar1/p130Cas protein and primary breast cancer: prognosis and response to tamoxifen treatment. *J.Natl.Cancer Inst.* 92 120-127.

Vassilev LT 2007 MDM2 inhibitors for cancer therapy. *Trends Mol.Med.* 13 23-31.

Verbeek BS, Vroom TM, Adriaansen-Slot SS, Ottenhoff-Kalff AE, Geertzema JG, Hennipman A & Rijksen G 1996 c-Src protein expression is increased in human breast cancer. An immunohistochemical and biochemical analysis. *J Pathol.* 180 383-388.

Vogler M, Dinsdale D, Dyer MJ & Cohen GM 2009 Bcl-2 inhibitors: small molecules with a big impact on cancer therapy. *Cell Death.Differ.* 16 360-367.

Vultur A, Buettner R, Kowolik C, Liang W, Smith D, Boschelli F & Jove R 2008 SKI-606 (bosutinib), a novel Src kinase inhibitor, suppresses migration and invasion of human breast cancer cells. *Mol.Cancer Ther.* 7 1185-1194.

Wang FM, Liu HQ, Liu SR, Tang SP, Yang L & Feng GS 2005 SHP-2 promoting migration and metastasis of MCF-7 with loss of E-cadherin, dephosphorylation of FAK and secretion of MMP-9 induced by IL-1beta in vivo and in vitro. *Breast Cancer Res.Treat.* 89 5-14.

Wang X, Weng LP & Yu Q 2000 Specific inhibition of FGF-induced MAPK activation by the receptor-like protein tyrosine phosphatase LAR. *Oncogene* 19 2346-2353.

Webster MA, Cardiff RD & Muller WJ 1995 Induction of mammary epithelial hyperplasias and mammary tumors in transgenic mice expressing a murine mammary tumor virus/activated c-src fusion gene. *Proc.Natl.Acad.Sci.U.S.A* 92 7849-7853.

Wells JA & McClendon CL 2007 Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. *Nature* 450 1001-1009.

Wendt MK, Smith JA & Schiemann WP 2009 p130Cas is required for mammary tumor growth and transforming growth factor-beta-mediated metastasis through regulation of Smad2/3 activity. *J Biol.Chem.* 284 34145-34156.

Weng LP, Wang X & Yu Q 1999 Transmembrane tyrosine phosphatase LAR induces apoptosis by dephosphorylating and destabilizing p130Cas. *Genes Cells* 4 185-196.

Wheeler DL, Iida M & Dunn EF 2009 The role of Src in solid tumors. *Oncologist.* 14 667-678.

Yamakita Y, Totsukawa G, Yamashiro S, Fry D, Zhang X, Hanks SK & Matsumura F 1999 Dissociation of FAK/p130(CAS)/c-Src complex during mitosis: role of mitosis-specific serine phosphorylation of FAK. *J Cell Biol.* 144 315-324.

Yamashita H 2008 Current research topics in endocrine therapy for breast cancer. *Int.J.Clin.Oncol.* 13 380-383.

Yo K, Iwata S, Hashizume Y, Kondo S, Nomura S, Hosono O, Kawasaki H, Tanaka H, Dang NH & Morimoto C 2009 SHP-2 inhibits tyrosine phosphorylation of Cas-L and regulates cell migration. *Biochem Biophys.Res.Commun.* 382 210-214.

Yu H, Chen JK, Feng S, Dalgarno DC, Brauer AW & Schreiber SL 1994 Structural basis for the binding of proline-rich peptides to SH3 domains. *Cell* 76 933-945.

Zhang Y, Su H, Rahimi M, Tochihara R & Tang C 2009 EGFRvIII-induced estrogen-independence, tamoxifen-resistance phenotype correlates with PgR expression and modulation of apoptotic molecules in breast cancer. *Int.J Cancer* 125 2021-2028.

Zheng M & McKeown-Longo PJ 2002 Regulation of HEF1 expression and phosphorylation by TGF-beta 1 and cell adhesion. *J Biol.Chem.* 277 39599-39608.

4

Breast Cancer and Current Therapeutic Approaches: From Radiation to Photodynamic Therapy

Peter Ferenc, Peter Solár, Jaromír Mikeš, Ján Kovaľ and Peter Fedoročko
Faculty of Science, Institute of Biology and Ecology, P. J. Šafárik University in Košice, Slovakia

1. Introduction

Breast cancer is one of the oldest known forms of cancer in humans and it has been mentioned in almost every period of human history. Since the time of the ancient Egyptians and Greeks, there has been no cure but only treatment for this disease. In the 18th century, different theories about the origin of breast cancer were developed. During this period, an important link between breast cancer and the lymph nodes was established. The assumption that cancer was a localized disease led to the rise of the surgical approach in breast cancer treatment. Since the work of William Halstead (1882), radical mastectomy (removal of breast tissue, lymph nodes and chest tissue) remained the standard for almost 100 years (Leopold, 1999; Olson, 2002). With the advance in science, novel therapeutic and diagnostic opportunities came into use in breast cancer treatment. Introduction of radiation at the beginning of the 20th century enabled tumour size to be reduced before surgery. Another major breakthrough came with the use of chemotherapy in the 1940s. Their combination with surgery offers another powerful treatment modality. The discovery by Beatson in 1895 that removal of the ovaries results (in some cases) in reduction of breast tumours led to the later elucidation of oestrogen`s role in breast cancer growth (Forrest, 1982). Research in pharmaceutical approaches to breast cancer/oestrogen management ended in the development of aromatase inhibitors (AIs) and selective oestrogen receptor modifiers. An important step came in 1998, when the US Food and Drug Administration (FDA) approved trastuzumab for the treatment of HER2 positive metastatic breast cancer. Treatment with trastuzumab has a major impact on the survival of a subset of patients with resistant and hard to treat breast tumours (Shepard et al., 2008). With the introduction of mammography, early detection of breast cancer was made possible. Mammography screening combined with more precise therapy was shown to reduce breast cancer mortality between 24.9 and 38.3% (Berry et al., 2005). Several other detection methods including magnetic resonance, ultrasound and 3D digital mammography have been developed and are now used in the fight against breast cancer (Gilbert, 2008; Hellerhoff, 2010).

2. Current therapeutic approaches

2.1 Radiotherapy

Radiation therapy uses high-energy x-rays to destroy cancer cells. This therapy usually follows lumpectomy to eliminate any microscopic cancer cells in the remaining breast tissue.

Sometimes radiation therapy is also given after a mastectomy, but only if there is a high risk of cancer recurring in that area. Early studies on the use of adjuvant radiotherapy are difficult to interpret owing to poor radiotherapy techniques, inappropriate dosage or a variety of confounding variables within a particular trial. The results of clinical studies have confirmed that adjuvant radiotherapy will reduce the risk of local recurrence and produce a reduction in breast cancer deaths for tumours of <5 cm with involved nodes (Fernando, 2000).

Furthermore, adjuvant radiotherapy combined with tamoxifen has been shown to produce an improvement in both local control and survival in postmenopausal node-positive patients who have undergone mastectomy. Adjuvant radiation combined with systemic chemotherapy has a significant effect on local recurrence and probably on survival in node-positive patients after mastectomy (Fernando, 2000).

Radiotherapy has undergone significant technological advances during the last 20 years, although its use in breast cancer was relatively limited until recently. The major recent changes in the use of radiotherapy for breast cancer have been the following: the establishment of partial breast irradiation as an option for therapy in early stage disease; the revival of hypofractionated therapies for breast-only therapy; the clearer definition of the role of post-mastectomy irradiation; and the continuing investigation as to which patients having conservative surgery do not need radiation therapy (Powell, 2010). Nowadays, Memorial Sloan-Kettering Cancer Center (New York, NY, USA) offers several newer forms of radiation therapy for breast cancer, which include intensity-modulated radiation therapy, radiation delivered in the prone position and image-guided radiation therapy.

In addition to cytocidal effects, ionizing radiation has been shown to cause a plethora of changes on both the cancer cells and tumour stroma, critical in determining its therapeutic success (Formenti & Demaria, 2008). Many of these changes have been proven in experimental systems to affect the ability of the immune system to reject the tumour (Demaria & Formenti, 2007). In this regard, radiation-induced upregulation of Fas/CD95 (Chakraborty et al., 2003) and MHC 1 (Reits et al., 2006) on cancer cells and VCAM 1 (Lugade et al., 2008) on tumour-associated endothelia must be considered. Moreover, Matsumura et al. (2008) showed that radiation enhances the release of chemokine CXCL16 by human and mouse breast cancer cells, which is very important for efficient recruitment of antitumour T cells and tumour inhibition following treatment with radiation and CTL-associated antigen 4 blockade.

Recently, targeted intraoperative radiotherapy impaired the stimulation of breast cancer cell proliferation and invasion caused by surgical wounding. Indeed, the fluid from wound drainage stimulated proliferation, migration and invasion of breast cancer cells. The observed effect was negated when wound drainage fluid was obtained from patients who had undergone intraoperative radiotherapy (Belletti et al., 2008). More clinical studies are needed to support the hypothesis that immune mechanisms underlie the effect of local control on systemic outcome (Formenti & Demaria, 2008).

2.2 Chemotherapy

Many specialists recommend chemotherapy following surgery to kill cancer cells that may have spread outside the breast (adjuvant therapy). Chemotherapy might be recommended before surgery (neoadjuvant therapy) if the breast tumour is large, the lymph nodes are involved or the tumour is attached to the chest wall muscles, and also in the cases of inflammatory breast cancer.

Anthracyclines were considered the gold standard of adjuvant chemotherapy until the late 1990s. However, long-term treatment with side effects such as cardiac toxicity and leukemia/myelodysplastic syndrome can negate their benefits. The real benefit from anthracyclines could be felt by patients with topoisomerase II α amplification, which is usually associated with HER2 amplification. Overall, the anthracycline regimens (for example 5-fluorouracil, doxorubicin and cyclophosphamide – FAC; 5-fluorouracil, epirubicin and cyclophosphamide – FEC, or doxorubicin and cyclophosphamide – AC) are associated with reduction in the risk of recurrence by 11.2% and in the risk of death by 16 %, compared with combinations including cyclophosphamide, methotrexate and 5-fluorouracil (Lopez-Tarruella & Martin, 2009).

Although the precise role of taxanes is uncertain, based upon the data from first-generation taxane trials it is reasonable to consider taxane therapy in women with an elevated risk of relapse where endocrine sensitivity is absent or incomplete (Bedard & Cardoso, 2008). As the number of treatment options increases, the need to define a set of criteria to select those patients who will benefit from each treatment regimen or strategy becomes a priority (Lopez-Tarruella & Martin, 2009).

About three quarters of breast cancer cells express oestrogen and/or progesterone receptors, therefore the first targeted breast cancer therapy was the antioestrogen one. The first such therapy approved for the treatment of breast tumours was the therapy involving tamoxifen. Although first studies showed positive effects of tamoxifen, adverse effects causing endometrial cancer and thromboembolism were later shown by Fisher et al. (1994) and Jordan (1995).

Because of higher production of oestrogens in breast cancer tissues in comparison to noncancerous ones, another very attractive target for breast cancer treatment is aromatase (Harada, 1997). Multiple clinical studies have demostrated the efficacy and reduced side effects of AIs vs. tamoxifen. However, their benefit is limited by the resistance induced through the crosstalk between oestrogen receptor and other signalling pathways, particularly MAPK and PI3K/Akt. Interfering with these other signalling pathways is an attractive strategy to circumvent the resistance to AIs in breast cancer. Several clinical trials are under way to evaluate the role of these novel target therapies to reverse resistance to AIs. These agents include MEK inhibitors, Raf inhibitors, PI3K inhibitors, mTOR inhibitors and Akt inhibitors (Chumsri et al., 2011).

Fulvestrant (selective oestrogen receptor downregulator) is recommended for second-line therapy after failure of tamoxifen, and for third-line therapy after failure of tamoxifen and AIs. Other third-line agents used after the failure of other options include progestins, androgens or high-dose oestrogens (Beslija et al., 2007).

Several multigene markers that predict relapse more accurately than classical clinicopathologic features have been developed. The 21-gene assay was developed specifically for patients with oestrogen receptor ER-positive breast cancer, and has been shown to predict distant recurrence more accurately that classical clinicopathologic features in patients with ER-positive breast cancer and negative axillary nodes treated with adjuvant tamoxifen (Sparano & Paik, 2008). Another 70-gene profile is a new prognostic tool that has the potential to greatly improve risk assessment and treatment decision-making for early breast cancer. Its prospective validation is currently under way through the MINDACT (Microarray in Node-Negative Disease May Avoid Chemotherapy), a 6000-patient randomized, multicentric trial (Cardoso et al., 2008).

2.3 Therapy of HER2 positive breast cancers

The HER2 oncoprotein is an important therapeutic target in the treatment of invasive breast cancers associated with poor disease-free survival and resistance to chemotherapy (Nahta et al., 2006). HER2 status is a significant prognostic factor for local-regional disease progression. Patients with positive HER2 status had a local-regional disease progression-free rate of 59% compared with 92% for patients with negative HER2 status (Haffty et al., 2004).

Although the application of monoclonal antibody against HER2 – trastuzumab showed beneficial effect when combined with docetaxel and platinum salts (Pegram et al., 2004) or paclitaxel and carboplatin (Perez et al., 2005; Robert et al., 2006), its use beyond first-line therapy might develop resistance to this agent. In this regard, inhibition of PTEN (Nagata et al., 2004), overexpression of IGF-IR (Lu et al., 2001) and MUC4 (Nagy et al., 2005) and increased level of VEGF protein (du Manoir et al., 2006) could play a significant role. In order to make trastuzumab treatment more effective after disease progression, new agents targeting the HER2 pathway have been developed. The number of HER-targeting agents include antibody, small tyrosine kinase inhibitor (TKI) molecules, mTOR inhibitors, Hsp90 inhibitor, farnesyltransferase inhibitor and PI3K inhibitor (Morrow et al., 2009). One of the TKI small molecules, lapatinib, has been approved (in combination with capecitabine) by the FDA in the treatment of patients with advanced or metastatic HER2 positive breast cancer which progresses after trastuzumab, anthracyclines and taxanes (Morrow et al., 2009).

2.4 Therapy of HER2 negative breast cancers

Trastuzumab has improved outcomes in breast cancer patients with HER2 overexpressing tumours. However, systemic treatment for patients with HER2 negative diseases is still limited to endocrine and cytotoxic therapies. Anthracyclines and taxanes used in early-stage disease reduce the available therapeutic options for patients with relapsed disease. Treatment choices are limited in patients with triple-negative breast cancer (do not express HER2 and hormone receptors), where the prognosis is usually poor (Miles, 2009). These tumours are sensitive to platinum compounds and their DNA damaging effect, because of downregulation of BRCA-1 (DNA repair protein) (James et al., 2007). The results of combined platinum and taxane (docetaxel) therapy in patients with triple-negative metastatic breast cancer ongoing from phase III trial are expected in 2012 (Miles, 2009).

There are some novel chemotherapeutic agents in clinical development. One of them, nab-paclitaxel (nanoparticle albumin-bound paclitaxel), has been approved for metastatic breast cancer patients with failed first-line therapy. The second very interesting group of agents consists of microtubule stabilizing anticancer drugs, epothilones (ixabepilone has been approved only in the USA), which are desirable for patients with anthracycline and taxane resistant tumours (Thomas et al., 2007). Another special group of drugs comprises anti-angiogenic agents which target and inhibit VEGF (bevacizumab) (Miller et al., 2007) or VEGF receptor, as well as other receptor tyrosine kinases (e.g. sunitinib, pazopanib, axitinib, sorafenib) (Miller et al., 2005). Other very attractive candidates for single or combined therapy of patients with metastatic breast cancer are also EGFR inhibitors (von Minckwitz et al., 2005), mTOR inhibitors (Chan et al., 2005), Ras cascade inhibitors (Normanno et al., 2005) and PARP inhibitors (Bryant et al., 2005; Nguewa et al., 2006).

Nowadays, targeted therapy with anti-sense nucleotides, inhibitors of apoptosis proteins, proteasome system inhibitors as well as cyclin-dependent kinase inhibitors are in phase I-III of clinical studies (Schlotter et al., 2008). One of the greatest challenges in breast cancer

treatment is the delivery of miRNA inhibitors or miRNA mimics specifically to tumour cells, which will probably become reality in the near future (O'Day & Lal, 2010).

2.5 Therapeutic potential of natural compound genistein

Genistein (GE) belongs in the isoflavone class of flavonoids, with soy beans as a major source (Akiyama et al., 1987). The flavonoids display a wide spectrum of pharmacological activities, but their anticancer activity is the most important (Lee et al., 2002). In particular, GE has proven to be a valuable tool for the inhibition of cancer metastasis, exerting effects on both the initial step of primary tumour growth as well as the later steps of the metastatic cascade. This isoflavonoid inhibits cell growth and induces cell death in numerous types of cancer cells (Yeh et al., 2007). Data obtained to date suggest that the anticancer effects of GE result from various mechanisms, including the regulation of cell cycle progression (Constantinou et al., 1998), inhibition of tyrosine kinases (Akiyama et al., 1987) and inhibition of matrix metalloproteinase (Xu & Bergan, 2006).

A number of studies have suggested that GE may induce apoptosis in several breast cancer cell lines and produce synergistic inhibitory effects when combined with cancer therapies. GE has been shown to induce apoptosis in the high invasive MDA-MB-231 and low invasive MCF-7 breast cancer cell lines at relatively high concentrations of 10 – 100 μM (Li et al., 2008; Nomoto et al., 2002). The concentration as well as the cell type are critical determinants of the isoflavone effect (Pavese et al., 2010). In accord with this fact, GE has been shown to have biphasic proliferative effect in breast cancer cells, inhibiting *in vitro* cell proliferation at high concentrations (>10 μmol/l), while stimulating proliferation of oestrogen receptor positive cells (but not oestrogen receptor negative cells) at lower concentrations (<10 μmol/l) (Zava & Duwe, 1997). A number of studies have shown that GE at higher concentrations affects multiple intracellular targets and has impact on tumour cells independently of the oestrogen receptors (Constantinou et al., 1998), but as a phytooestrogen, GE can bind to both oestrogen receptors (ERα/ERβ), though it has a higher affinity for ERβ than ERα (Muthyala et al., 2004). Concerning the role of oestrogen receptors, Liu et al. (2002) demonstrated that both RT-PCR and immunohistochemical staining showed significantly higher ERα expression in cancerous human breast than in normal breast, while ERβ was higher in normal human breast than in cancerous breast. On the other hand, up-regulation of ERβ in breast cancer cells by trichostatin A, a histone deacetylase inhibitor, led to induced sensitivity to tamoxifen (Jang et al., 2004). GE could therefore be used as a potential chemotherapeutic agent against breast cancer of the ERα-negative and ERβ-positive type (Rajah et al., 2009).

The study undertaken by Xu et al. (2009) was the first to demonstrate the inhibition of prometastatic processes in humans through therapeutic application of GE, even with low blood concentration of GE (approximately 140 nM).

According to studies concerned with GE's weak oestrogenic activity (Messina et al., 2006), it seems that the effect of GE on breast cancer depends on the nature of the oestrogenic environment in which the study is conducted. In this regard, if endogenous oestrogen is low, GE can bind the ER receptor and exert progrowth effects upon responsive systems. On the other hand, if oestrogen is high and potent, GE can act as a competitor to oestrogen and thus antagonize this hormone´s effect. In addition, gene expression levels of BRCA-1 and BRCA-2, breast tumour suppressor genes, were maintained over the 3-year period in the group administered with GE, whereas the placebo group showed decreased levels of both BRCA-1 and BRCA-2 gene products (Marini et al., 2008). The recent nested case control

study by the Japan Public Health Center shows statistically significant inverse association between GE and the risk of breast cancer over a 10-year period. Furthermore data from this study suggest that even at the relatively low concentrations achievable from dietary intake alone (highest plasma level 353.9 ng/ml), GE poses a risk-reducing rather than a risk-enhancing effect on breast cancer (Iwasaki et al., 2008). Similarly, a prospective study in the Dutch population examined the association between plasma levels of isoflavones (daidzein, GE, glycitein, O-desmethylangolensin and equol) or lignans (enterodiol and enterolactone) and breast cancer risk. The result of the study was that high circulating GE levels are associated with reduced breast cancer risk (Verheus et al., 2007). In contrast, a prospective study of European women found no protective effect of high levels of GE and other phytooestrogens (in the blood and urine) against breast cancer (Ward et al., 2008).

3. Photodynamic therapy (PDT)

There is a plethora of approaches to cancer therapy that may be sorted into various categories in many different ways. But generally speaking, there are treatments based on biologically relevant actions of chemical compounds or physical effects. Irradiation, either in the form of electromagnetic waves or accelerated particles, has earned its stable position in the oncological armoury, and γ-irradiation has been successfully used for decades. However electromagnetic radiation with longer wavelengths and lower energy is also used in modern medicine for various intents. Direct exposure to non-ionizing radiation for therapeutic use (natural light, UVB or UVA radiation) known as a phototherapy is usually applied for treatment of skin conditions such as dermatitis, psoriasis or vitiligo. However, it has also found its place in other medical areas, with particular applications in psychiatry in the treatment of internal depressions, sleeping changes, or the circadian rhythm (Ledo & Ledo, 2000).

The physical and chemical "approaches" may also be combined together to ensure higher therapeutic efficiency, or work together for diagnostic purpose. Combination of a photosensitizing chemical substance followed by electromagnetic non-ionizing radiation is known as a photochemotherapy, and is typically administered using psoralen (as the photosensitizer) and long-wavelength ultraviolet radiation (UVA). Furthermore, photosensitizer and light may also be combined with oxygen to get a highly effective therapeutic paradigm named as "photodynamic" therapy (PDT). This might be subsumed into the photochemotherapy subset, and together they belong in the phototherapy family. Photodynamic therapy employs visible light, often in the red or near IR part of the spectrum. The energy of photons absorbed by the photosensitizer is generally used for transformation of oxygen into highly-reactive intermediate oxygen radicals. The main advantage of this approach is the combination of three inoffensive entities which together create a highly toxic conjunction, and so it has also found applications in the treatment of a wide range of malignancies (Ledo & Ledo, 2000).

By its nature, PDT is a flexible and versatile therapeutic approach depending on the nature of the photosensitive compound, its concentration and incubation time, on the wavelength of light radiation, fluence rate and light dose, the time between drug administration and its activation (Kuliková et al., 2010), as well as on the histological origin of tissue and the oxygen pressure in it (Agostinis et al., 2002). All these factors modulate three independent processes contributing to tumour destruction by PDT: direct cell death, destruction of tumour vasculature causing tumour ischemia, and activation of an immune response

(Buytaert et al., 2007). Practical application of PDT is straightforward and based on three elementary steps: administration of a photosensitive compound, its selective accumulation in neoplastic tissue, and irradiation of the tissue with visible light of an appropriate wavelength (Oleinick et al., 2002). Depending on the part of a body being treated, the photosensitizing agent may be either injected into the bloodstream or applied locally to the skin. After the drug is absorbed by the cancer cells, a light source is applied only to the area to be treated. *In vivo* studies have shown that the PDT can work as well as surgery or radiation therapy, but unlike both of them PDT can also alert the immune system and stimulate specific immune responses for treatment of malignant as well as non-malignant diseases (Qiang et al., 2008). Topical PDT is well tolerated and leads to excellent aesthetic results with only minor side effects (Fritsch & Ruzicka, 2006), so it is an excellent choice for non-malignant applications such as psoriasis, viral-induced diseases or acne vulgaris. As the photosensitizers are also fluorescent, they are applicable as a highly efficient contrasting method in detection of tumours *via* so-called "photodynamic diagnostics" (PDD). Hypericin for example has proved to be very effective in fluorescence cystoscopy of bladder cancer (Jichlinski & Leisinger, 2005).

Until recently, all the advantages of PDT were believed to be compromised by the weak penetration of visible light into body tissues. It was therefore considered effective only for treatment of superficial cancers (Hopper, 1996) located on or just under the skin or in the lining of internal organs. Although the limited penetration issue cannot be eliminated, the use of specially-designed catheters and fibre optics can distribute light in three-dimensional space. Simultaneous irradiation with a set of catheters accurately combined in space can efficiently irradiate a large tumour mass. Moreover, using fibre optics, visible light is much easier to distribute in comparison to high-energy particle or γ-radiation. Moreover, the lower penetration of visible light may even be advantageous in the case of anatomically complex tumours, and may help to protect sensitive histological structures. Slow body clearance and therefore long-lasting skin sensitivity to light is another drawback of PDT that may be managed with special precautions (Reddy et al., 2006). Furthermore, new prospects have been introduced lately thanks to experiments with different delivery systems such as nanoparticles (Simon et al., 2010).

Since PDT may be targeted precisely, it is in many cases less invasive than surgery, and unlike radiation it can be repeated several times at the same site, if necessary. Thanks to these attributes, PDT does not demonstrate any long-term side effects when used properly.

3.1 Mechanism of PDT

The molecular mechanism of PDT is based on absorption of photons, which transforms the photosensitizer from the ground singlet into the excited state. Release of accumulated energy and consequent relaxation of the molecules back to the ground state might be accomplished either by emitting fluorescence that can be used by PDD for diagnostic purposes (Berg et al., 2005) or by intersystem crossing to a relatively stable (in range μs – ms) excited triplet state followed by generation of radicals (Takemura et al., 1989).

Relaxation from the triplet state can generate either free radicals or radical ions by hydrogen atom extraction or electron transfer to biological substrates (such as membrane lipids), solvent molecules or oxygen (Berg et al., 2005). The radicals generated by the photosensitizer can interact with ground-state molecular oxygen to produce superoxide anion (O^{2-}) radicals, hydrogen peroxides (H_2O_2) and hydroxyl radicals (·OH) (so-called "Type I reaction"). Direct transfer of energy from the triplet state photosensitizer to the

ground state molecular oxygen forms non-radical but highly reactive singlet oxygen ($^1O_2$) (so-called "Type II reaction"), which is of higher significance for PDT action (Niedre et al., 2002). On the other hand, production of superoxide anions in the Type I reaction can form hydrogen peroxide able to diffuse through the membranes, so it might be toxic for neighbouring cells. Addition of another electron can lead to generation of two hydroxyl radicals, the most dangerous member of the reactive oxygen species (ROS) family with ability to attack and oxidize any compound of biological origin (Plaetzer et al., 2005). Both oxygen-dependent reactions occur simultaneously, but the ratio between them depends on the photosensitizer and available substrate molecules (Berg et al., 2005). Oxidative damage in the cell induced by ROS generated *via* PDT also depends on the intracellular localization, affects different cell organelles and induces cell death (Ahmad & Mukhtar, 2000).

Since the photogenerated singlet oxygen has a very short life and very limited diffusion in biological systems (half-life: <0.04 μs, radius of action: <0.02 μm), the primary molecular targets of the photodynamic process have to reside within a few nanometers from the dye (Moan & Berg, 1991). Therefore it is generally accepted that subcellular localization of the photosensitizer coincides with the primary site of photodamage. The plasma membrane, mitochondria, lysosomes, Golgi apparatus and endoplasmic reticulum (ER) are the most frequent targets of PDT. Moreover, since most dyes do not accumulate in cell nuclei, PDT has generally much lower potential of causing DNA damage, mutations and carcinogenesis as compared to that induced by X-radiation at equitoxic fluencies/doses (Oleinick et al., 2002). Even so, some studies have reported the relocalization of certain photosensitizers after irradiation (Berg et al., 1991; Marchal et al., 2007), suggesting that besides the primary site, photodamage can be rapidly propagated to other subcellular locations. Since photo-generation of singlet oxygen and radicals is limited to the light period when the photosensitizer is activated, the fluency rate of the light source and therefore the time frame of the administration might also affect the PDT efficiency, as the photosensitizer moving during light administration may generate different damage patterns.

Disregarding this issue, photosensitizers localized in the mitochondria and ER tend to promote apoptosis, while those targeting the plasma membrane or lysosomes can either delay or even block apoptosis and thereby also any arising predisposition for necrosis (Kessel et al., 1997). Necrosis (apart from massive cellular destruction leading to bioenergetic catastrophe) may under given circumstances be considered, regarding the concept of programmed necrosis (Proskuryakov et al., 2003), as a form of programmed cell death. Similarly the autophagic repair process may transform into a programmed event, possibly executable after irreparable photodamage to crucial cellular structures (Buytaert et al., 2006b; Buytaert et al., 2006a). Photoactivated photosensitizers with a prevalent mitochondrial localization, (e.g. porphyrogenic sensitizers and phthalocyanine-related compounds) rapidly mediate $\Delta\Psi m$ dissipation accompanied by cytochrome *c* release and a drop in intracellular ATP levels (Almeida et al., 2004; Oleinick et al., 2002). However, the mitochondria are also critical executers of lethal pathways emanating from photodamage to other subcellular sites or organelles, although in this case the release of apoptogenic proteins from the mitochondria is delayed (Buytaert et al., 2007). On the other hand, in some cases (e.g. hypericin) PDT may trigger ER Ca^{2+} store emptying as a consequence of sarco(endo)plasmic-reticulum Ca^{2+}-ATPase (SERCA2) protein level loss, initiated by ER-associating hypericin (HY) irradiation (Buytaert et al., 2006a). Intracellular Ca^{2+} overload, with consequent mitochondrial Ca^{2+}-uptake, increased cellular pro-oxidant state and the

generation of free fatty acids, such as those produced by phospholipase A2, are known factors favouring permeability transition pore (PTP) opening (Rasola & Bernardi, 2007).
Nevertheless, necrosis as a type of programmed cell death is not a result of one well-described signalling cascade but is the consequence of extensive crosstalk between several biochemical and molecular events at different cellular levels. It seems that serine/threonine kinase RIP1 (receptor interacting protein), which contains a death domain, may act as a central initiator. Fluctuations in calcium level and ROS accumulation may directly or indirectly provoke damage to proteins, lipids and DNA, culminating in disruption of organelle and cell integrity (Festjens et al., 2006).
It has been shown as well that PDT may induce non-apoptotic cell death associated with the induction of autophagy (Buytaert et al., 2006b). Due to the high reactivity of photogenerated ROS, it is not surprising that autophagy is initiated in an attempt to remove heavily-damaged organelles or to degrade large aggregates of cross-linked proteins produced by photochemical reactions, which cannot be removed by the ubiquitin–proteasome system or by the degradation associated with ER. Since autophagy is a self-limiting process, it is possible that its persistence results in metabolic and bioenergetic collapse, which is causative for cell death (Buytaert et al., 2006b).
It is evident that the type of ROS and site of their production within the cell represents the vital death switch mechanism which regulates transition among cell death types. However, apoptosis is a highly-regulated event and there are often various changes in cell signalling pathways which are present primarily in the cell or evoked by PDT itself. For example increased expression of anti-apoptotic proteins from the Bcl-2 family, often found in about half of the various human cancers (Reed, 1998), could impose a certain resistance to apoptosis and switch the balance towards necrosis in some cell types (Agostinis et al., 2002). Likewise, we have documented that p53-deficient cells, although similarly sensitive to PDT with HY as their wild-type p53-expressing opposites, tend to die by necrosis (Mikeš et al., 2009).

3.2 Hypericin

Hypericin, a naturally-occurring photosensitive compound, is a naphthodianthrone derivative synthesized by the plant St. John's Wort. Among others it possesses properties suitable for PDT (Čavarga et al., 2005; Chan et al., 2009) and PDD (Thong et al., 2009). Peculiar attributes of this photosensitizer are high efficiency in production of singlet oxygen (Redmond & Gamlin, 1999) and superoxide anions after irradiation with light wavelength around 600 nm and low or no toxicity in the dark (Jacobson et al., 2001; Miadoková et al., 2009). Photoactivated HY is known to induce changes at cellular as well as vascular level or even affect CD8+ T cell-mediated cytotoxicity (Lavie et al., 2000). At the cellular level, activated HY induces many events, more or less specific, such as membrane lipid peroxidation (Chaloupka et al., 1999), increased activity of superoxide dismutase, decreased glutathione concentration (Hadjur et al., 1996) or injury to the mitochondria (Vantieghem et al., 2001). One relatively specific example seems to be its ability to inhibit various enzymes. HY, whether light-activated or not, has been found to inhibit an extensive spectrum of Ser/Thr protein kinases (Blank et al., 2001), protein tyrosine kinases or even HIV-1 reverse transcriptase (Schinazi et al., 1990), and it also seems to play a role in the onset of multidrug resistance phenotype (Jendželovský et al., 2009). Its fluorescence is applicable in the detection of tumours *via* PDD and has proved to be very effective in fluorescence cystoscopy of bladder cancer (Jichlinski & Leisinger, 2005).

The cytotoxic effects of HY are generally considered to be oxygen- and light-dependent (Huygens et al., 2005), as the absolute elimination of HY photocytotoxicity in a hypoxic environment (Delaey et al., 2000) together with the absence of effect on mitochondrial function have been documented (Utsumi et al., 1995). On the other hand, light-independent inhibition of some enzymes (Johnson & Pardini, 1998) as well as anti-metastatic and cytotoxic activity of HY in the dark have been demonstrated both *in vitro* (Blank et al., 2001) and *in vivo* (Blank et al., 2004). However, the light-independent action of HY generally requires markedly higher doses. The significance of proper light regime has also been suggested by us (Kuliková et al., 2010; Sačková et al., 2005) and it is now beyond doubt that low light doses induce photo-tolerance. Discontinuity time proved to be crucial.

The mode of cell death may be significantly governed by HY uptake and intracellular localization, too. It is mostly reported as localizing in the endoplasmic reticulum and/or Golgi apparatus, as well as in the lysosomes and mitochondria (Agostinis et al., 2002; Kaščáková et al., 2008). For this reason, rapid loss of $\Delta\Psi_m$, subsequent cytochrome *c* release, caspase-3 activation and apoptosis all occur as a result of PDT with hypericin (HY-PDT). Since the photocytotoxic action of HY represents a massive impact on various cellular targets, cytochrome *c* release as well as caspase-3 activation and apoptosis can be suppressed, for example in cells over-expressing Bcl-2, but not $\Delta\Psi$m loss (Hadjur et al., 1996; Vantieghem et al., 2001).

Although cells sensitized by activated HY show all of the elementary signs of apoptosis, recent studies have revealed that cell death may proceed *via* both caspase-dependent or -independent pathways. Initial experiments linked HY-induced apoptosis with inhibition of protein kinase C (PKC) (Couldwell et al., 1994); however, inhibition of PKC was later proven to be insufficient to cause apoptosis (Weller et al., 1997). On the other hand, HY also activates rescuing responses, chiefly governed by activation of p38MAPK (Hendrickx et al., 2003) and the genes that are under its control (Buytaert et al., 2008; Chan et al., 2009).

Hypericin's cytotoxicity or photocytotoxicity may also be a result of its interaction with expression and/or activity of some specific enzymes. Some of them, like PI3K, PKC, protein tyrosine kinase activities (PTK) of the epidermal growth factor receptor (EGF-R) and the insulin receptor are closely related to tumourigenesis, survival or proliferation regulation. The Ser/Thr protein kinases (e.g. protein kinase CK-2 or mitogen-activated kinase) are also extremely sensitive to inhibition even in nanomolar concentrations, and have also proved to be irreversible after irradiation (Agostinis et al., 1995). As the light-dependent action of HY is based on induction of oxidative stress, the action of antioxidant enzymes has been tested *in vivo*. The inhibition of glutathione reductase was highly effective even in the nanomolar range of HY, whether light-activated or not (Johnson & Pardini, 1998). The inhibition of selenium-dependent glutathione peroxidase, glutathione S-transferase and superoxide dismutase proved to be efficient in micromolar concentrations and light-dependent.

Evaluation of the inhibitory effect of St. John's Wort towards human cytochromes P450 (CYP) has revealed possible interactions of its constituents. HY *per se* proved to be a competitive inhibitor of CYP2C9, CYP2D6 and CYP3A4 with IC50 below 10 μM (Obach, 2000). Besides CYP3A4, the inhibition of P-glycoprotein (P-gp) has also been intensively studied (Pal & Mitra, 2006), since both participate significantly in multidrug resistance fenotype of many tumours. Our recently-published results show that HY could be a substrate of another ABC-transporting protein, the BCRP (ABCG2) (Jendželovský et al., 2009). We demonstrated that HY affects the expression of these proteins without activation as well.

3.3 The impact of Akt pathway on breast cancer therapy

Akt kinases are downstream components of PI3K derived signals from receptor tyrosine kinases (RTK). It is also the major convergence point for RTK signalling in breast cancer. Several studies have found Akt2 to be amplified or overexpressed at the mRNA level in various tumour cell lines (Miwa et al., 1996) and in a number of human malignancies, such as colon, pancreatic and breast cancers (Bacus et al., 2002; Roy et al., 2002). However, activation of Akt1, Akt2 and Akt3 by phosphorylation appears to be more clinically relevant than detection of Akt2 amplification or overexpression (Cicenas et al., 2005).

Generally, Akt kinase can regulate the proliferation, metabolism as well as survival of cancer cells by modulation of various signalling molecules. The role of Akt protein in surviving cells through inhibition of apoptotic protein suggests that Akt activity may influence the sensitivity of tumour cells to chemotherapy. There have been many studies showing the correlation between chemoresistance and level of phosforylation of Akt in tumours. In their study Cicenas et al. (2005) found that high levels of phosphorylated Akt correlated with poor prognosis in primary breast cancer, and the significance of this correlation increased in the subset of patients with HER2 overexpressing tumours. Moreover phosphorylated Akt contributes to the development of breast cancer, so inhibiting the phosphorylation process could provide a new therapeutic approach (Kucab et al., 2005).

Important data about the role of Akt in cancer cell motility were produced in the study by Yoeli-Lerner et al. (2005), where activation of Akt inhibited carcinoma migration and invasion by breast cancer cells. Their results indicate that Akt can promote tumour progression through increased cell survival mechanism, and it can block breast cancer cell motility and invasion by a mechanism that depends, at least in part, on the nuclear factor of activated T-cells.

The Ras cascade as well as Akt pathways have a major impact on regulation of apoptosis, and moreover they are mutually linked (McCubrey et al., 2006). Both Erk1/2 and EGFR-PI3K-Akt pathways seem to be involved in cellular survival after PDT. The effect of PDT is associated with inactivation of the EGFR-PI3K-Akt pathway. Since EGFR inhibitors and PDT act synergistically, this combination is highly relevant for clinical use (Martinez-Carpio & Trelles, 2010).

Molecule	Modifications	Cell line	References
Akt	inhibition	HT-29	Sačková et al., 2006
	activation/ inhibition	human dermal fibroblasts	Schieke et al., 2004
	activation	BA, BT-474, NIH 3T3, MCF-7	Bozkulak et al., 2007, Zhuang and Kochevar, 2003, Ferenc et al., 2010
	depletion	SKBR-3	Solár et al., 2011
Erk	irreversible inhibition	A431, HaCaT, L929, HeLa	Assefa et al., 1999
	moderate attenuation	human dermal fibroblasts	Schieke et al., 2004
	insignificant modulation	LY-R	Xue et al., 1999
	transient activation	LFS087, GM38A	Tong et al., 2002
	no effect	HaCaT	Klotz et al., 1998
	inhibition /depletion	NCTC 2544	Silva et al., 2010
	activation	MCF-7	Ferenc et al., 2010
	depletion	SKBR-3	Solár et al., 2011

Table 1. Regulation of Akt and Erk by PDT.

A downstream event in the mitogenic Ras pathway is Erk activation through binding of ligands to extracellular growth factor receptors involved in regulation of growth and cell cycle progression. The Ras/Raf/Erk activation pathway can promote opposite prosurvival or anti-proliferative cellular responses, such as apoptosis and autophagy. This wide variety of processes triggered by the activation of a single pathway depends on the timing, duration and strength of activation, on subcellular localization and on the presence of ROS (Cagnol & Chambard, 2010). It is known that ROS induce activation of Ras cascade with increased Erk1/2 activity in various type of cells as a consequence of oxidative stress (Conde de la Rosa et al., 2006).

Available data suggest that the photooxidative stress induced by PDT may modulate Erk activity as does other ROS such as H_2O_2, which is produced in a variety of tumour cell lines by 1,3-dibutyl-2-thiooxoimidazolidine- 4,5-dione (Wong et al., 2010). Decreased phosphorylation status of Akt at Ser 473 without change in Akt level in MCF-7 and MDA-MB-231 cell lines, was observed after application of GE (Chinni et al., 2003). Moreover, GE eliminated irradiation-induced activation of Akt and Erk1/2 (Akimoto et al., 2001).

Application of GE or HY-PDT alone in the study by Ferenc et al. (2010) demonstrated both types of reaction; stimulated Akt and Erk1/2 phosphorylation in MCF-7 cells as well as no effect (Erk1/2; PDT) or even dephosphorylation in MDA-MB-231 cells. Moreover pre-treatment with GE prior to PDT led to suppression of phosphorylation status of Akt and Erk1/2 in both cell lines. Furthermore, Akt protein levels depleted after HY-PDT with GE pre-treatment did not correlated well with mRNA level, which was unaffected. Theoretically, post-translation modification of Akt and Erk1/2 could be partly responsible for effective reduction of proliferarion and clonogenic ability as well as induction of apoptosis recorded in breast adenocarcinoma cells (Ferenc et al., 2010).

One interesting fact revealed in another study (Solár et al., 2011) was a drop in Akt and Erk1/2 activity after elevated oxidative stress achieved by high dose of HY-PDT. Using such high oxidative stress, the upstream molecular target of Erk kinase could be damaged, which might as a final result prevent activation of Erk protein (Lee et al., 2006).

It is well recognized that the majority of cancer-related deaths, including those from breast cancer, is caused by metastatic diseases. To date many new genes and signal pathways involved in this process have been identified. Some genes hold great promise as potential drug targets. Reactivation of metastasis-suppressor genes and their signal pathways such as MKK/JNK, PTEN/Akt and NDRG/ATF is also a rational strategy (Iiizumi et al., 2008).

In accord with the PTEN studies undertaken in the last decade, we would like to point out the very important role of lipid phosphatase in suppression of tumour growth. One of the functions of this tumour suppressor protein is related to negative control of the PI3K/Akt signalling pathway, through dephosphorylation of phosphatidilinositol 3,4,5-triphosphate. Dave et al. (2005) detected induction of apoptosis with elevation of PTEN gene expression in the MCF-7 cell line after application of GE. Furthermore, induced programmed cell death was blocked by using PTEN siRNA. DeGraffenried et al. (2004) observed an interesting result when they detected increased levels of Akt phosphorylation after inhibition of PTEN gene expression. These results were also confirmed by Kikuno et al. (2008) when the elevation of PTEN expression caused silencing of Akt activity. In this regard, significant increases in PTEN expression (MDA-MB-231) and PTEN protein levels have been recorded, and simultaneously decreased phosphorylation of Ser380, Thr382 and Thr383 (important for PTEN protein opening, its translocation to membrane structure

and inhibition of PI3K) has been found after PDT with GE pre-treatment (Ferenc, unpublished data).

3.4 HER2 and photodynamic therapy

An alternative form of treatment, at least for chest wall recurrence of breast carcinoma, is PDT. Allison et al. (2001) succeeded in using PDT to control recurrent breast cancer that had failed to respond to conventional therapy. PDT offers patients with chest wall progression a treatment option with an excellent clinical response and allows opportunities for good long-term local tumour control (Cuenca et al., 2004). One experimental study with ALA-PDT resulted in the downregulation of EGFR mRNA as well as protein levels in a treatment-cycle and light-dose dependent manner in CL1-5, A375 and MDA-MB-231 cells (Tsai et al., 2009). Our recent study showed a decline in HER2 mRNA levels a short time after photoactivation of HY in breast adenocarcinoma cell lines, but no changes in HER2 mRNA were found in dark conditions (Solár et al., 2011). Furthermore, we have also demonstrated HY-PDT mediated degradation of HER2 receptor *via* lysosomal activity (Kovaľ et al., 2010). The efficacy of PDT may be increased using combinations of PDT and anti-VEGF antibody (Bhuvaneswari et al., 2007), or of PDT and EGFR inhibitor (Kovaľ et al., 2010; Weyergang et al., 2008), or using a triple combination of PDT + VEGF inhibitor + EGFR inhibitor. More investigations in animal models to evaluate the efficacy and safety of these combinations are needed (Martinez-Carpio & Trelles, 2010).

4. Conclusion

The aim of this chapter was to summarize the current therapeutic approaches to breast cancer with regard to alternative methods such as PDT. Although great advances have been made during the last 20 years in the treatment of breast cancer and the number of deaths has fallen since the late 1980s, no significant improvement in the survival rates of patients with distant metastases have been observed. The inability to inhibit the resistance of cancer cells, or the development of metastases that may result in the death of the patient represent the principal problems linked with the management of breast cancers. PDT is a relatively new method used for destruction of cutaneous malignancies, but it has been found to be highly efficient against recurrent breast cancer cells. Painless and repeatable treatment is one of the benefits accompanying PDT, which may be used with other regimens or as a single therapy. Significant results in tumour therapies are rarely achieved by the application of a single therapeutic method, and combinations of variable approaches with different mechanisms of action are commonly more efficient. For example, the conjunction of PDT with pharmacological modulators of signalling pathways can either enhance injury of malignant cells, or protect surrounding normal cells.

5. Acknowledgement

This work was supported by the Slovak Research and Development Agency under contract nos. VVCE-0001-07 and APVV-0321-07; the Scientific Grant Agency of the Ministry of Education of the Slovak Republic under contract nos. VEGA 1/0240/08, VEGA 1/0475/10 and VEGA 1/0296/09; and the NEXO 2 (Network of Excellence in Oncology) under contract no. 049/2009/2.1/OPVaV. Thanks are also due to Andrew J. Billingham for proofreading the manuscript.

6. References

Agostinis, P., Vandenbogaerde, A., Donella-Deana, A., Pinna, L.A., Lee, K.T., Goris, J., Merlevede, W., Vandenheede, J.R. & De Witte, P. (1995). Photosensitized inhibition of growth factor-regulated protein kinases by hypericin. *Biochem Pharmacol*, Vol.49, No.11, pp. 1615-1622. ISSN 0006-2952

Agostinis, P., Vantieghem, A., Merlevede, W. & de Witte, P.A. (2002). Hypericin in cancer treatment: more light on the way. *Int J Biochem Cell Biol*, Vol.34, No.3, pp. 221-241. ISSN 1357-2725

Ahmad, N. & Mukhtar, H. (2000). Mechanism of photodynamic therapy-induced cell death. *Methods Enzymol*, Vol.319, pp. 342-358. ISSN 0076-6879

Akimoto, T., Nonaka, T., Ishikawa, H., Sakurai, H., Saitoh, J.I., Takahashi, T. & Mitsuhashi, N. (2001). Genistein, a tyrosine kinase inhibitor, enhanced radiosensitivity in human esophageal cancer cell lines in vitro: possible involvement of inhibition of survival signal transduction pathways. *Int J Radiat Oncol Biol Phys*, Vol.50, No.1, pp. 195-201. ISSN 0360-3016

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. & Fukami, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem*, Vol.262, No.12, pp. 5592-5595. ISSN 0021-9258

Allison, R., Mang, T., Hewson, G., Snider, W. & Dougherty, D. (2001). Photodynamic therapy for chest wall progression from breast carcinoma is an underutilized treatment modality. *Cancer*, Vol.91, No.1, pp. 1-8. ISSN 0008-543X

Almeida, R.D., Manadas, B.J., Carvalho, A.P. & Duarte, C.B. (2004). Intracellular signaling mechanisms in photodynamic therapy. *Biochim Biophys Acta*, Vol.1704, No.2, pp. 59-86. ISSN 0006-3002

Assefa, Z., Vantieghem, A., Declercq, W., Vandenabeele, P., Vandenheede, J.R., Merlevede, W., de Witte, P. & Agostinis, P. (1999). The activation of the c-Jun N-terminal kinase and p38 mitogen-activated protein kinase signaling pathways protects HeLa cells from apoptosis following photodynamic therapy with hypericin. *J Biol Chem*, Vol.274, No.13, pp. 8788-8796. ISSN 0021-9258

Bacus, S.S., Altomare, D.A., Lyass, L., Chin, D.M., Farrell, M.P., Gurova, K., Gudkov, A. & Testa, J.R. (2002). AKT2 is frequently upregulated in HER-2/neu-positive breast cancers and may contribute to tumor aggressiveness by enhancing cell survival. *Oncogene*, Vol.21, No.22, pp. 3532-3540. ISSN 0950-9232

Bedard, P.L. & Cardoso, F. (2008). Recent advances in adjuvant systemic therapy for early-stage breast cancer. *Ann Oncol*, Vol.19, Suppl 5, pp. v122-127. ISSN 1569-8041

Belletti, B., Vaidya, J.S., D'Andrea, S., Entschladen, F., Roncadin, M., Lovat, F., Berton, S., Perin, T., Candiani, E., Reccanello, S., Veronesi, A., Canzonieri, V., Trovo, M.G., Zaenker, K.S., Colombatti, A., Baldassarre, G. & Massarut, S. (2008). Targeted intraoperative radiotherapy impairs the stimulation of breast cancer cell proliferation and invasion caused by surgical wounding. *Clin Cancer Res*, Vol.14, No.5, pp. 1325-1332. ISSN 1078-0432

Berg, K., Madslien, K., Bommer, J.C., Oftebro, R., Winkelman, J.W. & Moan, J. (1991). Light induced relocalization of sulfonated meso-tetraphenylporphines in NHIK 3025 cells and effects of dose fractionation. *Photochem Photobiol*, Vol.53, No.2, pp. 203-210. ISSN 0031-8655

Berg, K., Selbo, P.K., Weyergang, A., Dietze, A., Prasmickaite, L., Bonsted, A., Engesaeter, B.O., Angell-Petersen, E., Warloe, T., Frandsen, N. & Hogset, A. (2005). Porphyrin-related photosensitizers for cancer imaging and therapeutic applications. *J Microsc*, Vol.218, No.Pt 2, pp. 133-147. ISSN 0022-2720

Berry, D.A., Cronin, K.A., Plevritis, S.K., Fryback, D.G., Clarke, L., Zelen, M., Mandelblatt, J.S., Yakovlev, A.Y., Habbema, J.D. & Feuer, E.J. (2005). Effect of screening and adjuvant therapy on mortality from breast cancer. *N Engl J Med*, Vol.353, No.17, pp. 1784-1792. ISSN 1533-4406

Beslija, S., Bonneterre, J., Burstein, H., Cocquyt, V., Gnant, M., Goodwin, P., Heinemann, V., Jassem, J., Kostler, W.J., Krainer, M., Menard, S., Petit, T., Petruzelka, L., Possinger, K., Schmid, P., Stadtmauer, E., Stockler, M., Van Belle, S., Vogel, C., Wilcken, N., Wiltschke, C., Zielinski, C.C. & Zwierzina, H. (2007). Second consensus on medical treatment of metastatic breast cancer. *Ann Oncol*, Vol.18, No.2, pp. 215-225. ISSN 0923-7534

Bhuvaneswari, R., Yuen, G.Y., Chee, S.K. & Olivo, M. (2007). Hypericin-mediated photodynamic therapy in combination with Avastin (bevacizumab) improves tumor response by downregulating angiogenic proteins. *Photochem Photobiol Sci*, Vol.6, No.12, pp. 1275-1283. ISSN 1474-905X

Blank, M., Mandel, M., Hazan, S., Keisari, Y. & Lavie, G. (2001). Anti-cancer activities of hypericin in the dark. *Photochem Photobiol*, Vol.74, No.2, pp. 120-125. ISSN 0031-8655

Blank, M., Lavie, G., Mandel, M., Hazan, S., Orenstein, A., Meruelo, D. & Keisari, Y. (2004). Antimetastatic activity of the photodynamic agent hypericin in the dark. *Int J Cancer*, Vol.111, No.4, pp. 596-603. ISSN 0020-7136

Bozkulak, O., Wong, S., Luna, M., Ferrario, A., Rucker, N., Gulsoy, M. & Gomer, C.J. (2007). Multiple components of photodynamic therapy can phosphorylate Akt. *Photochem Photobiol*, Vol.83, No.5, pp. 1029-1033. ISSN 0031-8655

Bryant, H.E., Schultz, N., Thomas, H.D., Parker, K.M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N.J. & Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature*, Vol.434, No.7035, pp. 913-917. ISSN 1476-4687

Buytaert, E., Callewaert, G., Hendrickx, N., Scorrano, L., Hartmann, D., Missiaen, L., Vandenheede, J.R., Heirman, I., Grooten, J. & Agostinis, P. (2006a). Role of endoplasmic reticulum depletion and multidomain proapoptotic BAX and BAK proteins in shaping cell death after hypericin-mediated photodynamic therapy. *Faseb J*, Vol.20, No.6, pp. 756-758. ISSN 1530-6860

Buytaert, E., Callewaert, G., Vandenheede, J.R. & Agostinis, P. (2006b). Deficiency in apoptotic effectors Bax and Bak reveals an autophagic cell death pathway initiated by photodamage to the endoplasmic reticulum. *Autophagy*, Vol.2, No.3, pp. 238-240. ISSN 1554-8627

Buytaert, E., Dewaele, M. & Agostinis, P. (2007). Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. *Biochim Biophys Acta*, Vol.1776, No.1, pp. 86-107. ISSN 0006-3002

Buytaert, E., Matroule, J.Y., Durinck, S., Close, P., Kocanova, S., Vandenheede, J.R., de Witte, P.A., Piette, J. & Agostinis, P. (2008). Molecular effectors and modulators of hypericin-mediated cell death in bladder cancer cells. *Oncogene*, Vol.27, No.13, pp.1916-1929. ISSN 0950-9232

Cagnol, S. & Chambard, J.C. (2010). ERK and cell death: mechanisms of ERK-induced cell death--apoptosis, autophagy and senescence. *Febs J*, Vol.277, No.1, pp. 2-21. ISSN 1742-4658

Cardoso, F., Van't Veer, L., Rutgers, E., Loi, S., Mook, S. & Piccart-Gebhart, M.J. (2008). Clinical application of the 70-gene profile: the MINDACT trial. *J Clin Oncol*, Vol.26, No.5, pp. 729-735. ISSN 1527-7755

Cicenas, J., Urban, P., Vuaroqueaux, V., Labuhn, M., Kung, W., Wight, E., Mayhew, M., Eppenberger, U. & Eppenberger-Castori, S. (2005). Increased level of phosphorylated akt measured by chemiluminescence-linked immunosorbent assay is a predictor of poor prognosis in primary breast cancer overexpressing ErbB-2. *Breast Cancer Res*, Vol.7, No.4, pp. R394-401. ISSN 1465-542X

Conde de la Rosa, L., Schoemaker, M.H., Vrenken, T.E., Buist-Homan, M., Havinga, R., Jansen, P.L. & Moshage, H. (2006). Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: involvement of JNK and ERK MAP kinases. *J Hepatol*, Vol.44, No.5, pp. 918-929. ISSN 0168-8278

Constantinou, A.I., Kamath, N. & Murley, J.S. (1998). Genistein inactivates bcl-2, delays the G2/M phase of the cell cycle, and induces apoptosis of human breast adenocarcinoma MCF-7 cells. *Eur J Cancer*, Vol.34, No.12, pp. 1927-1934. ISSN 0959-8049

Couldwell, W.T., Gopalakrishna, R., Hinton, D.R., He, S., Weiss, M.H., Law, R.E. & Apuzzo, M.L. (1994). Hypericin: a potential antiglioma therapy. *Neurosurgery*, Vol.35, No.4, pp. 705-709; discussion 709-710. ISSN 0148-396X

Cuenca, R.E., Allison, R.R., Sibata, C. & Downie, G.H. (2004). Breast cancer with chest wall progression: treatment with photodynamic therapy. *Ann Surg Oncol*, Vol.11, No.3, pp. 322-327. ISSN 1068-9265

Čavarga, I., Brezáni, P., Fedoročko, P., Miškovský, P., Bobrov, N., Longauer, F., Rybárová, S., Miroššay, L. & Štubňa, J. (2005). Photoinduced antitumour effect of hypericin can be enhanced by fractionated dosing. *Phytomedicine*, Vol.12, No.9, pp. 680-683. ISSN 0944-7113

Dave, B., Eason, R.R., Till, S.R., Geng, Y., Velarde, M.C., Badger, T.M. & Simmen, R.C. (2005). The soy isoflavone genistein promotes apoptosis in mammary epithelial cells by inducing the tumor suppressor PTEN. *Carcinogenesis*, Vol.26, No.10, pp. 1793-1803. ISSN 0143-3334

DeGraffenried, L.A., Fulcher, L., Friedrichs, W.E., Grunwald, V., Ray, R.B. & Hidalgo, M. (2004). Reduced PTEN expression in breast cancer cells confers susceptibility to inhibitors of the PI3 kinase/Akt pathway. *Ann Oncol*, Vol.15, No.10, pp. 1510-1516. ISSN 0923-7534

Delaey, E., Vandenbogaerde, A., Merlevede, W. & de Witte, P. (2000). Photocytotoxicity of hypericin in normoxic and hypoxic conditions. *J Photochem Photobiol B*, Vol.56, No.1, pp. 19-24. ISSN 1011-1344

Demaria, S. & Formenti, S.C. (2007). Sensors of ionizing radiation effects on the immunological microenvironment of cancer. *Int J Radiat Biol*, Vol.83, No.11-12, pp. 819-825. ISSN 0955-3002

du Manoir, J.M., Francia, G., Man, S., Mossoba, M., Medin, J.A., Viloria-Petit, A., Hicklin, D.J., Emmenegger, U. & Kerbel, R.S. (2006). Strategies for delaying or treating in

vivo acquired resistance to trastuzumab in human breast cancer xenografts. *Clin Cancer Res*, Vol.12, No.3 Pt 1, pp. 904-916. ISSN 1078-0432

Ferenc, P., Solár, P., Kleban, J., Mikeš, J. & Fedoročko, P. (2010). Down-regulation of Bcl-2 and Akt induced by combination of photoactivated hypericin and genistein in human breast cancer cells. *J Photochem Photobiol B*, Vol.98, No.1, pp. 25-34. ISSN 1873-2682

Fernando, I.N. (2000). The role of radiotherapy in patients undergoing mastectomy for carcinoma of the breast. *Clin Oncol (R Coll Radiol)*, Vol.12, No.3, pp. 158-165. ISSN 0936-6555

Festjens, N., Vanden Berghe, T. & Vandenabeele, P. (2006). Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response. *Biochim Biophys Acta*, Vol.1757, No.9-10, pp. 1371-1387. ISSN 0006-3002

Fisher, B., Costantino, J.P., Redmond, C.K., Fisher, E.R., Wickerham, D.L. & Cronin, W.M. (1994). Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. *J Natl Cancer Inst*, Vol.86, No.7, pp. 527-537. ISSN 0027-8874

Formenti, S.C. & Demaria, S. (2008). Local control by radiotherapy: is that all there is? *Breast Cancer Res*, Vol.10, No.6, pp. 215. ISSN 1465-542X

Forrest, A.P. (1982). Beatson: hormones and the management of breast cancer. *J R Coll Surg Edinb*, Vol.27, No.5, pp. 253-263. ISSN 0035-8835

Fritsch, C. & Ruzicka, T. (2006). Fluorescence diagnosis and photodynamic therapy in dermatology from experimental state to clinic standard methods. *J Environ Pathol Toxicol Oncol*, Vol.25, No.1-2, pp. 425-439. ISSN 0731-8898

Gilbert, F.J. (2008). Breast cancer screening in high risk women. *Cancer Imaging*, Vol.8, Spec No A, pp. S6-9. ISSN 1470-7330

Hadjur, C., Richard, M.J., Parat, M.O., Jardon, P. & Favier, A. (1996). Photodynamic effects of hypericin on lipid peroxidation and antioxidant status in melanoma cells. *Photochem Photobiol*, Vol.64, No.2, pp. 375-381. ISSN 0031-8655

Haffty, B.G., Hauser, A., Choi, D.H., Parisot, N., Rimm, D., King, B. & Carter, D. (2004). Molecular markers for prognosis after isolated postmastectomy chest wall recurrence. *Cancer*, Vol.100, No.2, pp. 252-263. ISSN 0008-543X

Harada, N. (1997). Aberrant expression of aromatase in breast cancer tissues. *J Steroid Biochem Mol Biol*, Vol.61, No.3-6, pp. 175-184. ISSN 0960-0760

Hellerhoff, K. (2010). [Digital breast tomosynthesis: technical principles, current clinical relevance and future perspectives]. *Radiologe*, Vol.50, No.11, pp. 991-998. ISSN 1432-2102

Hendrickx, N., Volanti, C., Moens, U., Seternes, O.M., de Witte, P., Vandenheede, J.R., Piette, J. & Agostinis, P. (2003). Up-regulation of cyclooxygenase-2 and apoptosis resistance by p38 MAPK in hypericin-mediated photodynamic therapy of human cancer cells. *J Biol Chem*, Vol.278, No.52, pp. 52231-52239. ISSN 0021-9258

Hopper, C. (1996). The role of photodynamic therapy in the management of oral cancer and precancer. *Eur J Cancer B Oral Oncol*, Vol.32B, No.2, pp. 71-72. ISSN 0964-1955

Huygens, A., Kamuhabwa, A.R., Van Laethem, A., Roskams, T., Van Cleynenbreugel, B., Van Poppel, H., Agostinis, P. & De Witte, P.A. (2005). Enhancing the photodynamic effect of hypericin in tumour spheroids by fractionated light delivery in

combination with hyperoxygenation. *Int J Oncol*, Vol.26, No.6, pp. 1691-1697. ISSN 1019-6439

Chakraborty, M., Abrams, S.I., Camphausen, K., Liu, K., Scott, T., Coleman, C.N. & Hodge, J.W. (2003). Irradiation of tumor cells up-regulates Fas and enhances CTL lytic activity and CTL adoptive immunotherapy. *J Immunol*, Vol.170, No.12, pp. 6338-6347. ISSN 0022-1767

Chaloupka, R., Obsil, T., Plasek, J. & Sureau, F. (1999). The effect of hypericin and hypocrellin-A on lipid membranes and membrane potential of 3T3 fibroblasts. *Biochim Biophys Acta*, Vol.1418, No.1, pp. 39-47. ISSN 0006-3002

Chan, P.S., Koon, H.K., Wu, Z.G., Wong, R.N., Lung, M.L., Chang, C.K. & Mak, N.K. (2009). Role of p38 MAPKs in hypericin photodynamic therapy-induced apoptosis of nasopharyngeal carcinoma cells. *Photochem Photobiol*, Vol.85, No.5, pp. 1207-1217. ISSN 0031-8655

Chan, S., Scheulen, M.E., Johnston, S., Mross, K., Cardoso, F., Dittrich, C., Eiermann, W., Hess, D., Morant, R., Semiglazov, V., Borner, M., Salzberg, M., Ostapenko, V., Illiger, H.J., Behringer, D., Bardy-Bouxin, N., Boni, J., Kong, S., Cincotta, M. & Moore, L. (2005). Phase II study of temsirolimus (CCI-779), a novel inhibitor of mTOR, in heavily pretreated patients with locally advanced or metastatic breast cancer. *J Clin Oncol*, Vol.23, No.23, pp. 5314-5322. ISSN 0732-183X

Chinni, S.R., Alhasan, S.A., Multani, A.S., Pathak, S. & Sarkar, F.H. (2003). Pleotropic effects of genistein on MCF-7 breast cancer cells. *Int J Mol Med*, Vol.12, No.1, pp. 29-34. ISSN 1107-3756

Chumsri, S., Howes, T., Bao, T., Sabnis, G. & Brodie, A. (2011). Aromatase, aromatase inhibitors, and breast cancer. *J Steroid Biochem Mol Biol*, Vol.125, No.1-2, pp. 13-22.

Iiizumi, M., Liu, W., Pai, S.K., Furuta, E. & Watabe, K. (2008). Drug development against metastasis-related genes and their pathways: a rationale for cancer therapy. *Biochim Biophys Acta*, Vol.1786, No.2, pp. 87-104. ISSN 0006-3002

Iwasaki, M., Inoue, M., Otani, T., Sasazuki, S., Kurahashi, N., Miura, T., Yamamoto, S. & Tsugane, S. (2008). Plasma isoflavone level and subsequent risk of breast cancer among Japanese women: a nested case-control study from the Japan Public Health Center-based prospective study group. *J Clin Oncol*, Vol.26, No.10, pp. 1677-1683. ISSN 1527-7755

Jacobson, J.M., Feinman, L., Liebes, L., Ostrow, N., Koslowski, V., Tobia, A., Cabana, B.E., Lee, D., Spritzler, J. & Prince, A.M. (2001). Pharmacokinetics, safety, and antiviral effects of hypericin, a derivative of St. John's wort plant, in patients with chronic hepatitis C virus infection. *Antimicrob Agents Chemother*, Vol.45, No.2, pp. 517-524. ISSN 0066-4804

James, C.R., Quinn, J.E., Mullan, P.B., Johnston, P.G. & Harkin, D.P. (2007). BRCA1, a potential predictive biomarker in the treatment of breast cancer. *Oncologist*, Vol.12, No.2, pp. 142-150. ISSN 1083-7159

Jang, E.R., Lim, S.J., Lee, E.S., Jeong, G., Kim, T.Y., Bang, Y.J. & Lee, J.S. (2004). The histone deacetylase inhibitor trichostatin A sensitizes estrogen receptor alpha-negative breast cancer cells to tamoxifen. *Oncogene*, Vol.23, No.9, pp. 1724-1736. ISSN 0950-9232

Jendželovský, R., Mikeš, J., Kovaľ, J., Souček, K., Procházková, J., Kello, M., Sačková, V., Hofmanová, J., Kozubík, A. & Fedoročko, P. (2009). Drug efflux transporters, MRP1

and BCRP, affect the outcome of hypericin-mediated photodynamic therapy in HT-29 adenocarcinoma cells. *Photochem Photobiol Sci*, Vol.8, No.12, pp. 1716-1723. ISSN 1474-9092

Jichlinski, P. & Leisinger, H.J. (2005). Fluorescence cystoscopy in the management of bladder cancer: a help for the urologist! *Urol Int*, Vol.74, No.2, pp. 97-101. ISSN 0042-1138

Johnson, S.A. & Pardini, R.S. (1998). Antioxidant enzyme response to hypericin in EMT6 mouse mammary carcinoma cells. *Free Radic Biol Med*, Vol.24, No.5, pp. 817-826. ISSN 0891-5849

Jordan, V.C. (1995). Tamoxifen: toxicities and drug resistance during the treatment and prevention of breast cancer. *Annu Rev Pharmacol Toxicol*, Vol.35, pp. 195-211. ISSN 0362-1642

Kaščáková, S., Naďová, Z., Mateasik, A., Mikeš, J., Huntošová, V., Refregiers, M., Sureau, F., Maurizot, J.C., Miškovský, P. & Jancura, D. (2008). High level of low-density lipoprotein receptors enhance hypericin uptake by U-87 MG cells in the presence of LDL. *Photochem Photobiol*, Vol.84, No.1, pp. 120-127. ISSN 0031-8655

Kessel, D., Luo, Y., Deng, Y. & Chang, C.K. (1997). The role of subcellular localization in initiation of apoptosis by photodynamic therapy. *Photochem Photobiol*, Vol.65, No.3, pp. 422-426. ISSN 0031-8655

Kikuno, N., Shiina, H., Urakami, S., Kawamoto, K., Hirata, H., Tanaka, Y., Majid, S., Igawa, M. & Dahiya, R. (2008). Genistein mediated histone acetylation and demethylation activates tumor suppressor genes in prostate cancer cells. *Int J Cancer*, Vol.123, No.3, pp. 552-560. ISSN 1097-0215

Klotz, L.O., Fritsch, C., Briviba, K., Tsacmacidis, N., Schliess, F. & Sies, H. (1998). Activation of JNK and p38 but not ERK MAP kinases in human skin cells by 5-aminolevulinate-photodynamic therapy. *Cancer Res*, Vol.58, No.19, pp. 4297-4300. ISSN 0008-5472

Kovaľ, J., Mikeš, J., Jendželovský, R., Kello, M., Solár, P. & Fedoročko, P. (2010). Degradation of HER2 receptor through hypericin-mediated photodynamic therapy. *Photochem Photobiol*, Vol.86, No.1, pp. 200-205. ISSN 1751-1097

Kucab, J.E., Lee, C., Chen, C.S., Zhu, J., Gilks, C.B., Cheang, M., Huntsman, D., Yorida, E., Emerman, J., Pollak, M. & Dunn, S.E. (2005). Celecoxib analogues disrupt Akt signaling, which is commonly activated in primary breast tumours. *Breast Cancer Res*, Vol.7, No.5, pp. R796-807. ISSN 1465-542X

Kuliková, L., Mikeš, J., Hýždalová, M., Palumbo, G. & Fedoročko, P. (2010). NF-kappaB is not directly responsible for photoresistance induced by fractionated light delivery in HT-29 colon adenocarcinoma cells. *Photochem Photobiol*, Vol.86, No.6, pp. 1285-1293. ISSN 1751-1097

Lavie, G., Meruelo, D., Aroyo, K. & Mandel, M. (2000). Inhibition of the CD8+ T cell-mediated cytotoxicity reaction by hypericin: potential for treatment of T cell-mediated diseases. *Int Immunol*, Vol.12, No.4, pp. 479-486. ISSN 0953-8178

Ledo, E. & Ledo, A. (2000). Phototherapy, photochemotherapy, and photodynamic therapy: unapproved uses or indications. *Clin Dermatol*, Vol.18, No.1, pp. 77-86. ISSN 0738-081X

Lee, J.S., Kim, S.Y., Kwon, C.H. & Kim, Y.K. (2006). EGFR-dependent ERK activation triggers hydrogen peroxide-induced apoptosis in OK renal epithelial cells. *Arch Toxicol*, Vol.80, No.6, pp. 337-346. ISSN 0340-5761

Lee, W.R., Shen, S.C., Lin, H.Y., Hou, W.C., Yang, L.L. & Chen, Y.C. (2002). Wogonin and fisetin induce apoptosis in human promyeloleukemic cells, accompanied by a decrease of reactive oxygen species, and activation of caspase 3 and Ca(2+)-dependent endonuclease. *Biochem Pharmacol*, Vol.63, No.2, pp. 225-236. ISSN 0006-2952

Leopold, E. (1999). *A Darker Ribbon: Breast Cancer, Women, and their Doctors in the Twentieth Century.*, Beacon Press, ISBN 978-0807065136, Boston

Li, Z., Li, J., Mo, B., Hu, C., Liu, H., Qi, H., Wang, X. & Xu, J. (2008). Genistein induces cell apoptosis in MDA-MB-231 breast cancer cells via the mitogen-activated protein kinase pathway. *Toxicol In Vitro*, Vol.22, No.7, pp. 1749-1753. ISSN 0887-2333

Liu, S., Sugimoto, Y., Kulp, S.K., Jiang, J., Chang, H.L., Park, K.Y., Kashida, Y. & Lin, Y.C. (2002). Estrogenic down-regulation of protein tyrosine phosphatase gamma (PTP gamma) in human breast is associated with estrogen receptor alpha. *Anticancer Res*, Vol.22, No.6C, pp. 3917-3923. ISSN 0250-7005

Lopez-Tarruella, S. & Martin, M. (2009). Recent advances in systemic therapy: advances in adjuvant systemic chemotherapy of early breast cancer. *Breast Cancer Res*, Vol.11, No.2, art.no.204.. ISSN 1465-542X

Lu, Y., Zi, X., Zhao, Y., Mascarenhas, D. & Pollak, M. (2001). Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *J Natl Cancer Inst*, Vol.93, No.24, pp. 1852-1857. ISSN 0027-8874

Lugade, A.A., Sorensen, E.W., Gerber, S.A., Moran, J.P., Frelinger, J.G. & Lord, E.M. (2008). Radiation-induced IFN-gamma production within the tumor microenvironment influences antitumor immunity. *J Immunol*, Vol.180, No.5, pp. 3132-3139. ISSN 0022-1767

Marchal, S., Francois, A., Dumas, D., Guillemin, F. & Bezdetnaya, L. (2007). Relationship between subcellular localisation of Foscan and caspase activation in photosensitised MCF-7 cells. *Br J Cancer*, Vol.96, No.6, pp. 944-951. ISSN 0007-0920

Marini, H., Minutoli, L., Polito, F., Bitto, A., Altavilla, D., Atteritano, M., Gaudio, A., Mazzaferro, S., Frisina, A., Frisina, N., Lubrano, C., Bonaiuto, M., D'Anna, R., Cannata, M.L., Corrado, F., Cancellieri, F., Faraci, M., Marini, R., Adamo, E.B., Wilson, S. & Squadrito, F. (2008). OPG and sRANKL serum concentrations in osteopenic, postmenopausal women after 2-year genistein administration. *J Bone Miner Res*, Vol.23, No.5, pp. 715-720. ISSN 1523-4681

Martinez-Carpio, P.A. & Trelles, M.A. (2010). The role of epidermal growth factor receptor in photodynamic therapy: a review of the literature and proposal for future investigation. *Lasers Med Sci*, Vol.25, No.6, pp. 767-771. ISSN 1435-604X

Matsumura, S., Wang, B., Kawashima, N., Braunstein, S., Badura, M., Cameron, T.O., Babb, J.S., Schneider, R.J., Formenti, S.C., Dustin, M.L. & Demaria, S. (2008). Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells. *J Immunol*, Vol.181, No.5, pp. 3099-3107. ISSN 1550-6606

McCubrey, J.A., Steelman, L.S., Abrams, S.L., Lee, J.T., Chang, F., Bertrand, F.E., Navolanic, P.M., Terrian, D.M., Franklin, R.A., D'Assoro, A.B., Salisbury, J.L., Mazzarino, M.C., Stivala, F. & Libra, M. (2006). Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv Enzyme Regul*, Vol.46, No.1, pp. 249-279. ISSN 0065-2571

Messina, M., McCaskill-Stevens, W. & Lampe, J.W. (2006). Addressing the soy and breast cancer relationship: review, commentary, and workshop proceedings. *J Natl Cancer Inst*, Vol.98, No.18, pp. 1275-1284. ISSN 1460-2105

Miadoková, E., Chalupa, I., Vlčková, V., Ševčovičová, A., Naďová, S., Kopasková, M., Hercegová, A., Gašperová, P., Alfoldiová, L., Komjatiová, M., Czanyiová, Z., Gálová, E., Čellárová, E. & Vlček, D. (2009). Genotoxicity and antigenotoxicity evaluation of non-photoactivated hypericin. *Phytother Res*, Vol.24, No.1, pp. 90-95. ISSN 1099-1573

Mikeš, J., Kovaľ, J., Jendželovský, R., Sačková, V., Uhrínová, I., Kello, M., Kuliková, L. & Fedoročko, P. (2009). The role of p53 in the efficiency of photodynamic therapy with hypericin and subsequent long-term survival of colon cancer cells. *Photochem Photobiol Sci*, Vol.8, No.11, pp. 1558-1567. ISSN 1474-9092

Miles, D.W. (2009). Recent advances in systemic therapy. When HER2 is not the target: advances in the treatment of HER2-negative metastatic breast cancer. *Breast Cancer Res*, Vol.11, No.4, pp. 208. ISSN 1465-542X

Miller, K., Wang, M., Gralow, J., Dickler, M., Cobleigh, M., Perez, E.A., Shenkier, T., Cella, D. & Davidson, N.E. (2007). Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med*, Vol.357, No.26, pp. 2666-2676. ISSN 1533-4406

Miller, K.D., Chap, L.I., Holmes, F.A., Cobleigh, M.A., Marcom, P.K., Fehrenbacher, L., Dickler, M., Overmoyer, B.A., Reimann, J.D., Sing, A.P., Langmuir, V. & Rugo, H.S. (2005). Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. *J Clin Oncol*, Vol.23, No.4, pp. 792-799. ISSN 0732-183X

Miwa, W., Yasuda, J., Murakami, Y., Yashima, K., Sugano, K., Sekine, T., Kono, A., Egawa, S., Yamaguchi, K., Hayashizaki, Y. & Sekiya, T. (1996). Isolation of DNA sequences amplified at chromosome 19q13.1-q13.2 including the AKT2 locus in human pancreatic cancer. *Biochem Biophys Res Commun*, Vol.225, No.3, pp. 968-974. ISSN 0006-291X

Moan, J. & Berg, K. (1991). The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. *Photochem Photobiol*, Vol.53, No.4, pp. 549-553. ISSN 0031-8655

Morrow, P.K., Zambrana, F. & Esteva, F.J. (2009). Recent advances in systemic therapy: Advances in systemic therapy for HER2-positive metastatic breast cancer. *Breast Cancer Res*, Vol.11, No.4, art. no.207. ISSN 1465-542X

Muthyala, R.S., Ju, Y.H., Sheng, S., Williams, L.D., Doerge, D.R., Katzenellenbogen, B.S., Helferich, W.G. & Katzenellenbogen, J.A. (2004). Equol, a natural estrogenic metabolite from soy isoflavones: convenient preparation and resolution of R- and S-equols and their differing binding and biological activity through estrogen receptors alpha and beta. *Bioorg Med Chem*, Vol.12, No.6, pp. 1559-1567. ISSN 0968-0896

Nagata, Y., Lan, K.H., Zhou, X., Tan, M., Esteva, F.J., Sahin, A.A., Klos, K.S., Li, P., Monia, B.P., Nguyen, N.T., Hortobagyi, G.N., Hung, M.C. & Yu, D. (2004). PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell*, Vol.6, No.2, pp. 117-127. ISSN 1535-6108

Nagy, P., Friedlander, E., Tanner, M., Kapanen, A.I., Carraway, K.L., Isola, J. & Jovin, T.M. (2005). Decreased accessibility and lack of activation of ErbB2 in JIMT-1, a herceptin-resistant, MUC4-expressing breast cancer cell line. *Cancer Res*, Vol.65, No.2, pp. 473-482. ISSN 0008-5472

Nahta, R., Yu, D., Hung, M.C., Hortobagyi, G.N. & Esteva, F.J. (2006). Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. *Nat Clin Pract Oncol*, Vol.3, No.5, pp. 269-280. ISSN 1743-4254

Nguewa, P.A., Fuertes, M.A., Cepeda, V., Alonso, C., Quevedo, C., Soto, M. & Perez, J.M. (2006). Poly(ADP-ribose) polymerase-1 inhibitor 3-aminobenzamide enhances apoptosis induction by platinum complexes in cisplatin-resistant tumor cells. *Med Chem*, Vol.2, No.1, pp. 47-53. ISSN 1573-4064

Niedre, M., Patterson, M.S. & Wilson, B.C. (2002). Direct near-infrared luminescence detection of singlet oxygen generated by photodynamic therapy in cells in vitro and tissues in vivo. *Photochem Photobiol*, Vol.75, No.4, pp. 382-391. ISSN 0031-8655

Nomoto, S., Arao, Y., Horiguchi, H., Ikeda, K. & Kayama, F. (2002). Oestrogen causes G2/M arrest and apoptosis in breast cancer cells MDA-MB-231. *Oncol Rep*, Vol.9, No.4, pp. 773-776. ISSN 1021-335X

Normanno, N., Di Maio, M., De Maio, E., De Luca, A., de Matteis, A., Giordano, A. & Perrone, F. (2005). Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. *Endocr Relat Cancer*, Vol.12, No.4, pp. 721-747. ISSN 1351-0088

O'Day, E. & Lal, A. (2010). MicroRNAs and their target gene networks in breast cancer. *Breast Cancer Res*, Vol.12, No.2, art. no.201. ISSN 1465-542X

Obach, R.S. (2000). Inhibition of human cytochrome P450 enzymes by constituents of St. John's Wort, an herbal preparation used in the treatment of depression. *J Pharmacol Exp Ther*, Vol.294, No.1, pp. 88-95. ISSN 0022-3565

Oleinick, N.L., Morris, R.L. & Belichenko, I. (2002). The role of apoptosis in response to photodynamic therapy: what, where, why, and how. *Photochem Photobiol Sci*, Vol.1, No.1, pp. 1-21. ISSN 1474-905X

Olson, J. (2002). *Bathsheba's Breast: Women, Cancer, and History.*, John Hopkins Press, ISBN 978-0801869365, Baltimore

Pal, D. & Mitra, A.K. (2006). MDR- and CYP3A4-mediated drug-herbal interactions. *Life Sci*, Vol.78, No.18, pp. 2131-2145. ISSN 0024-3205

Pavese, J.M., Farmer, R.L. & Bergan, R.C. (2010). Inhibition of cancer cell invasion and metastasis by genistein. *Cancer Metastasis Rev*, Vol.29, No.3, pp. 465-482. ISSN 1573-7233

Pegram, M.D., Pienkowski, T., Northfelt, D.W., Eiermann, W., Patel, R., Fumoleau, P., Quan, E., Crown, J., Toppmeyer, D., Smylie, M., Riva, A., Blitz, S., Press, M.F., Reese, D., Lindsay, M.A. & Slamon, D.J. (2004). Results of two open-label, multicenter phase II studies of docetaxel, platinum salts, and trastuzumab in HER2-positive advanced breast cancer. *J Natl Cancer Inst*, Vol.96, No.10, pp. 759-769. ISSN 1460-2105

Perez, E.A., Suman, V.J., Rowland, K.M., Ingle, J.N., Salim, M., Loprinzi, C.L., Flynn, P.J., Mailliard, J.A., Kardinal, C.G., Krook, J.E., Thrower, A.R., Visscher, D.W. & Jenkins, R.B. (2005). Two concurrent phase II trials of paclitaxel/carboplatin/trastuzumab (weekly or every-3-week schedule) as first-line therapy in women with HER2-

overexpressing metastatic breast cancer: NCCTG study 983252. *Clin Breast Cancer*, Vol.6, No.5, pp. 425-432. ISSN 1526-8209

Plaetzer, K., Kiesslich, T., Oberdanner, C.B. & Krammer, B. (2005). Apoptosis following photodynamic tumor therapy: induction, mechanisms and detection. *Curr Pharm Des*, Vol.11, No.9, pp. 1151-1165. ISSN 1381-6128

Powell, S. (2010). Radiotherapy for breast cancer in the 21st Century. *Breast J*, Vol.16, Suppl 1, pp. S34-38. ISSN 1524-4741

Proskuryakov, S.Y., Konoplyannikov, A.G. & Gabai, V.L. (2003). Necrosis: a specific form of programmed cell death? *Exp Cell Res*, Vol.283, No.1, pp. 1-16. ISSN 0014-4827

Qiang, Y.G., Yow, C.M. & Huang, Z. (2008). Combination of photodynamic therapy and immunomodulation: current status and future trends. *Med Res Rev*, Vol.28, No.4, pp. 632-644. ISSN 0198-6325

Rajah, T.T., Du, N., Drews, N. & Cohn, R. (2009). Genistein in the presence of 17beta-estradiol inhibits proliferation of ERbeta breast cancer cells. *Pharmacology*, Vol.84, No.2, pp. 68-73. ISSN 1423-0313

Rasola, A. & Bernardi, P. (2007). The mitochondrial permeability transition pore and its involvement in cell death and in disease pathogenesis. *Apoptosis*, Vol.12, No.5, pp. 815-833. ISSN 1360-8185

Reddy, G.R., Bhojani, M.S., McConville, P., Moody, J., Moffat, B.A., Hall, D.E., Kim, G., Koo, Y.E., Woolliscroft, M.J., Sugai, J.V., Johnson, T.D., Philbert, M.A., Kopelman, R., Rehemtulla, A. & Ross, B.D. (2006). Vascular targeted nanoparticles for imaging and treatment of brain tumors. *Clin Cancer Res*, Vol.12, No.22, pp. 6677-6686. ISSN 1078-0432

Redmond, R.W. & Gamlin, J.N. (1999). A compilation of singlet oxygen yields from biologically relevant molecules. *Photochem Photobiol*, Vol.70, No.4, pp. 391-475. ISSN 0031-8655

Reed, J.C. (1998). Bcl-2 family proteins. *Oncogene*, Vol.17, No.25, pp. 3225-3236. ISSN 0950-9232

Reits, E.A., Hodge, J.W., Herberts, C.A., Groothuis, T.A., Chakraborty, M., Wansley, E.K., Camphausen, K., Luiten, R.M., de Ru, A.H., Neijssen, J., Griekspoor, A., Mesman, E., Verreck, F.A., Spits, H., Schlom, J., van Veelen, P. & Neefjes, J.J. (2006). Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. *J Exp Med*, Vol.203, No.5, pp. 1259-1271. ISSN 0022-1007

Robert, N., Leyland-Jones, B., Asmar, L., Belt, R., Ilegbodu, D., Loesch, D., Raju, R., Valentine, E., Sayre, R., Cobleigh, M., Albain, K., McCullough, C., Fuchs, L. & Slamon, D. (2006). Randomized phase III study of trastuzumab, paclitaxel, and carboplatin compared with trastuzumab and paclitaxel in women with HER-2-overexpressing metastatic breast cancer. *J Clin Oncol*, Vol.24, No.18, pp. 2786-2792. ISSN 1527-7755

Roy, H.K., Olusola, B.F., Clemens, D.L., Karolski, W.J., Ratashak, A., Lynch, H.T. & Smyrk, T.C. (2002). AKT proto-oncogene overexpression is an early event during sporadic colon carcinogenesis. *Carcinogenesis*, Vol.23, No.1, pp. 201-205. ISSN 0143-3334

Sačková, V., Kulíková, L., Mikeš, J., Kleban, J. & Fedoročko, P. (2005). Hypericin-mediated photocytotoxic effect on HT-29 adenocarcinoma cells is reduced by light

fractionation with longer dark pause between two unequal light doses. *Photochem Photobiol*, Vol.81, No.6, pp. 1411-1416. ISSN 0031-8655

Sačková, V., Fedoročko, P., Szilárdiová, B., Mikeš, J. & Kleban, J. (2006). Hypericin-induced phototoxicity is connected with G2/M arrest in HT-29 and S phase arrest in U937 cells. *Photochem Photobiol*, Vol.82, No.5, pp. 1285-1291. ISSN 0031-8655

Shepard, H.M., Jin, P., Slamon, D.J., Pirot, Z. & Maneval, D.C. (2008). Herceptin. *Handb Exp Pharmacol*, No.181, pp. 183-219. ISSN 0171-2004

Schieke, S.M., von Montfort, C., Buchczyk, D.P., Timmer, A., Grether-Beck, S., Krutmann, J., Holbrook, N.J. & Klotz, L.O. (2004). Singlet oxygen-induced attenuation of growth factor signaling: possible role of ceramides. *Free Radic Res*, Vol.38, No.7, pp. 729-737. ISSN 1071-5762

Schinazi, R.F., Chu, C.K., Babu, J.R., Oswald, B.J., Saalmann, V., Cannon, D.L., Eriksson, B.F. & Nasr, M. (1990). Anthraquinones as a new class of antiviral agents against human immunodeficiency virus. *Antiviral Res*, Vol.13, No.5, pp. 265-272. ISSN 0166-3542

Schlotter, C.M., Vogt, U., Allgayer, H. & Brandt, B. (2008). Molecular targeted therapies for breast cancer treatment. *Breast Cancer Res*, Vol.10, No.4, pp. 211. ISSN 1465-542X

Silva, J.N., Galmiche, A., Tome, J.P., Boullier, A., Neves, M.G., Silva, E.M., Capiod, J.C., Cavaleiro, J.A., Santus, R., Maziere, J.C., Filipe, P. & Morliere, P. (2010). Chain-dependent photocytotoxicity of tricationic porphyrin conjugates and related mechanisms of cell death in proliferating human skin keratinocytes. *Biochem Pharmacol*, Vol.80, No.9, pp. 1373-1385. ISSN 1873-2968

Simon, V., Devaux, C., Darmon, A., Donnet, T., Thienot, E., Germain, M., Honnorat, J., Duval, A., Pottier, A., Borghi, E., Levy, L. & Marill, J. (2010). Pp IX silica nanoparticles demonstrate differential interactions with in vitro tumor cell lines and in vivo mouse models of human cancers. *Photochem Photobiol*, Vol.86, No.1, pp. 213-222. ISSN 1751-1097

Solár, P., Ferenc, P., Kovaľ, J., Mikeš, J., Solárová, Z., Hrčková, G., Fulton, B.L. & Fedoročko, P. (2011). Photoactivated hypericin induces downregulation of HER2 gene expression. *Radiat Res*, Vol.175, No.1, pp. 51-56. ISSN 1938-5404

Sparano, J.A. & Paik, S. (2008). Development of the 21-gene assay and its application in clinical practice and clinical trials. *J Clin Oncol*, Vol.26, No.5, pp. 721-728. ISSN 1527-7755

Takemura, T., Ohta, N., Nakajima, S. & Sakata, I. (1989). Critical importance of the triplet lifetime of photosensitizer in photodynamic therapy of tumor. *Photochem Photobiol*, Vol.50, No.3, pp. 339-344. ISSN 0031-8655

Thomas, E.S., Gomez, H.L., Li, R.K., Chung, H.C., Fein, L.E., Chan, V.F., Jassem, J., Pivot, X.B., Klimovsky, J.V., de Mendoza, F.H., Xu, B., Campone, M., Lerzo, G.L., Peck, R.A., Mukhopadhyay, P., Vahdat, L.T. & Roche, H.H. (2007). Ixabepilone plus capecitabine for metastatic breast cancer progressing after anthracycline and taxane treatment. *J Clin Oncol*, Vol.25, No.33, pp. 5210-5217. ISSN 1527-7755

Thong, P.S., Olivo, M., Chin, W.W., Bhuvaneswari, R., Mancer, K. & Soo, K.C. (2009). Clinical application of fluorescence endoscopic imaging using hypericin for the diagnosis of human oral cavity lesions. *Br J Cancer*, Vol.101, No.9, pp. 1580-1584. ISSN 1532-1827

Tong, Z., Singh, G. & Rainbow, A.J. (2002). Sustained activation of the extracellular signal-regulated kinase pathway protects cells from photofrin-mediated photodynamic therapy. *Cancer Res*, Vol.62, No.19, pp. 5528-5535. ISSN 0008-5472

Tsai, T., Ji, H.T., Chiang, P.C., Chou, R.H., Chang, W.S. & Chen, C.T. (2009). ALA-PDT results in phenotypic changes and decreased cellular invasion in surviving cancer cells. *Lasers Surg Med*, Vol.41, No.4, pp. 305-315. ISSN 1096-9101

Utsumi, T., Okuma, M., Kanno, T., Takehara, Y., Yoshioka, T., Fujita, Y., Horton, A.A. & Utsumi, K. (1995). Effect of the antiretroviral agent hypericin on rat liver mitochondria. *Biochem Pharmacol*, Vol.50, No.5, pp. 655-662. ISSN 0006-2952

Vantieghem, A., Xu, Y., Declercq, W., Vandenabeele, P., Denecker, G., Vandenheede, J.R., Merlevede, W., de Witte, P.A. & Agostinis, P. (2001). Different pathways mediate cytochrome c release after photodynamic therapy with hypericin. *Photochem Photobiol*, Vol.74, No.2, pp. 133-142. ISSN 0031-8655

Verheus, M., van Gils, C.H., Keinan-Boker, L., Grace, P.B., Bingham, S.A. & Peeters, P.H. (2007). Plasma phytoestrogens and subsequent breast cancer risk. *J Clin Oncol*, Vol.25, No.6, pp. 648-655. ISSN 1527-7755

von Minckwitz, G., Jonat, W., Fasching, P., du Bois, A., Kleeberg, U., Luck, H.J., Kettner, E., Hilfrich, J., Eiermann, W., Torode, J. & Schneeweiss, A. (2005). A multicentre phase II study on gefitinib in taxane- and anthracycline-pretreated metastatic breast cancer. *Breast Cancer Res Treat*, Vol.89, No.2, pp. 165-172. ISSN 0167-6806

Ward, H., Chapelais, G., Kuhnle, G.G., Luben, R., Khaw, K.T. & Bingham, S. (2008). Breast cancer risk in relation to urinary and serum biomarkers of phytoestrogen exposure in the European Prospective into Cancer-Norfolk cohort study. *Breast Cancer Res*, Vol.10, No.2, art. no.R32. ISSN 1465-542X

Weller, M., Trepel, M., Grimmel, C., Schabet, M., Bremen, D., Krajewski, S. & Reed, J.C. (1997). Hypericin-induced apoptosis of human malignant glioma cells is light-dependent, independent of bcl-2 expression, and does not require wild-type p53. *Neurol Res*, Vol.19, No.5, pp. 459-470. ISSN 0161-6412

Weyergang, A., Kaalhus, O. & Berg, K. (2008). Photodynamic targeting of EGFR does not predict the treatment outcome in combination with the EGFR tyrosine kinase inhibitor Tyrphostin AG1478. *Photochem Photobiol Sci*, Vol.7, No.9, pp. 1032-1040. ISSN 1474-905X

Wong, C.H., Iskandar, K.B., Yadav, S.K., Hirpara, J.L., Loh, T. & Pervaiz, S. (2010). Simultaneous induction of non-canonical autophagy and apoptosis in cancer cells by ROS-dependent ERK and JNK activation. *PLoS One*, Vol.5, No.4, pp. e9996. ISSN 1932-6203

Xu, L. & Bergan, R.C. (2006). Genistein inhibits matrix metalloproteinase type 2 activation and prostate cancer cell invasion by blocking the transforming growth factor beta-mediated activation of mitogen-activated protein kinase-activated protein kinase 2-27-kDa heat shock protein pathway. *Mol Pharmacol*, Vol.70, No.3, pp. 869-877. ISSN 0026-895X

Xu, L., Ding, Y., Catalona, W.J., Yang, X.J., Anderson, W.F., Jovanovic, B., Wellman, K., Killmer, J., Huang, X., Scheidt, K.A., Montgomery, R.B. & Bergan, R.C. (2009). MEK4 function, genistein treatment, and invasion of human prostate cancer cells. *J Natl Cancer Inst*, Vol.101, No.16, pp. 1141-1155. ISSN 1460-2105

Xue, L., He, J. & Oleinick, N.L. (1999). Promotion of photodynamic therapy-induced apoptosis by stress kinases. *Cell Death Differ*, Vol.6, No.9, pp. 855-864. ISSN 1350-9047

Yeh, T.C., Chiang, P.C., Li, T.K., Hsu, J.L., Lin, C.J., Wang, S.W., Peng, C.Y. & Guh, J.H. (2007). Genistein induces apoptosis in human hepatocellular carcinomas via interaction of endoplasmic reticulum stress and mitochondrial insult. *Biochem Pharmacol*, Vol.73, No.6, pp. 782-792. ISSN 0006-2952

Yoeli-Lerner, M., Yiu, G.K., Rabinovitz, I., Erhardt, P., Jauliac, S. & Toker, A. (2005). Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. *Mol Cell*, Vol.20, No.4, pp. 539-550. ISSN 1097-2765

Zava, D.T. & Duwe, G. (1997). Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro. *Nutr Cancer*, Vol.27, No.1, pp. 31-40. ISSN 0163-5581

Zhuang, S. & Kochevar, I.E. (2003). Singlet oxygen-induced activation of Akt/protein kinase B is independent of growth factor receptors. *Photochem Photobiol*, Vol.78, No.4, pp.361-371. ISSN 0031-8655

Part 2

Anti-Tumor Compounds

5

Boron Compounds in the Breast Cancer Cells Chemoprevention and Chemotherapy

Ion Romulus Scorei
Biochemistry Department, University of Craiova,
Craiova, Dolj,
Romania

1. Introduction

Various biological functions of Boron (B) compounds are known (Blevins & Lukaszewski, 1994; Tariq & Mott, 2007; Nielsen, 2008). Boron is found in nuts, vegetables, dried/fresh fruits and red wine (Brown & Shelp, 1997). Boron is also present in bacterial antibiotics, such as tartrolon, borophycin, boromycin and aplasmomycin (Rezanka & Sigler, 2008); in the bacterial quorum sensing molecule *auto-inducer AI-2* (Bemd et al., 2002); and in vibrioferrin, a B-containing siderophore produced by particular marine bacteria (Shady et al., 2007). In plants, the rigidity of the cell wall depends on the rhamnogalacturonan II complex (RG-II) formation, a pectic polysaccharide covalently linked by cis-diol bonds to apiosil residues of borate-esters (Ishii & Matsunaga, 1996, 2001). Several articles have provided information about transporters responsible for efficient B uptake by roots, xylem loading and B distribution among leaves. The transporters are required under B limitation for efficient acquisition and utilisation of B. Two types of transporters are involved in these processes: NIPs (nodulin-26-like intrinsic proteins) for boric acid channels and boron exporters encoded by BOR1 (Miwa & Fujiwara, 2010). The expression of the genes encoding these transporters has been shown to be finely regulated in the B availability response to ensure tissue B homeostasis. Furthermore, the tolerance of plants to the stress produced by low B or high B in the environment can be generated by altering the expression of these transporters (Tanaka & Fujiwara, 2007). All of these transporters are involved in boron transport regulation in plants. B is an essential element not only for vascular plants but also for diatoms, cyanobacteria and a number of marine algal flagellate species (Rezanka & Sigler, 2008). Recently, ATR1 has been found to be responsible for the high B tolerance in *S. Cerevisiae*. ATR1 encodes a multidrug resistance transporter and it is widely distributed in bacteria, archaea and lower eukaryotes (Miwa & Fujiwara, 2010). Animals such as zebra, fish, trout and frogs also require boron (Rowe & Eckert, 1999; Fort et al., 1999). Borate ions activate the mitogen-activated protein kinases pathway and stimulate the growth and the proliferation of human embryonic kidney 293 cells (Park et al., 2005). The B-transporter, NaBCl, controls plasma borate levels in human kidney cells (Park at al., 2004). The fact that B has such a broad range of physiological functions is not surprising. The electron structure of B and its position in the periodic table (adjacent to carbon) make B-containing molecules electrophilic with the trigonal

planar structures that are isoelectronic neutral relative to carbocations. The additional bond with B allows the formation of anionic tetravalent compounds with tetrahedral structures, which behave as nucleophiles (Petasis, 2007). Various types of B-containing molecules already exist and have been investigated as therapeutic agents. These molecules include B-containing analogues of natural biomolecules (Morin, 1994), the antibacterial and antimalarial agent diazaborine (Baldock et al., 1998), antibacterial oxazaborolidines (Jabbour et al., 2004; Jabbour et al., 2006), antibacterial diphenyl borinic esters (Benkovic et al., 2005), the antifungal agent benzoxaborole AN2690 (Baker et al., 2006) and a B-N bond containing an estrogen receptor modulator (Zhou et al., 2007). Except for the drug Bortezomib, the majority of B compounds currently used in cancer treatment are in the Neutron Capture Therapy (BNCT) class (Beddoe, 1997; Endo et al., 2003). The discovery of many B-containing molecules is predicted, and these molecules will be useful in applications involving cell surface signalling (Bolanos et al. 2004; Redondo-Nieto et al., 2008). The main objective of this review is to reveal other promising research directions for B-based chemicals in chemoprevention and chemotherapy, particularly in breast cancer.

2. Boron compounds in cancer prevention

2.1 Dietary boron and cancer risk

A diet with low B has been found to lead to a number of general health problems and to increase cancer risk. The most common symptoms of B deficiency include arthritis, memory loss, osteoporosis, degenerative and soft cartilage diseases, hormonal disequilibria and a drop in libido (Scorei & Popa, 2010). The daily uptake of B varies as a function of food selection, the use of some specific personal products and the water B content. Reported values for the overall B uptake vary as follows: 0.8-1.9 mg/day in the European Union, 1.7-7 mg/day in the United States, ~0.93 mg/day in Korea, 2.16-2.28 mg/day in Australia, 1.75-2.12 mg/day in Mexico and 1.8-1.95 mg/day in Kenya (Rainey & Nyquist, 1998). These dissimilarities may be correlated with regional differences in the abundance of high-energy food and in food products rich in fibres and plant proteins. The actual B requirements for the human body remain unclear. Thus, more knowledge about the biological functions of B and the regulation of its exchange is required (Nielsen, 2009). The B Tolerable Upper Intake Level (UL) for adults of ~18 years is ~20 mg B per day (Scorei & Rotaru, 2011).

2.1.1 Lung cancer

The mortality due to lung cancer has reached higher values for men than for women (Espey et al., 2007). A negative correlation has been found between the amount of B intake and the incidence of lung cancer, although the underlying mechanism remains unclear (Meacham et al., 2007). Experimental evidence has shown that nutrition with some B-compounds (such as boric acid, borax and calcium fructoborate) has had anti-oxidant or/and anti-inflammatory consequences (Nielsen, 2000; Hunt, 1998; Scorei et al., 2005). Correlations exist between some lung cancers and 17-beta-estradiol, and the treatment includes 17-beta-estradiol-based Hormone Replacement Therapy (HRT) (Schabath et al., 2004). Dietary supplementation with B has been shown to increase the concentration of 17-beta-estradiol (Wang et al., 2008), mimic the HRT effect and, in the case of postmenopausal women, it may be used to decrease the cancer risks associated with low estrogen levels (Devirian & Volpe, 2003). Low dietary B

(alone or together with HRT) has been correlated with an increase in lung cancer risk for women (Mahabir et al., 2008). The reduction of lung cancer risk may involve estrogen receptor binding substrates, other than estrogen, including carcinogenic Polycyclic Aromatic Hydrocarbons (PAHs) from cigarette smoke condensate (Pike et al., 1999). Women with high dietary B intake and HRT users may present higher hormone levels that compete with cigarette smoke carcinogens for estrogen receptors. If this model is correct, increasing the B intake during HRT will also limit the carcinogenic potential of PAHs from cigarette smoke. Recently, the highest quartile of B intake has been confirmed to be associated with the lowest lung cancer risks for smokers, while the highest risk exists in smokers with low dietary B and no HRT (Mahabir et al., 2008).

2.1.2 Prostate cancer

Dietary B is inversely correlated with the occurrence of prostate cancer (Yan et al., 2004), even if the source of this correlation remains unclear. The prostate cancer risk was one third smaller for men ingesting more than 1.8 mg B per day from food, relative to only 0.9 mg B/day. A relatively high correlation (r = 0.63) was found between the B concentration from the subsurface water and the prostate cancer distribution in Texas (Barranco et al., 2007). A broader understanding of the cellular mechanisms that involve B have shown that boric acid (BA) inhibits prostate cancer cell growth by decreasing cyclin A-E expression, though B does not induce cell death (Barranco et al., 2009). Furthermore, cells treated with BA demonstrate diminished adhesion and migration, which indicates a low metastatic potential. B has been hypothesised to have effects on prostate cancers through its influence on steroid hormones (particularly androgens, which are involved in prostate carcinogenesis) (Gann et al., 1996). Three research directions have been followed to study the relationship between B and prostate cancer risk: steroid hormone regulation, anti-cancer metabolites and cell proliferation. Several potential BA binding sites may be involved in prostate cancer. For example, Prostate Serum Antigen (PSA), a serine protease, is a potential site for direct boration (Gallardo-Williams et al., 2003). Boric acid decreases the expression of five major cyclin proteins (A, B_1, C, D_1 and E), which have significant roles in the cell cycle (Barranco & Eckhert, 2006) and inhibit the release of Ca(II) stored by the NAD+cADPR system. This regulation of cyclins could explain the effects of B on prostate cancer cells. When B consumption was ~1.17 mg per day, no correlation with prostate cancer frequency was observed (Gonzalez et al., 2007).

2.1.3 Cervical cancer

Cervical cancer is the second most frequent cancer in women worldwide (Parkin et al., 1993). The cause of this discrepancy is still unclear and can involve a combination of environmental, genetic, social and infectious factors. For example, Human papillomavirus (HPV) is the primary cause of cervical cancer. HPV 16 and HPV 18 are responsible for ~95% of cervical cancers. Many other factors also correlate with the incidence of cervical cancer (Ursin et al., 1996; Ylitalo et al., 1999; Castellsague et al., 2002). According to one hypothesis, the low cervical cancer incidence in Turkey correlates with its B-enriched soil (Sayli et al., 2001; Simsek et al., 2003). Indeed, the ingestion of B via drinking water prevents cervical cancer risk (Korkmaz et al., 2007). This effect has been suggested to be due to the B interference chemistry in the life cycle of HPVs, but no correlation could be found regarding the incidence of oral cancers, which are also induced by HPVs. Serine protease inhibitors

have been found to reduce the immortalisation and transforming capacity of the HPV E7 oncogene (Stoppler et al., 1996). Because B exists in the human body mostly in the form of BA, which is an inhibitor of serine proteases, ingestion of high amounts of B through drinking water has been hypothesised as able to inhibit HPV transformation, thus reducing the incidence of cervical cancer (Korkmaz et al., 2007).

2.2 Dietary boron and breast cancer prevention

Today, breast cancer is the most common cancer type diagnosed in women, excluding non-melanoma skin cancers (Greenlee et al., 2000). Breast cancer is related to endogenous hormones. Many studies have linked breast cancer risk to the age of menarche, menopause and first pregnancy (Harris et al., 1992). Postmenopausal obesity has been observed to increase the risk (Stoll, 1998; Pujol et al., 1997), perhaps due to increased peripheral estrogen production. However, this relationship between weight and risk does not appear in premenopausal women (Kelsey, 1979). In fact, some studies have reported an inverse relationship between weight and risk at a younger age (Holmes et al., 1999). In Japan, breast cancer is a rare disease compared to the Western countries (Tominaga & Kuroishi, 1999). When Japanese women immigrated to the USA, they acquired the same risk for breast cancer as that in the general population of women in the USA (Probst-Hensch et al., 2000). This increased risk occurred due to environmental and dietary factors (Maskarinec et al., 2001; Probst-Hensch et al., 2000). Proliferative breast disease has been observed more frequently in women with a significant family history of breast cancer (Vogel, 2000). A number of environmental factors have also been linked to breast cancer risk. Exposure to ionising radiation, whether after a nuclear explosion or during medical procedures, has clearly been demonstrated to correlate with an increase in the risk of breast cancer (Spiegelman et al., 1994). The risk level varies with the age of the subject. The risk was observed to be lower for exposures in the case of women over 40 years. The role of diet in the aetiology of the breast cancer seems to be extremely important. This importance has been suggested by the international variation in breast cancer incidence rates and by the observation that national per capita fat consumption correlates with breast cancer incidence and mortality (Armstrong & Doll, 1975). However, prospective studies regarding diet and breast cancer risk have failed to identify a relationship between dietary fat intake and breast cancer incidence during 10 years of follow-up (Hunter & Willett, 1996). The absence of a link between dietary fat intake and cancer risk within the context of a Western diet has been confirmed by a pooled analysis of seven cohort studies involving 337,816 women. The analysis has demonstrated no risk differences between women with the lowest and those with the highest quintile of fat intake (Hunter et al., 1996). However, all of these studies have addressed fat intake during adult life and do not exclude the possibility that fat intake during childhood and adolescence could subsequently influence breast cancer risk. Strong evidence exists that supports an association between alcohol and breast cancer. A meta-analysis of 12 cases of control studies has demonstrated a relative risk (RR) of 1.4 for each 24 g of alcohol consumed daily (Longnecker et al., 1988). However, defining a relationship between the age at which alcohol consumption began and breast cancer risk is difficult.

2.2.1 Dietary boron intake, sex steroid hormones and breast cancer

Several reports have indicated that 17-beta-estradiol levels increase with dietary boron supplementation in human subjects (Nielsen et al., 1987; Naghii & Samman, 1997).

Therefore, dietary boron might mimic the actions of Hormone Replacement Therapy (HRT). After one week, supplementation of healthy males with 10 mg B/day resulted in a significant rise in the plasma free testosterone concentration, which is an observation based on recent clinical data (Naghii et al., 2010). According to this recent study, the free testosterone level increases and the estradiol level decreases after short-term boron consumption. Breast cancer patients appear to have relative sex steroid hormone imbalance, in favour of estrogens (McTiernan et al., 2003). High bio-available testosterone counteracts the proliferative effects of estrogens on the mammary tissues and exerts a protective role to the breast, inhibiting cancer development and/or tumour growth (Hofling et al., 2007). However, preclinical studies have suggested that testosterone serves as a natural, endogenous protector of the breast. Worldwide data from prospective studies regarding the connection between the endogenous sex hormones levels and breast cancer risk in postmenopausal women have also shown multiple and complex relationships. Nine prospective studies of women who had not taken exogenous sex hormones when samples from their blood were collected for the determination of hormone levels have shown that the breast cancer risk increases significantly with an increased concentration of all examined sex hormones: total estradiol, free estradiol, non-sex hormone binding globulin (SHBG)–bound estradiol, estrone, estrone sulphate, androstenedione, dehydroepiandrosterone, dehydroepiandrosterone sulphate and testosterone. High SHBG has been associated with a decrease in breast cancer risk (Dimitrakakis et al., 2010). Investigations regarding the association between premenopausal estrogen levels and breast cancer risk are complicated due to the cyclic estrogen variation during the menstrual cycle. The breast cancer risk among premenopausal women is directly related to the circulating levels of testosterone and androstenedione.

In conclusion, daily dietary B intake at a minimum of 10 mg of B per day decreases the estradiol level and increases the testosterone level (Naghii et al., 2010). This regulation assures a true hormone-dependent protection against breast cancer.

2.2.2 Boron, vitamin D and breast cancer

Vitamin D is a steroid hormone that is synthesised in human skin from 7-dehydrocholesterol in the presence of the UV light. Vitamin D is primarily metabolised in the liver and subsequently in the kidney in the form of calcitriol, the most biologically active metabolite of vitamin D (Zehnder et al., 2001; Tangpricha et al., 2001). In addition, epidemiologic, clinical and animal studies have demonstrated that vitamin D is important as a protective agent against the development of breast cancer (Buras et al., 1994; Mehta & Mehta, 2002). Boron has a role in energy substrate metabolism due to its involvement in vitamin D metabolism (Hegsted et al., 1991). Various boron-containing compounds have been discovered to be effective in transiently and acutely raising calcitriol levels to a significant degree in mammalian blood. Indeed, calcitriol levels could be increased almost 80% upon the oral administration of a single dose of a boron-containing complex (15-20 mg boron as calcium fructoborate per day) (Pietrzkowski, 2010). Calcitriol in turn controls calcium and phosphate homeostasis and is essential for the development and maintenance of healthy bones. Calcitriol, or 1,25-DihydroxyvitaminD_3, is the biologically active form of vitamin D and interacts with the Vitamin D Receptor (VDR). Calcitriol is a coordinate regulator of proliferation, differentiation and survival of breast cancer cell (Colston & Hansen, 2002). Therefore, vitamin D compounds that bind and activate VDRs have become

established therapeutic agents for breast cancer treatment. Various *in vitro* and *in vivo* studies have shown that vitamin D inhibits cell proliferation of a wide range of cell types, including carcinomas of the breast, prostate, colon, skin and brain, myeloid leukaemia cells and others (Mehta & Mehta, 2002; Ingraham et al., 2008). However, the processes mediating this inhibition have still not been entirely elucidated. Recently, vitamin D has been demonstrated to induce apoptosis and inhibit angiogenesis, tumour invasion and metastases. These preclinical data suggest that vitamin D (alone or in combination with other agents) has potential applications in cancer prevention and treatment. Low levels of plasma calcitriol have been associated with high rates of colorectal, breast, lung and prostate cancer incidence and mortality in men (Garland et al., 2006). The broad-spectrum anti-tumour effects of calcitriol and its analogues are mostly based on the inhibition of cancer cell proliferation and invasiveness, induction of differentiation and apoptosis and promotion of angiogenesis.

In a NHANES III (National Health and Nutrition Examination Survey III) cohort, Freedman has reported that women with serum calcitriol levels higher than 62 nmol/L present a 75% decrease in breast cancer mortality (Freedman et al., 2007). In two other studies, the authors have concluded that the breast cancer risk for women is 58% lower when vitamin D levels are higher than 95 nmol/L, in comparison with women with a calcitriol level lower than 37.5 nmol/L (Simard et al., 1991; Bemd & Chang, 2002; Gissel et al., 2008). In a dose-response meta-analysis, it has been reported that women with the highest calcitriol levels in their blood have a reduced breast cancer risk (Garland et al., 2006). In another study, 1760 women were divided into 5 groups, from the lowest to the highest calcitriol levels (Garland et al., 2007). A dose-response association was evident. The highest breast cancer rates were found in the group with the lowest calcitriol levels (less than 32 nmol/L). The cancer rates were the lowest in women with serum calcitriol levels higher than 130 nmol/L. If the serum calcitriol levels are high enough, they reduce the breast cancer risk by 35%. Thus, boron could become an effective preventive diet for breast cancer by its action in inhibiting calcitriol degradation (Miljkovic, 2004).

2.2.3 Boron, omega-3 fatty acids and breast cancer

Laboratory animals subjected to a diet rich in both omega-3 fatty acids and boron demonstrate a high bone mineral density and their bones become stronger compared to animals fed with other dietary fats and boron. These findings suggest that omega-3 fatty acids and boron can work together to support dense, strong bones (Nielsen & Penland, 2006). A diet rich in omega-3 α-linolenic acid promotes femur strength, especially when the dietary boron is adequate. In recent research studies, the bone health benefits of omega-3 fatty acids have been discovered to be greatly amplified when these essential fats are combined with the critical trace of mineral boron (Nielsen, 2008). Intriguing new research findings have suggested that bone-supporting effects of boron can be the greatest when omega-3 fatty acids are available. For instance, as mentioned earlier, a diet rich in both omega-3 fatty acids and boron give laboratory animals higher bone mineral density and stronger bones in comparison to animals that are fed other fats.

The association between ω-3 fatty acids and breast cancer risk has been examined in several studies (Rose & Connoly, 1993; Deckere, 1999; Saadatian et al., 2004). The results have demonstrated that this correlation varies according to the study design. A meta-analysis of biomarker studies based on three cohorts and seven case-control studies has found a

significant protective effect of total ω-3 PUFAs (Kim et al., 2009). At the same time, only an inverse association with a borderline significance for α-linolenic acid in case-control studies has been demonstrated (Fritsche & Johnston, 1990). However, according to a recent systematic review, only one study has shown a significantly increased breast cancer risk, three studies have presented a decreased risk and seven other studies have failed to demonstrate a significant relationship with ω-3 fatty acid intake (MacLean et al., 2006). In the UK, fish oil consumption has been associated with protection against breast carcinogenesis (Caygill et al., 1995; Caygill et al., 1996). A postmenopausal study, which took place in the USA, has found a significant inverse correlation between fish intake (canned, fried, fresh and shellfish) and breast cancer risk (Shannon et al., 2003). This investigation identified fish and fish ω-3 fatty acid intake as an important potential protective factor in the nutritional aetiology of breast cancer. Moreover, recent studies have shown that boron protects omega-3 fatty acids (Nielsen, 2004; Nielsen & Penland, 2006) and consequently, a diet rich in boron and omega-3 will ensure a low breast cancer risk for women, especially because boron stimulates calcitriol synthesis, an anti-tumour agent that has been extremely well studied during recent years.

3. Boron-mediated chemoprevention of breast cancer

Cancer chemoprevention uses natural, synthetic or biological chemical agents to reverse, suppress or prevent carcinogenic progression (Sporn & Suh, 2000). Epithelial carcinogenesis is a multi-step process in which an accumulation of genetic events within a single cell line leads to a progressively dysplastic cellular appearance, deregulated cell growth, and, finally, carcinoma. In fact, the initial proposed definition for chemoprevention strictly refers to cancer prevention with pharmacological agents that inhibit or reverse the carcinogenesis process. This concept differs from that of cancer prevention, which refers mostly to removal or avoidance of factors such as fat, tobacco or UV radiation (Malone et al., 1989). According to their mechanisms, chemopreventive agents are classified into two broad categories: effective compounds against complete carcinogens and effective compounds against tumour promoters. Some compounds belong to both categories. The inhibitors of carcinogen-induced tumours are further divided in three major groups according to their different mechanisms of action. The first group includes agents that interfere with the precursor compounds of metabolic reactions, which are converted into carcinogens. The second group comprises agents capable of preventing carcinogens by reaching or reacting with target sites. This mechanism is realised by scavenging the reactive form of carcinogens. The third group includes molecules that have an inhibitory action following the exposure to carcinogenic agents. For this reason, these molecules are called suppressing agents.

3.1 Boric acid

Boric acid (BA) is one of the most studied B-containing chemicals. BA has been demonstrated to control the proliferation of some cancer cell types (Barranco et al., 2009; Barranco & Eckhert, 2006; Acerbo &Miller, 2009; Scorei et al., 2008). BA is an inhibitor of peptidases, proteases, proteasomes, arginase, nitric oxide synthase and transpeptidases (Bradke et al., 2008; Hunt, 1998). Inhibition of serine protease and dehydrogenase activities can be explained by the capacity of BA to bind OH groups from NAD and serine (Gallardo-Williams et al., 2003). The Prostatic Serum Antigen (PSA) is a serine protease and a putative target for BA (Scorei & Popa, 2010). Based on the PSA inhibition, the use of BA in the

chemical therapy of prostate carcinoma has been proposed (Gallardo-Williams et al., 2003). BA inhibitory effects have also been found in androgen-independent cell lines (DU-145 and PC- 3l), suggesting that other (serine protease-independent) mechanisms could also exist (Barranco & Eckhert, 2006). BA inhibits the cell cycle control and proliferation of DU-145, acting against the agonist-stimulated release of Ca^{2+} from ryanodine receptor sensitive cell stores (Henderson et al., 2009). In the case of melanoma cells, BA slows down the proliferation, possibly by inhibiting the second step of pre-mRNA splicing (Shomron & Ast, 2003). A high dose of BA (12.5–50 mM) slows cell replication and induces apoptosis in both melanoma cells and MDA231 breast cancer cells (Acerbo & Miller, 2009; Scorei et al., 2008). Thus, the inhibition of cancer cells by BA involves a diversity of cellular targets, such as direct enzymatic inhibition, apoptosis, receptor binding and mRNA splicing.

Recently, 1 mM of BA has been experimentally demonstrated to inhibit the ZR-75-1 breast cancer cell line, but not the MCF-7 cell line (Meacham et al., 2010; Elegbede, 2007). The lack of BA-mediated inhibition of MCF-7 cellular growth could be due to the presence of the "sodium-boron co-transporter (NaBC1)". This co-transporter exists on the cell surface and can pump out boron molecules from the cell in exchange for Na^{+} ions. This co-transporter is not present in ZR-75-1 cells. ZR-75-1 is a non-metastatic epithelial breast cancer cell line, which is estrogen receptor– and progesterone receptor–positive. MCF-7 is metastatic epithelial cell lines of breast cancer. These cells are positive for estrogen receptor and progesterone receptor. If BA becomes an anti-cancer agent for breast cancer, these data will encourage women with increased cancer risk factors to raise their boron intake to reduce their chance of developing this disease.

3.2 Calcium fructoborate

Calcium fructoborate (CF) is a natural product that is extracted from plants, but it can also be produced by chemical synthesis (Scorei & Popa, 2010). CF is efficient in the prevention and treatment (as adjuvant) of osteoporosis and osteoarthritis (Peng et al., 2000; Miljkovic et al., 2009; Scorei & Rotaru, 2011). In addition, CF has shown inhibitory effects on MDA-MB-231 breast cancer cells (Scorei et al., 2008; Scorei & Rotaru, 2011). CF most likely enters the cell through a co-transport mechanism via a sugar transporter (Miljkovic et al., 2009). MBA-MD-231 is a metastatic cancer cell line and is negative for expression of estrogen receptor. Inside cells, CF acts as an antioxidant and induces the over-expression of apoptosis-related proteins and eventually apoptosis. In our recent study, we have demonstrated that CF and BA inhibit the proliferation of MDA-MB-231 breast cancer cells in a dose-dependent manner (Scorei et al., 2008). As revealed by different experiments (TUNEL, Bcl-2 and pro-caspase-3 protein expression and cytochrome c caspase-3 activities), the anti-proliferative effect of CF in MDA-MB-231 breast cancer cells appeared to be mediated by the induction of apoptosis. On the other hand, CF raises the calcitriol level in blood (Pietrzkowski, 2010), which induces apoptosis and inhibits angiogenesis, tumour invasion and metastasis of breast cancer cells.

3.3 Boronic acid and its esters

From the structural point of view, boronic acids are trivalent boron-containing organic compounds that possess one alkyl substituent (i.e., a C–B bond) and two hydroxyl groups to fill the remaining valences on the boron atom. Boronic acids are potent and selective inhibitors of cancer cell migration and viability. One potential mechanism of action is the inhibition of proteases. Due to the easy interconversion of boronic acids between the neutral

sp^2 (trigonal planar substituted) and the anionic sp^3 (tetrahedral substituted) hybridisation states, the B-OH unit replaces the C=O bond at a site where an acyl group transfer takes place (Groziak, 2001). Phenylboronic acid (PBA) and diphenylboronic esters (DPBE) are the most efficient types of boronic acid derivatives that act as serine protease inhibitors (Yang et al., 2003). PBA is more efficient than BA and decreases cancer cell viability in eight days. Non-tumorigenic cells are at least five times less sensitive to PBA at the effective dose for cancer cells. These data suggest that PBA could be a promising cancer treatment and could possibly be used prophylactically. Phenylboronic acid has more favourable *in vitro* properties than do other compounds (e.g., migrastatin and carboxyamido-triazole). PBA shows a selective inhibition of breast and prostate cancer migration *in vivo* and of tumour metastasis in mice (Bradke et al., 2008). The properties that phenylboronic acid shares with other anti-cancer drugs highlight the fact that it is more effective than BA for cancer chemoprevention (Groziak, 2001; Yang et al., 2003).

4. Boron chemotherapy of breast cancer

4.1 Bortezomib

The drug Bortezomib (PS-341) (Teicher et al., 1999) is a boronic acid derivative and a proteasome inhibitor, which is a novel target in cancer therapy. This compound disrupts cell cycle regulation and induces apoptosis. Strong cytotoxic effects of PS-341 have been seen in prostate cancer cells and MCF-7 and EMT-6 breast carcinoma cells. In cell cultures, Bortezomib induces apoptosis in both haematologic and solid tumour malignancies, including myeloma, mantle cell lymphoma, cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, breast cancer, and head and neck cancers (Palumbo et al., 2008; Adams et al., 1999; Boccadoro et al., 2005; MacLaren et al., 2001). A good correlation has been observed between the Bortezomib dose, proteasome inhibition and positive modulation of serum PSA. Bortezomib has been approved by the US Food and Drug Administration for the treatment of chemorefractory multiple myeloma patients (Kane et al., 2003) and for some forms of non-Hodgkin's lymphoma (Goy et al., 2005). This inhibitor is still in clinical studies for multiple tumour types, including breast cancer (Boccadoro et al., 2005; Dees et al., 2004; Cardoso et al., 2004; Codony-Servat, 2006). In a phase II trial, the Bortezomib efficacy in patients with metastatic breast cancer was evaluated (Albenell et al., 2003). Although Bortezomib inhibits proteasome activity and reduces the circulating levels of IL6, these biological effects have not been associated with a meaningful clinical activity; no objective clinical response has been observed. Therefore, we do not recommend further investigations for Bortezomib as a single agent in the treatment of metastatic breast cancer (Yang et al., 2006). Until now, clinical experiments with Bortezomib have demonstrated only a limited activity against solid tumours when it is used as a single agent. However, Bortezomib could have a significant anti-tumour activity when it is used in combination with other active conventional agents (Agyin et al., 2009). Numerous trials using Bortezomib combination regimens are currently pending. Regarding breast cancer, the potential efficacy of Bortezomib with taxanes and anthracyclines is of particular interest. In a recent phase I trial that used Bortezomib together with docetaxel in anthracycline-pretreated advanced breast cancer, six of nine patients achieved partial response (Yang et al., 2006). *In vitro* and *in vivo* (murine xenograft) studies have revealed that Bortezomib is active against a variety of malignancies, including haematologic malignancies and solid tumours (i.e., breast, prostate, lung, pancreas, colon, ovarian, and head and neck cancers). Bortezomib has demonstrated

its activity as a single agent and in combination with several other cytotoxic agents, such as 5-fluorouracil, irinotecan, gemcitabine, doxorubicin and docetaxel, and with radiation, enhancing both chemotherapy- and radiation therapy (RT)-induced apoptosis. Bortezomib has also shown activity in some cell lines resistant to standard therapies. Bortezomib has been demonstrated to decrease the survival of cultured MCF-7 cells derived from human breast cancer and of EMT-6 parent mouse mammary carcinoma xenograft tumours in a dose-dependent manner (Teicher et al., 1999). In addition, combinations of Bortezomib with anthracyclines have been investigated, proving the prominent role of these agents in breast cancer therapy. Using a BT-474–based xenograft model of human breast cancer, the researchers found that the combination of Bortezomib with liposomal doxorubicin enhances anti-tumour efficacy and increases apoptosis, compared with the results obtained using each agent alone (Orlowski & Dees, 2003). Recently, a new proteasome inhibitor has been designed and synthesised; this compound, named BU-32, is a bisdipeptidyl boronic acid, a Bortezomib analogue, that contains an additional dipeptide boronic acid moiety on the pyrazine ring to potentially achieve a stronger binding affinity and an increased potency (Agyin et al., 2009). Divalent proteasome inhibitors, either hetero-bivalent or homo-bivalent, have been reported to increase the potency of inhibition by two orders of magnitude compared to the monovalent analogues, although a linker of 18 to 22 carbon atoms typically separates the active moieties in these compounds. A novel diboronated Bortezomib analogue, named BU-32 (NSC D750499-S), has been shown to be a potent and selective inhibitor of the chymotrypsin-like activity of the 20S proteasome. This compound has *in vitro* anti-tumour activity against different breast cancer cells and *in vivo* efficacy in mouse xenograft and metastasis models.

Paclitaxel (PAC), an anticancer drug, is used for the treatment of breast, ovarian, lung and head and neck cancer (Von Hoff, 1997; Rowinsky, 1997). However, this drug is known to cause adverse effects in different organs, such as heart and brain (Lachkar et al., 2006). Recently, results have clearly indicated that boron (as BA) supplementation to lymphocyte cultures ameliorates PAC-induced DNA damage (Turkez et al., 2010). BA plays a similar role in cancer patients after chemotherapy. However, specific protective agents for chemotherapy induce adverse effects after PAC or treatment with other anti-cancer drugs, although they should not interfere with the anti-tumour activity of the drugs (Pisano et al., 2003). The *in vivo* BA and PAC interactions are still unknown. At this point, further *in vivo* investigations are necessary to justify boron daily intake to minimise the adverse effects of anti-cancer drugs.

5. Mechanisms involving the activity of Boron compounds on cancer cells

5.1 Boron cross-talk with calcitriol

Assuming that boron and vitamin D are linked in a metabolic pathway, how does boron increase calcitriol levels? It seems unlikely that boron status would influence endogenous cholecalciferol synthesis, which is a nonenzymatic dermal reaction in which 7-dehydrocholesterol, an intermediate in cholesterol synthesis, is cleaved by ultraviolet light and then undergoes a spontaneous rearrangement. On the contrary, it seems likely that boron either up-regulates the 25-hydroxylation step or suppresses the major pathway of 25-OH-D catabolism, 24-hydroxylation (Miljkovic et al., 2004), giving the hypothesis that boron acts to suppress the latter reaction. Boron readily forms covalent complexes with cis-vicinal dihydroxy compounds. Thus, it is conceivable that boron can form such a complex with

24,25-dihydroxyvitamin D, the final product of the 25-OH-D reaction with 24-hydroxylase. Either this postulated complex acts as a competitive inhibitor for the 24-hydroxylase reaction, or, alternatively, acts to down-regulate the expression of this enzyme. Another possibility is that boron is a direct enzyme inhibitor at very modest concentrations. Indeed, boron can inhibit numerous enzymes, usually at supraphysiological concentrations (Hunt, 1994). Testing these hypotheses *in vitro* are mandatory, using hepatocytes or other cells capable of expressing the 24-hydroxylase activity. Clinically, the testable implication of this hypothesis is that boron supplementation should increase 25-OH-D serum levels, while 24,25-dihydroxyvitamin D serum levels remain constant or decline. This implication would strengthen the interesting possibility by which boron is a potent inhibitor for a range of microsomal enzymes, which catalyse the insertion of the hydroxyl group vicinal to the existing hydroxyl groups in steroids; specific examples of such enzymes are 24-hydroxylase and the estradiol hydroxylases (Miljkovic et al., 2004). Epidemiological studies have shown an inverse relationship between exposure to solar radiation and high breast cancer incidence and mortality (Garland et al., 1990; John et al., 1999). Another plausible link between 1,25-$(OH)_2D_3$ and breast cancer has been uncovered by the observation that the 20q13.2 chromosomal region, which contains 24-hydroxylase (CYP24), is amplified in breast cancer. Because 24-hydroxylase is involved in the degradation of 1,25-$(OH)_2D_3$, its amplification may lead to a decrease in 1,25-$(OH)_2D_3$ serum levels, thus providing a conducive microenvironment for cell growth in the absence of vitamin D-mediated growth control (Guryev et al., 2003). Furthermore, 1,25-$(OH)_2D_3$ serum levels have been found to be reduced to a greater extent in advanced bone metastatic breast cancer patients than in early stage patients (Mawer et al., 1997). The VDR is expressed in most breast cancer cell lines, carcinogen-induced rat mammary tumours, normal breast tissues and primary breast cancer tumours. Furthermore, increased RXR and VDR protein levels have been found in breast cancer tissues, compared with levels in normal breast tissues (Bortman et al., 2002; Friedrich et al., 2002). Several studies have demonstrated that a high proportion of breast cancer biopsy specimens contain vitamin D receptors (VDR) (Freake et al., 1984; Eisman et al., 1986; Berger et al., 1987). Moreover, an association between VDR levels and prognosis appears to exist; tumour receptor status has been positively related to disease-free survival (Colston et al., 1989; Berger et al., 1991).

Several studies have indicated that the activation of the cell death pathway is an important aspect of the anti-tumour effects of vitamin D analogues in breast cancer cells. Further characterisations of the apoptosis-related genes, which are regulated directly or indirectly by vitamin D derivatives, will provide the basis for the design of new compounds able to target these pathways in breast cancer cells. Understanding how the apoptotic pathway, mediated by the vitamin D, can modulate or overlap with more-established pathways leading to cell death is likely to provide clinically useful information. When biologically active vitamin D is needed, 25(OH)D in the kidney is enzymatically converted to its active form, 1,25$(OH)_2D_3$ (calcitriol). This conversion is mediated by the 25(OH) vitamin D 1α-hydroxylase, a cytochrome P450 protein, and 1,25$(OH)_2D_3$ is then conducted to the tissues. The actions of calcitriol in calcium homeostasis control of cell growth and differentiation, cell adhesion and apoptosis (controlled cell death) are mediated by its interaction with the VDR, which is a member of the nuclear receptor super-family (Thorne & Campbel, 2008).

In a recent study (Liu et al., 2006), it was reported that one of the first observed responses upon infection of human monocytes with tuberculosis (*M. tuberculosis*) is the activation of

the cytochrome P450 enzyme encoded by the gene *Cyp27B1,* which converts vitamin D to its active form. When calcitriol is available for the cell, it is able to synthesise cathelicidin, an antimicrobial peptide that can destroy the tuberculosis bacteria. When calcitriol is not available, cathelicidin cannot be synthesised and the defence mechanism fails. The protective effects of calcitriol against the development of cancer are mainly due to its role in regulating the cell cycle (Gombart et al., 2005). Calcitriol and functional VDR are required for normal control of the cell cycle. Normal cell growth is controlled by regulating the levels and activity of cyclins and their dependent kinases. Calcitriol and boron affect the cyclin pathways by regulating gene expression of the proteins p27 and p21; the consequence of this regulation is inhibition of the cyclin dependent kinases (CDKs). Calcitriol and its active complexes interact with cyclin D and have a protective effect by blocking cell proliferation (Holick, 2007; Ingraham et al., 2008).

5.2 Boron and proteasome inhibition

The proteasome inhibitor, Bortezomib, exhibits anti-proliferative, pro-apoptotic, anti-angiogenic and anti-tumour activities in several cancer models (Cardoso et al., 2004). The mechanism of action of Bortezomib involves the stabilisation of NF-κB, p21, p27, p53, Bid and Bax, the inhibition of caveolin-1 activation, the activation of JNK and the endoplasmic reticulum stress response (Boccadoro et al., 2005). These preclinical evaluations have revealed that Bortezomib is well tolerated at doses that demonstrate an anti-tumour activity in xenograft models of multiple myeloma, adult T-cell leukaemia, lung, breast, prostate, pancreas, head and neck, colon cancers and melanoma (Yang et al., 2003). The proteasome is an adenosine triphosphate (ATP)–dependent multi-catalytic protease that is present in the cytoplasm and the nucleus of all eukaryotic cells, from those in yeast to those in humans. The proteasome represents approximately 1% of the cellular proteins and is responsible for the non-lysosomal degradation of most intracellular proteins. The 26S proteasome consists of two functional entities: the 20S core catalytic complex and the 19S regulatory subunits. The proteasome has three catalytic activity types: chymotryptic-like, tryptic-like and caspase-like or postglutamyl cleavage activity. The boronic acid group forms a complex with the threonine hydroxyl group in the chymotrypsin-like active site and acts as a reversible inhibitor of the chymotryptic-like activity of the proteasome, which is sufficient to inhibit proteolysis (Boccadoro et al., 2005). In addition to the removal of damaged or unnecessary proteins, proteasome-mediated proteolysis is also an important mechanism for regulating the levels of some key regulatory proteins and their inhibitors. This regulation is crucial for controlling many cellular processes, including the activation of transcription factors, cell cycle progression, angiogenesis, cell adhesion, cytokine production and apoptosis. Many processes that rely on proteasome function contribute to the growth and survival of cancer cells. Thus, the critical role of the ubiquitin–proteasome pathway has led to the investigation of proteasome inhibition as a potential anti-cancer therapy (D'Alessandro et al., 2009). In cancer, the ubiquitin–proteasome pathway plays a number of important roles, including the regulation of tumour growth through multiple targets influencing cell cycle progression and apoptosis, cell adhesion, invasion and metastasis. The ubiquitin–proteasome pathway is also required for transcriptional regulation. Nuclear factor–κB (NF-κB) is a critical transcription factor involved in immune responses and cellular growth. Constitutive activation of NF-κB has been shown to be involved in the development of many human malignancies, including breast cancer (Cardoso et al., 2004).

NF-κB activation is regulated by the proteasome-mediated degradation of the IκB inhibitor protein. After IκB degradation, NF-κB moves to the nucleus and regulates genes encoding cytokine-like tumour necrosis factor (TNF), interleukin (IL)–1, IL-2, and IL-630, pro-inflammatory enzymes (nitric oxide synthase, cyclooxygenase-2), chemotactic factors (IL-8 and the monocyte chemoattractant protein–1) and cell adhesion molecules, such as E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), which are involved in tumour metastasis and angiogenesis (Boccadoro et al., 2005). NF-κB also regulates genes involved in the expression of anti-apoptotic proteins, such as members of the Bcl-2 and inhibitors of apoptosis families, which mediate resistance to chemotherapy and radiation therapy (RT) (Dong et al., 2002). Malignancies with high-activated NF-κB levels, such as breast cancer, should be sensitive to the interruption of this pathway. The level of NF-κB in tumour cells is high in the estrogen receptor (ER)–negative human breast cancers. Inhibition of NF-κB activation has been suggested to be a potential therapeutic approach in such tumours (Ling et al., 2003). The ubiquitin–proteasome pathway is also involved in ER turnover and in the regulation of growth factor receptors such as HER2/*neu* and EGFR (Tikhomirov et al., 2000; Mimnaugh et al., 1996; Magnifico et al., 1998) and oncoproteins, such as c-fos/c-jun, c-myc and N-myc. Although proteasome inhibition stabilises or increases the levels of these growth factors, preclinical studies have demonstrated that this stabilisation does not lead to activation of proliferation or increases in tumour growth.

6. Conclusion

Many scientific data exist that have shown that boron is an essential microelement in animal cells. With the knowledge that borate linkages function in cell-to-cell adhesion, it has been hypothesised that boronates target structural glycoproteins located along the cytoskeleton-plasma membrane-cell wall assembly. The latter are normally cross-linked by boron, and results confirm that boronates can indeed disrupt a boron-glycoprotein linkage. Therefore, any biological function of boron represents the result of its role as a cross-linking molecule.
Deficiency of boron in the diet has been linked to several pathological conditions, including some forms of cancer, osteoporosis and osteoarthritis.
Breast cancer affects many women all over the world and has a favourable prognosis when it is discovered in time (in its initial phase). Diets rich in boron could significantly reduce some cancer types, especially breast, prostate, lung and cervical forms of cancer. Discovering the role of boron in animal cell metabolism will have a great significance in the "war" against cancer. Establishing boron as an anti-cancer agent in breast cancer will encourage women with increased risk factors for this disease. Thus, these women will increase their intake of boron-rich food (i.e., avocado, broccoli, raisins and nuts) and dietary boron supplements to reduce their chance of developing this disease.
The present paper highlights the role of boron in the prevention, chemoprevention and chemotherapy of some forms of cancer, including breast cancer.
The major elements in breast cancer prevention, chemoprevention and chemotherapy with boron include:

1. for prevention, keep a diet rich in boron (around 20 mg per day) and an adequate 4:1 balance between omega-6 and omega-3 in nutrition;
2. in chemoprevention, BA and CF are the main "fighters" against breast cancer. Based on experimental evidence, BA induces apoptosis in both melanoma cells and MDA231

breast cancer cells and inhibits growth of breast cancer cell lines, like ZR-75-1 cells. CF has shown inhibitory effects on MDA-MB-231 breast cancer cells. In addition, it raises the calcitriol level in blood, thus increasing the level of protection against the development of breast cancer;

3. in chemotherapy, Bortezomib is a novel target in cancer therapy, being approved by the US Food and Drug Administration. It exhibits anti-proliferative, pro-apoptotic, anti-angiogenic and anti-tumour activities in several cancer models, including breast cancer.

In conclusion, even though the actual B requirements for the human body remain unclear and further researches are necessary, the amount of B in prevention and chemoprevention of breast cancer up to the 'Tolerable Upper Intake Level' (~ 20 mg B per day) is probable the adequate amount of boron in the body that might diminish the incidence of some forms of cancer, including breast cancer. However, it is compulsory to discover and develop new boron-containing compounds with anti-tumour activity.

7. References

Acerbo, A.S. & Miller, L. (2009). Assessment of the chemical changes induced in human melanoma cells by boric acid treatment using infrared imaging. *Analyst*, Vol. 134, pp. 1669-1674.

Adams, J.; Palombella, V.J.; Sausville, E.A.; Johnson, J.; Destree, A.; Lazarus, D.D.; Maas, J.; Pien, C.S.; Prakash, S. & Elliott, P.J. (1999). Proteasomes inhibitors: a novel class of potent and effective antitumor agents. *Cancer Research,* Vol. 59, pp. 2615-2622.

Agyin, J.K.; Santhamma, B.; Nair, H.B.; Roy, S.S. & Tekmal, R.R. (2009). BU-32: a novel proteasomes inhibitor for breast cancer. *Breast Cancer Research*, Vol. 11, No. 5, pp. 1-13.

Albenell, J.; Baselga, J. & Guix, M. (2003). Phase I study of bortezomib in combination with docetaxel in anthrcycline-pretreated advanced breast cancer. *Proceedings of American Society of Clinical Oncology,* Vol.22, No.16, (abstr.63), pp. 16.

Armstrong, B. & Doll, R. (1975). Environmental factors and cancer incidence and mortality in different countries with special reference to dietary practices. *International Journal of Cancer*, Vol. 15, pp. 617–625.

Baker, S.J.; Zhang, Y.K.; Akama, T.; Lau, A.; Zhou, H.; Hernandez, V.; Mao, W.; Alley, M.R.K.; Sanders, V. & Plattner, J.J. (2006). Discovery of a new boron-containing antifungal agent, 5-fluoro-1,3-dihydro-1-hydroxy-2,1-benzoxaborole (AN2690), for the potential treatment of onychomycosis. *Journal of Medical Chemistry*, Vol. 49, pp. 4447-4450.

Baldock, C.; Boer, G.J.D.; Rafferty, J.B.; Stuitje, A.R. & Rice, D.W. (1998). Mechanism of action of diazaborines. *Biochemical Pharmacy*, Vol. 55, pp. 1541-1549.

Barranco, W.T. & Eckhert, C.D. (2006). Cellular changes in boric acid treated DU-145 prostate cancer cells. *British Journal of Cancer*, Vol. 94, pp. 884- 890.

Barranco, W.T.; Hudak, P. & Eckhert, C.D. (2007). Evaluation of ecological and *in vitro* effects of boron on prostate cancer risk (United States). *Cancer Cause Control*, Vol. 18, pp. 71-77.

Barranco, W.T.; Kim, H.T; Stella Jr., S.L. & Eckhert, C.D. (2009). Boric acid inhibits stored Ca^{2+} release in DU-145 prostate cancer cells. *Cell Biology and Toxicology,* Vol. 25, pp. 309-320.

Beddoe, A.H. (1997). Boron neutron capture therapy. *British Journal of Radiology*, Vol. 70, pp. 665-667.

Bemd, G.J. & Chang, G.T. (2002). Vitamin D and vitamin D analogs in cancer treatment. *Current Drug Targets*, Vol. 3, pp. 85–94.

Benkovic, S.J.; Baker, S.J.; Alley, M.R.K.; Woo, Y.H.; Zhang, Y.K.; Akama, T.; Mao, W.; Baboval, J.; Ravi-Rajagopalan, P.T.; Wall, M.; Kahng, L.S.; Tavassoli, A. & Shapiro, L. (2005). Identification of boronic esters as inhibitors of bacterial cell growth and bacterial methyltransferases, CcrM and MenH. *Journal of Medicinal Chemistry*, Vol. 48, pp. 7468-7476.

Berger, U.; Wilson, P.; McClelland, R.; Colston, K.; Haussler, M.R.; Pike, J.W. & Coombes, R.C. (1987). Immunocytochemical detection of 1,25-dihydroxyvitamin D3 receptor in primary breast cancer. *Cancer Research,* Vol. 47, pp. 6793–6795.

Berger, U.; McClelland, R.A.; Wilson, P.; Greene, G.L.; Haussler, M.R.; Pike, J.W.; Colston, K.; Easton, D. & Coombes, R.C. (1991). Immunocytochemical detection of estrogen receptor, progesterone receptor and 1,25-dihydroxyvitamin D3 receptor in breast cancer and relation to prognosis. *Cancer Research,* Vol. 51, pp. 239–244.

Blevins, D.G. & Lukaszewski, K.M. (1994). Proposed physiologic functions of boron in plants pertinent to animal and human metabolism. *Environmental Health Perspectives*, Vol. 102, pp. 31-33.

Boccadoro, M.; Morgan, G. & Cavenagh, J. (2005). Preclinical evaluation of the proteasomes inhibitor bortezomib in cancer therapy. *Cancer Cell International,* Vol. 5, No. 18.

Bolanos, L.; Lukaszewski, K.; Bonilla, I. & Blevins, D. (2004). Why boron? *Plant Physiology and Biochemistry*, Vol. 42, pp. 907-912.

Bortman, P.; Folgueira, M.A.K.; Katayama, M.L.H.; Snitcovsky, I.M.L. & Brentani, M.M. (2002). Antiproliferative effects of 1,25-dihydroxyvitamin D3 on breast cells: a mini review. *Brazilian Journal of Medical and Biological Research*, Vol. 35, pp. 1–9.

Bradke, T.; Hall, C.; Stephen, W.; Carper, S.W. & Plopper, G.E. (2008). Phenylboronic acid selectively inhibits human prostate and breast cancer cell migration and decreases viability. *Cell Adhesion and Migration,* Vol. 2, pp. 153-160.

Brown, P.H. & Shelp, B.J. (1997). Boron mobility in plants. *Plant Soil*, Vol. 193, pp. 85–101.

Buras, R.; Schumaker, L.M. & Davoodi, F. (1994). Vitamin D receptors in breast cancer cells. *Breast Cancer Research and Treatment*, Vol. 31, pp. 191–202.

Caygill, C.P. & Hill, M.J. (1995). Fish, n-3 fatty acids and human colorectal and breast cancer mortality. *European Journal of Cancer Prevention*, Vol. 4, No. 4, pp. 329-332.

Caygill, C.P.; Charlett, A. & Hill, M.J. (1996). Fat, fish, fish oil and cancer. *British Journal of Cancer*, Vol. 74, No.1, pp. 159-164.

Cardoso, F.; Ross, J.S.; Picart, M.J.; Sotiriou, C. & Durbecq, V. (2004). Targeting the ubiquitin-proteasomes pathway in breast cancer. *Clinical Breast Cancer,* Vol. 5, pp. 148-157.

Castellsague, X.; Bosch, X.F. & Munoz, N. (2002). Environmental cofactors in HPV carcinogenesis. *Virus Research*, Vol. 89, pp. 191-199.

Codony-Servat, J.; Tapia, M.A.; Bosch, M.; Oliva, C.; Domingo-Domenech, J.; Mellado, B.; Rolfe, M.; Ross, J.S.; Gascon, P.; Rovira, A. & Albanell, J. (2006). Differential cellular and molecular effects of bortezomib, a proteasomes inhibitor, in human breast cancer cells. *Molecular Cancer Therapeutics,* Vol. 5, pp. 665-675.

Colston, K.W.; Berger, U. & Coombes, R.C. (1989). Possible role for vitamin D in controlling breast cancer cell proliferation. *Lancet,* Vol. 1, pp. 185–191.

Colston, K.W. & Hansen, C.M. (2002). Mechanisms implicated in the growth regulatory effects of vitamin D in breast cancer, *Endocrine Related Cancer*, Vol. 9, pp. 45–59.

D'Alessandro, A.; Pieroni, L.; Ronci, M.; D'Aguanno, C.; Federici, G. & Urbani, A. (2009). Proteasomes Inhibitors Therapeutic Strategies for Cancer. *Recent Patents on Anti-Cancer Drug Discovery,* Vol. 4, pp. 73-82.

Deckere, E.A. (1999). Possible beneficial effect of fish and fish n-3 polyunsaturated fatty acids in breast and colorectal cancer. *European Journal of Cancer Prevention*, Vol. 8, No. 3, pp. 213-221.

Dees, E.; O'Neil, B. & Humes, E. (2004). Phase I clinical trial of the proteasome inhibitor bortezomib in combination with pegylated liposomal doxorubicin in patients with refractory solid tumors. *Proceedings of American Society of Clinical Oncology,* Vol. 22, No. 217, (abstr.868).

Devirian, T. & Volpe, S. (2003). The physiological effects of dietary boron. *Critical Reviews in Food Science and Nutrition*, Vol. 43, pp. 219-231.

Dimitrakakis, C.; Zava, D.; Marinopoulos, S.; Tsigginou, A.; Antsaklis, A. & Glaser, R. (2010). Low salivary testosterone levels in patients with breast cancer BMC. *Cancer*, Vol. 10, pp. 547.

Dong, Q.G.; Sclabas, G.M.; Fujioka, S.; Schmidt, C.; Peng, B.; Wu, T.; Tsao, M.S.; Evans, D.B.; Abbruzzese, J.L.; McDonnell, T.J. & Chiao, P.J. (2002). The function of multiple IkappaB: NF-kappaB complexes in the resistance of cancer cells to Taxol-induced apoptosis. *Oncogene*, Vol. 21, pp. 6510-6519.

Eisman, J.A.; Suva, L.J. & Martin, T.J. (1986). Significance of 1,25-dihydroxyvitamin D3 receptor in primary breast cancers. *Cancer Research,* 46, pp. 5406–5408.

Elegbede, A.F. (2007). Mechanism of boric acid analog cytotoxicity in breast cancer cells. M.S. Thesis, University of Nevada Las Vegas United States.

Endo, Y.; Yoshimi, T. & Miyaura, C. (2003). Boron clusters for medicinal drug design: Selective estrogen receptor modulators bearing carborane. *Pure and Applied Chemistry*, Vol. 75, pp. 1197-1205.

Espey, D.K.; Wu, X.C.; Swan, J.; Wiggins, C.; Jim, A.M.; Ward, E.; Wingo, P.A.; Howe, H.L.; Ries, L.A.G.; Miller, B.A.; Jemal, A.; Ahmed, F.; Cobb, N.; Kaur, J.S.; Edwards, B.K. (2007). Annual report to the nation on the status of cancer, 1975-2004, featuring cancer in American indians and Alaska natives. *Cancer*, Vol. 110, pp. 2119- 2152.

Fort, D.J.; Stover, E.L.; Strong, P.L.; Murray, F.J. & Keen, C.L. (1999). Chronic feeding of a low boron diet affects reproduction and development in Xenopus laevis. *Biological Trace Element Research*, Vol. 129, pp. 2055-2060.

Freake, H.C.; Abeasekekera, G.; Iwasaki, J.; Marocci, C.; MacIntyre, I.; McClelland, R.A.; Skilton, R.A.; Easton, D.F. & Coombes, R.C. (1984). Measurement of 1,25-

dihydroxyvitamin D3 receptors in breast cancer and relationship to biochemical and clinical indices. *Cancer Research,* Vol. 44, pp. 1677–1681.

Freedman, D. M.; Looker, A. C.; Chang, S.C. & Graubard B. I. (2007). Prospective Study of Serum Vitamin D and Cancer Mortality in the United States. *Journal of the National Cancer Institute*, Vol. 99, pp. 1594–1602.

Friedrich, M.; Axt-Fliedner, R.; Villena-Heinsen, C.; Tilgen, W.; Schmidt, W. & Reichrath, J. (2002). Analysis of vitamin D receptor (VDR) and retinoid X receptor in breast cancer. *Histochemistry Journal*, Vol. 34, pp. 35–40.

Fritsche, K.L. & Johnston, P.V. (1990). Effect of dietary alpha-linolenic acid on growth, metastasis, fatty acid profile and prostaglandin production of two murine mammary adenocarcinomas. *Journal of Nutrition,* Vol. 120, No. 12, pp. 1601-1609.

Gallardo-Williams, M.T.; Maronpot, R.R.; Wine, R.N.; Brunssen, S.H. & Chapin, R.E. (2003). Inhibition of the enzymatic activity of prostatespecific antigen by boric acid and 3-nitrophenyl boronic acid. *The Prostate*, Vol. 54, pp. 44-49.

Gann, P.H.; Hennekens, C.H.; Ma, J.; Longcope, C. & Stampfer, M.J. (1996). Prospective study of sex hormone levels and risk of prostate cancer. *Journal of the National Cancer Institute*, Vol. 88, pp. 1116-1126.

Garland, F.C.; Garland, C.F.; Gorham, E.D. & Young, J.F. (1990). Geographic variation in breast cancer mortality in the United States: a hypothesis involving exposure to solar radiation. *Preventive Medicine*, Vol. 19, pp. 614–622.

Garland, C.F.; Garland, F.C. & Gorham, E.D. (2006). The role of vitamin D in cancer prevention. *American Journal of Public Health*, Vol. 96, pp. 252–261.

Garland, C. F.; Grant, W. B.; Mohr, S.B.; Gorham, E.D. & Garland, F.C. (2007). What is the Dose-Response Relationship between Vitamin D and Cancer Risk? *Nutrition Reviews,* Vol. 65, No. 8, pp. S91-S95.

Gombart, A.F.; Borregaard, N. & Koeffler, H.P. (2005). Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *The FASEB Journal,* Vol. 19, pp. 1067-1077.

Gonzalez, A.; Peters, U.; Lampe, J.W. & White, E. (2007). Boron intake and prostate cancer risk. *Cancer Causes & Control,* Vol. 18, pp. 1131-1140.

Goy, A.; Younes, A. & McLaughlin, P. (2005). Phase II study of proteasomes inhibitor bortezomib in relapsed or refractory B-cell non-Hodgkin's lymphoma. *Journal of Clinical Oncology*, Vol. 23, pp. 667–675.

Greenlee, R.T.; Murray, T.; Bolden, S. & Wingo, P.A. (2000). Cancer statistics, 2000. *CA Cancer Journal of Clinicians*, Vol. 50, pp. 7-33.

Groziak, M.P. (2001). Boron therapeutics on the horizon. *American Journal of Therapeutics,*Vol. 8, pp. 321-328.

Guryev, O.; Carvalho, R.A.; Usanov, S.; Gilep, A. & Estabrook, R.W. (2003). A pathway for the metabolism of vitamin D3: Unique hydroxylated metabolites formed during catalysis with cytochrome P450scc (CYP11A1). *Proceedings of the National Academy of Sciences*, Vol. 100, No.25, pp. 14754-14759.

Gissel, T.; Rejnmark, L.; Mosekilde, L. & Vestergaard, P. (2008). Intake of vitamin D and risk of breast cancer—A meta-analysis. *Journal of Steroid Biochemistry and Molecular Biology*, Vol. 111, No. 3-5, pp. 195-199.

Harris, J.R.; Lippman, M.E.; Veronesi, U. & Willett, W. (1992). Breast cancer. *The New England Journal of Medicine*, Vol. 327, pp. 319-328.

Hegsted, M.; Keenan, M.J.; Siver, F. & Wozniak, P. (1991). Effect of boron on vitamin D deficient rats. *Biological Trace Elements Research*, Vol. 28, pp. 243-255.

Henderson, K.; Stella Jr., S.L.; Kobylewski, S. & Eckhert, C.D. (2009). Receptor activated Ca^{2+} release is inhibited by boric acid in prostate cancer cells. *Plos One*, Vol. 4, No. 6, pp. 1-10.

Hofling, M.; Hirschberg, A.L.; Skoog, L.; Tani, E.; Hagerstrom, T. & von Schoultz, B. (2007). Testosterone inhibits estrogen/progestogen-induced breast cell proliferation in postmenopausal women. *Menopause*, Vol. 14, No. 2, pp. 183-190.

Holick, M.F. (2007). Vitamin D deficiency. *The New England Journal of Medicine*, Vol. 357, pp. 266-81.

Holmes, M.D.; Hunter, D.J. & Colditz, G.A. (1999). Association of dietary intake of fat and fatty acids with risk of breast cancer. *Journal of the American Medical Association*, Vol. 281, pp. 914-920.

Hunt, C.D. (1994). The biochemical effects of physiologic amounts of dietary boron in animal nutrition models. *Environmental Health Perspectives*, Vol. 102, suppl. 7, pp. 35–43.

Hunt, C.D. (1998). Regulation of enzymatic activity: one possible role of dietary boron in higher animals and humans. *Biological Trace Element Researche*, Vol. 66, pp. 205-225.

Hunter, D.J. & Willett, W.C. (1996). Dietary factors. In: *Diseases of the Breast. Lippincott-Raven*, Philadelphia, PA, pp. 201–212.

Hunter, D.J.; Spiegelman, D.; Adami, H.O.; Beeson, L.; van den Brandt, P.A.; Folsom, A.R.; Fraser, G.E.; Goldbohm, R.A.; Graham, S. & Howe, G.R. (1996). Cohort studies of fat intake and the risk of breast cancer – a pooled analysis. *The New England Journal of Medicine*, Vol. 334, pp. 356–361.

Ingraham, B.A.; Bragdon, B. & Nohe A. (2008). Molecular basis of the potential of vitamin D to prevent cancer. *Current Medical Research and Opinion*, Vol. 24, No. 1, pp. 139–149.

Ishii, T. & Matsunaga, T. (1996). Isolation and characterization of a boron–rhamnogalacturonan-II complex from cell walls of sugar beet pulp. *Carbohydrates Research*, Vol. 284, pp. 1–9.

Ishii, T. & Matsunaga, T. (2001). Pectic polysaccharide rhamnogalacturonan II is covalently linked to homogalacturonan. *Phytochemistry*, Vol. 57, pp. 969–974.

Jabbour, A.; Steinberg, D.; Dembitsky, V.M.; Moussaieff, A.; Zaks, B. & Srebnik, M. (2004). Synthesis and evaluation of oxazaborolidines for antibacterial activity against *Streptococcus mutans*. *Journal of Medicinal Chemistry*, Vol. 47, pp. 2409-2410.

Jabbour, A.; Smoum, R.; Takrouri, K.; Shalom, E.; Zaks, B.; Steinberg, D.; Rubinstein, A.; Goldberg, I.; Katzhendler, J. & Srebnik, M. (2006). Pharmacologically active boranes. *Pure and Applied Chemistry*, Vol. 78, pp. 1425-1453.

John, E.M.; Schwartz, G.G.; Dreon, D.M. & Koo, J. (1999). Vitamin D and breast cancer risk: the NHANES epidemiologic follow-up study, 1971–1975 to 1992. National Health

and Nutrition Examination Survey. *Cancer Epidemiology, Biomarkers and Prevention*, Vol. 8, pp. 399–406.

Kane, R.C.; Bross, P.F.; Farrell, A.T.; Pazdur, R. & Velcade, U.S. (2003). FDA approval for the treatment of multiple myeloma progressing on prior therapy. *Oncologist*, Vol. 8, pp. 508-513.

Kelsey, J.L. (1979). A review of the epidemiology of human breast cancer. *Epidemiologic Reviews*, Vol. 1, pp. 74-109.

Kim, J.; Lim, S-Y.; Shin, A.; Sung, M-K.; Ro, J.; Kang, H-S.; Lee, K.S.; Kim, S-W. & Lee, E-S. (2009). Fatty fish and fish omega-3 fatty acid intakes decrease the breast cancer risk: a case-control study. *BMC Cancer,* Vol. 9, pp. 216.

Korkmaz, M.; Uzgo, E.; Bakirdere, S.; Aydin, F. & Ataman, Y. (2007). Effects of dietary boron on cervical cytopathology and on micronucleus frequency in exfoliated buccal cells. *Environmental Toxicology*, Vol. 22, pp. 17-25.

Lachkar, S.; Bota, S.; Nouvet, G. & Thiberville, L. (2006). Acute encephalopathy after infusion of paclitaxel. *Revue des Maladies Respiratoires,* Vol. 23, pp. 73–77.

Ling, Y.H.; Liebes, L.; Jiang, J.D.; Holland, J.F.; Elliott, P.J.; Adams. J.; Muggia, F.M. & Perez-Soler R. (2003). Mechanisms of proteasomes inhibitor PS-341- induced G(2)-M-phase arrest and apoptosis in human nonsmall cell lung cancer cell lines. *Clinical Cancer Research,* Vol. 9, pp. 1145-1154.

Liu, P.T.; Stenger, S. & Li, H. (2006). Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science,* Vol. 311, pp. 1770-3.

Longnecker, M.P.; Berllin, J.A.; Orza, M.J. & Chalmers, T.C. (1988). A metaanalysis of alcohol consumption in relation to breast cancer risk. *Journal of the American Medical Association*, Vol. 260, No. 5, pp. 652-6.

MacLaren, A.P.; Chapman, R.S.; Wyllie, A.H. & Watson, C.J. (2001). p53-dependent apoptosis induced by proteasomes inhibition in mammary epithelial cells. *Cell Death and Differentiation*, Vol. 8, pp. 210-218.

MacLean, C.H.; Newberry, S.J.; Mojica, W.A.; Khanna, P.; Issa, A.M.; Suttorp, M.J.; Lim, Y.W.; Traina, S.B.; Hilton, L. & Garland, R. (2006). Effects of omega-3 fatty acids on cancer risk: a systematic review. *Journal of the American Medical Association,* Vol. 295, No. 4, pp. 403-415.

Magnifico, A.; Tagliabue, E. & Ardini, E. (1998). Heregulin beta1 induces the down regulation and the ubiquitin-proteasome degradation pathway of p185HER2 oncoprotein. *FEBS Letters,* Vol. 422, pp. 129-131.

Mahabir, S.; Spitz, M.R.; Barrera, S.L.; Dong, Y.Q.; Eastham, C. & Forman, M.R. (2008). Dietary boron and hormone replacement therapy as risk factors for lung cancer in women. *American Journal of Epidemiology*, Vol. 167, pp. 1070-1080.

Malone, W.F.; Kelloff, G.J.; Boone, C. & Nixon, D.W. (1989). Chemoprevention and modern cancer prevention. *Preventive Medicine*, Vol. 18, pp. 2553-61.

Maskarinec, G.; Murphy, S.; Shumay, D.M. & Kakai, H. (2001). Dietary changes among cancer survivors. *European Journal of Cancer Care,* Vol. 10, No. 1, pp. 12–20.

Mawer, E.B.; Walls, J.; Howell, A.; Davies, M.; Ratcliffe, W.A. & Bundred, N.J. (1997). Serum 1,25-dihydroxyvitamin D may be related inversely to disease activity in breast

cancer patients with bone metastases. *Journal of Clinical Endocrinoly & Metabolism*, Vol. 82, pp. 118–122.

McTiernan, A.; Rajan, K.B.; Tworoger, S.S.; Irwin, M.; Bernstein, L.; Baumgartner, R.; Gilliland, F.; Stanczyk, F.Z.; Yasui, Y. & Ballard-Barbash, R. (2003). Adiposity and Sex Hormones in Postmenopausal Breast Cancer Survivors. *Journal of Clinical Oncology,* Vol. 21, No. 10, pp. 1961-1966.

Meacham, S.L.; Elwell, K.E.; Ziegler, S. & Carper, S.W. (2007). In: *Advances in Plant and Animal Boron Nutrition,* F. Xu, (Ed.), pp. 299-306, Springer: New York.

Meacham, S.; Karakas, S.; Walace, A. & Altun, F. (2010). Boron in human health evidence for dietary recomandations and public policies. *The Open Mineral Processing Journal*, Vol. 3, pp. 36-53.

Mehta, R.G. & Mehta, R.R. (2002). Vitamin D and cancer. *Journal of Nutritional Biochemistry*, Vol. 13, pp. 252–264.

Mimnaugh, E.G.; Chavany, C. & Neckers, L. (1996). Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. *Journal of Biological Chemistry,* Vol. 271, pp. 22796-22801.

Miwa, K. & Fujiwara, T. (2010). Boron transport in plants: co-ordinated regulation of transporters. *Annals of Botany*, Vol. 105, pp. 1103–1108.

Morin, C. (1994). The chemistry of boron analogues of biomolecules. *Tetrahedron*, Vol. 50, pp. 12521-12569.

Miljkovic, D.; Miljkovic, N. & McCartya, M.F. (2004). Up-regulatory impact of boron on vitamin D function – does it reflect inhibition of 24-hydroxylase? *Medical Hypotheses,* Vol. 63, pp. 1054–1056.

Miljkovic, D.; Scorei, I.R.; Cimpoiasu, V.M. & Scorei, I.D. (2009). Calcium fructoborate: plant-based dietary boron for human nutrition. *Journal of Dietary Supplements,* Vol. 6, pp. 211-226.

Naghii, M.R. & Samman, S. (1997). The effect of boron supplementation on its urinary excretion and selected cardiovascular risk factors in healthy male subjects. *Biological Trace Element Research*, Vol. 56, pp. 273–86.

Naghii, M.R.; Mofid, M.; Asgari, A.R.; Hedayati, M. & Daneshpour M-S. (2010). Comparative effects of daily and weekly boron supplementation on plasma steroid hormones and pro-inflammatory cytokines. *Journal of Trace Elements in Medicine and Biology*, [Epub ahead of print].

Nielsen, F.H.; Hunt, C.D.; Mullen, L.M. & Hunt, J.R. (1987). Effect of dietary boron on mineral, estrogen, and testosterone metabolism in postmenopausal women. *FASEB Journal*, Vol. 1, pp. 394–7.

Nielsen, F.H. (2000). The emergence of boron as nutritionally important throughout the life cycle. *Nutrition*, Vol. 16, pp. 512-514.

Nielsen, F.H. (2004). Dietary fat composition modifies the effect of boron on bone characteristics and plasma lipids in rats. *Biofactors,* Vol. 20, No. 3, pp. 1 -71.

Nielsen, F.H. & Penland, J.G. (2006). Boron deprivation alters rat behavior and brain mineral composition differently when fish oil instead of safflower oil is the diet fat source. *Neuroscience and Nutrition*, Vol. 9, No.1-2, pp. 105-12.

Nielsen, F.H. (2008). Is boron nutritionally relevant? *Nutrition Reviews*, Vol. 66, pp. 183-191.

Nielsen, F.H. (2009). Boron deprivation decreases liver S adenosylmethionine and spermidine and increases plasma homocysteine and cysteine in rats. *Journal of Trace Elements in Medicine and Biology*, Vol. 23, pp. 204-213.

Orlowski, R.Z. & Dees, E.C. (2003). The role of the ubiquitination-proteasome pathway in breast cancer: applying drugs that affect the ubiquitin-proteasome pathway to the therapy of breast cancer. *Breast Cancer Research*, Vol. 5, pp. 1-7.

Palumbo, A.; Gay, F.; Bringhen, S.; Falcone, A.; Pescosta, N.; Callea, V.; Caravita, T.; Morabito, F.; Magarotto, V.; Ruggeri, M.; Avonto, I.; Musto, P.; Cascavilla, N.; Bruno, B. & Boccadoro, M. (2008). Bortezomib, doxorubicin and dexamethasone in advanced multiple myeloma. *Annals of Oncology*, Vol. 19, pp. 1160-1165.

Park, M.; Li, Q.; Shcheynikov, N.; Muallen, S. & Zeng W. (2004). NaBC1 is a ubiquitous electrogenic Na^+-coupled borate transporter essential for cellular boron homeostasis and cell growth and proliferation. *Molecular Cell*, Vol. 16, No. 3, pp. 331-341.

Park, M.; Li, Q.; Shcheynikov, N.; Muallen, S. & Zeng W. (2005). Borate transport and cell growth and proliferation, *Cell Cycle*, Vol. 4, No. 1, pp. 24-26.

Parkin, D.M.; Muir, C.S.; Whelan, S.L.; Gao, Y.T. & Ferlay, J.J. (1993). In: *Cancer Incidence in Five Continents*, J. Powell, (Ed), Vol. VI, Lyon, France International Agency for Research on Cancer.

Peng, X.; Lingxil, Z.; Schrauzer, G.N. & Xiong, G. (2000). Selenium, boron and germanium deficiency in the etiology of Kashin-Beck disease. *Biological Trace Element Research*, Vol. 77, pp. 193-197.

Petasis, N.A. (2007). Expanding roles for organoboron compounds versatile and valuable molecules for synthetic. *Australian Journal of Chemistry*, Vol. 60, pp. 795-798.

Pike, A.C.W.; Brzozowski, A.M.; Hubbard, E.R.; Bonn, T.; Thorsell, A.G.; Engström, O.; Ljunggren, J.; Gustafsson, J.A. & Mats-Carlquist, M. (1999). Structure of the ligand-binding domain of estrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO Journal*, Vol. 18, pp. 4608-4618.

Pisano, C.; Pratesi, G.; Laccabue, D.; Zunino, F.; Giudice, P.; Bellucci, A.; Pacifici, L.; Camerini, B.; Vesci, L.; Castorina, M.; Cicuzza, S.; Tredici, G.; Marmiroli, P.; Nicolini, G.; Galbiati, S.; Calvani, M.; Carminati, P. & Cavaletti, G. (2003). Paclitaxel and cisplatin-induced neurotoxicity: a protective role of acetyl-l-carnitine. *Clinical Cancer Research,* Vol. 9, pp. 5756–576.

Probst-Hensch, N.M.; Pike, M.C.; McKean-Cowdin, R.; Stanczyk, F.Z.; Kolonel L.N. & Henderson B.E. (2000). Ethnic differences in post-menopausal plasma oestrogen levels: high oestrone levels in Japanese-American women despite low weight. *British Journal of Cancer*, Vol. 82, No. 11, pp. 1867–1870.

Pietrzkowski, Z. (oct.7, 2010). Compositions and methods related to calcitriol. *US patent 0256076A1*.

Pujol, P.; Galtier-Dereure, F. & Bringer, J. (1997). Obesity and breast cancer risk. *Human Reproduction*, Vol. 12, No. 1, pp. 116-125.

Rainey, C. & Nyquist, L. (1998). Multicountry estimation of dietary boron intake. *Biological Trace Element Research*, Vol. 66, pp. 79-86.

Redondo-Nieto, M.; Reguera, M.; Bonilla, I. & Bola, L. (2008). Boron dependent membrane glycoproteins in symbiosome development and nodule organogenesis. A model for a common role of boron in organogenesis. *Plant Signaling & Behavior*, Vol. 3, pp. 298-300.

Rezanka, T. & Sigler, K. (2008). Biologically active compounds of semi-metals. *Phytochemistry*, Vol. 69, pp. 585–606.

Rowe, R.I. & Eckert, C.D. (1999). Boron is required for zebracsh embryogenesis, *Journal of Experimental Biology*, Vol. 202, pp. 1649-1654.

Rose, D.P. & Connolly, J.M. (1993). Effects of dietary omega-3 fatty acids on human breast cancer growth and metastases in nude mice. *Journal of the National Cancer Institute,* Vol. 85, No. 21, pp. 1743-1747.

Rowinsky, E.K. (1997). The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. *Annual Review of Medicine,* Vol. 48, pp. 353–374.

Saadatian, E.M.; Norat, T.; Goudable, J. & Riboli, E. (2004). Biomarkers of dietary fatty acid intake and the risk of breast cancer: a metaanalysis. *International Journal of Cancer,* Vol. 111, No. 4, pp. 584-591.

Sayli, B.S.; Tuccar, E. & Ellan, A.H. (2001). An assessment of fertility and infertility in boron-exposed Turkish subpopulations, Part 3: Evaluation of fertility among sibs and in borate families. *Biological Trace Element Research*, Vol. 81, pp. 255-267.

Simsek, A.; Velioglu, S.Y.; Coskun, L.A. & Sayli, B.S. (2003). Boron concentrations in selected foods from borate-producing regions in Turkey. *Journal of the Science Food and Agriculture,* Vol. 83, pp. 586-592.

Schabath, M.B.; Wu, X.; Vassilopoulou-Sellin, R.; Vaporciyan, A.A. & Spitz, M.R. (2004). Hormone replacement therapy and lung cancer risk. A case-control analysis clinical. *Cancer Research*, Vol. 10, pp. 113-123.

Scorei, R.; Cimpoiasu, V.M. & Iordachescu, D. (2005). *In vitro* evaluation of the antioxidant activity of calcium fructoborate. *Biological Trace Element Research*, Vol. 107, pp. 127-134.

Scorei, R.; Ciubar, R.; Iancu, C.; Mitran, V.; Cimpean, A. & Iordachescu, D. (2007). *In vitro* effects of calcium fructoborate on fMLPstimulated human neutrophil granulocytes. *Biological Trace Element Research*, Vol. 118, pp. 27-37.

Scorei, R.; Ciubar, R.; Ciofrangeanu, C.M.; Mitran, V.; Cimpean, A. & Iordachescu, D. (2008). Comparative effects of boric acid and calcium fructoborate on breast cancer cells. *Biological Trace Element Research*, Vol. 122, pp. 197-205.

Scorei, R. & Popa, R. (2010). Boron-containing compounds as preventive and chemotherapeutic agents for cancer. *Anti-Cancer Agents in Medicinal Chemistry*, Vol. 10, pp. 346–351.

Scorei, R. & Rotaru, P. (2011). Calcium fructoborate-potential anti-inflammatory agent. *Biological Trace Element Research*, DOI: 10.1007/s12011-011-8972-6.

Simard, A.; Vobecky, J. & Vobecky, J.S. (1991). Vitamin D deficiency and cancer of the breast: an unprovocative ecological hypothesis. *Canadian Journal of Public Health*, Vol. 82, pp. 300–303.

Shady, A.A.; Frithjof, C.K.; Green, D.H.; Wesley, R.H. & Carrano, C.J. (2007). Boron Binding by a Siderophore Isolated from Marine Bacteria Associated with the Toxic Dinoflagellate Gymnodinium catenatum. *Journal of the American Chemical Society*, Vol. 129, pp. 478-479.

Shannon, J.; Cook, L.S. & Stanford, J.L. (2003). Dietary intake and risk of postmenopausal breast cancer (United States). *Cancer Causes Control*, Vol. 14, No. 1, pp. 19-27.

Shomron, N. & Ast, G. (2003). Boric acid reversibly inhibits the second step of pre-mRNA splicing. *FEBS Letters*, Vol. 552, pp. 219-224.

Spiegelman, D.; Colditz, G.A.; Hunter, D. & Hertzmark, E. (1994). Validation of the Gail et al: model for predicting individual breast cancer risk. *Journal of the National Cancer Institute*, Vol. 86, pp. 600-607.

Sporn, M.B. & Suh, N. (2000). Chemoprevention of cancer. *Carcinogenesis*, Vol. 21, No. 3, pp. 525-530.

Stoll, B.A. (1998). Teenage obesity in relation to breast cancer risk. *International Journal of Obesity Related Metabolism Disorders*, Vol. 22, pp. 1035-1040.

Stoppler, H.; Koval, D. & Schlegel, R. (1996). The serine protease inhibitors TLCK and TPCK inhibit the *in vitro* immortalization of primary human keratinocytes by HPV-18 DNA. *Oncogene*, Vol. 13, pp. 1545-1548.

Tanaka, M. & Fujiwara, T. (2007). Physiological roles and transport mechanisms of boron: perspectives from plants. Pflugers Archives - *European Journal of Physiology*, Vol. 456, pp. 671–677.

Tangpricha, V.; Flanagan, J.N.; Whitlatch, L.W.; Tseng, C.C.; Chen, T.C.; Holt, P.R.; Lipkin, M.S. & Holick, M.F. (2001). 25-Hydroxyvitamin D–1alpha-hydroxylase in normal and malignant colon tissue, *Lancet*, Vol. 357, pp. 1673–1674.

Tariq, M. & Mott, C.J.B. (2007). The significance of boron in plant nutrition and environment. A review. *Agronomy Journal*, Vol. 6, pp. 1-10.

Teicher, B.A.; Gulshan, A.; Herbst, R.; Palombella, V.J. & Adams, J. (1999). The proteasomes inhibitor PS-341. *Clinical Cancer Research*, Vol. 5, pp. 2638-2645.

Thorne, J. & Campbel, M.J. (2008). The vitamin D receptor in cancer, *Proceedings of the Nutrition Society*, Vol. 67, pp. 115-127.

Tikhomirov, O. & Carpenter, G. (2000). Geldanamycin induces ErbB-2 degradation by proteolytic fragmentation. *Journal of Biological Chemistry,* Vol. 275, pp. 26625-26631.

Tominaga, S. & Kuroishi, T. (1999). Epidemiology and prevention of Breast Cancer in the 21st century. *Breast Cancer*, Vol. 6, No. 4, pp. 283-288.

Turkez, H.; Tatar□, A.; Hacimuftuoglu, A. & Ozdemir, E. (2010). Boric acid as a protector against paclitaxel genotoxicity. *Acta Biochimica Polonica*, Vol. 57, No. 1, pp. 95-97.

Ursin, G.; Pike, M.C.; Preston-Martin, S.; d'Ablaing, G. & Peters, R.K. (1996). Sexual, reproductive and other risk factors for adenocarcinoma of the cervix: results from a population-based case control study (California, United States). *Cancer Causes and Control*, Vol. 7, pp. 391-401.

Vogel, V.G. (2000). Breast Cancer Prevention: A Review of Current Evidence. *CA Cancer Journal of Clinicians*, Vol. 50, pp. 156-170.

Von Hoff, D.D. (1997). The taxoids: same roots, different drugs. *Seminars in Oncology,* Vol. 2, pp. 3–10.

Wang, Y.; Zhao, Y. & Chen, X. (2008). Experimental study on the estrogen-like effect of boric acid. *Biological Trace Element Research*, Vol. 121, pp. 160- 170.

Yan, C.; Winton, M.I.; Zhang, Z.F.; Rainey, C.; Marshall, J.; De Kernion, J.B. & Eckhert, C.D. (2004). Dietary boron intake and prostate cancer risk. *Oncology Reports*, Vol. 11, pp. 887-892.

Yang, W.; Gao, X. & Wang, B. (2003). Boronic acid compounds as potential pharmaceutical agents. *Medicinal Research Reviews*, Vol. 23, pp. 346-368.

Yang, C.H.; Gonzalez-Angulo, A.M.; Reuben, J.M.; Booser, D. J.; Pusztai, L.; Krishnamurthy, S.; Esseltine, D.; Stec, J.; Broglio, K.R.; Islam, R.; Hortobagyi, G.N. & Cristofanilli, M. (2006). Bortezomib (VELCADE_R) in metastatic breast cancer: pharmacodynamics, biological effects, and prediction of clinical benefits. *Annals of Oncology*, Vol. 17, pp. 813–817.

Ylitalo, N.; Sorensen, P.; Josefsson, A.; Frisch, M.; Sparen, P.; Ponten, J.; Gyllensten, U.; Melbye, M. & Adami, H.O. (1999). Smoking and oral contraceptives as risk factors for cervical carcinoma *in situ*. *International Journal of Cancer,* Vol. 81, pp. 357-365.

Zehnder, D.; Bland, R.; Williams, M.C.; McNinch, R.W.; Howie, A.J.; Stewart, P.M. & Hewison, M. (2001). Extrarenal expression of 25-hydroxyvitamin D(3)–1alphahydroxylase. *Journal of Clinical Endocrinology & Metabolism*, Vol. 86, pp. 888–894.

Zhou, H.B.; Nettles, K.W.; Bruning, J.B.; Kim, Y.; Joachimiak, A.; Sharma, S.; Carlson, K.E.; Stossi, F.; Katzenellenbogen, J.A.; Greene, G.L. & Katzenellenbogen, J.A. (2007). Elemental isomerism: a boron-nitrogen surrogate for a carbon-carbon double bond increases the chemical diversity of estrogen receptor ligands. *Chemistry & Biology*, Vol. 14, pp. 659-669.

6

Benzo-Fused Seven- and Six-Membered Derivatives Linked to Pyrimidines or Purines Induce Apoptosis of Human Metastatic Breast Cancer MCF-7 Cells *In Vitro*

Joaquín M. Campos, M. Carmen Núñez,
Ana Conejo-García and Olga Cruz-López
Departamento de Química Farmacéutica y Orgánica, Facultad de Farmacia, Universidad de Granada, c/ Campus de Cartuja s/n, Granada, Spain

1. Introduction

In recent years, strategy in cancer therapy has been the use of high doses of toxic non-specific agents and to investigate a range of new agents that specifically target tumour-related molecules, in a variety of biological pathways. A basic knowledge of these pathways in the cancer cell is becoming fundamental for clinical practice since it can provide prognostic as well as predictive information for established therapies, and lead to the discovery of potential new targets. Two main therapeutic strategies may be followed to optimize cancer treatment: a better selection of patients who will benefit the most from a given hormonal o cytotoxic therapy, through the use of predictive markers determined by genomics and/or proteomics techniques and the development of new agents with innovative and tumour-specific mechanism of action. We have reviewed the consideration of Choline Kinase as a novel target for the development of new anticancer drugs (Campos et al., 2003).

Breast cancer is a common and often fatal disease. Excluding cancers of the skin, that of the breast is the most common cancer among women, accounting for nearly one out of every three cancers diagnosed in American women. Each year over 186,000 new cases and 46,000 deaths are reported in the United States alone (Harris et al., 1996). Five main molecular pathways are of particular interest in terms of new drug development in breast cancer: the estrogen receptor pathway, the tyrosine signal transduction pathway, the angiogenesis pathway, the cell cycle regulation pathway and the apoptosis (programmed cell death) pathway. We will focus in this review on new cytotoxic, apoptotic and cell-cycle-regulator agents, designed by our Group.

As part of their action on neoplastic cells, many anticancer drugs activate apoptosis that may be a primary mechanism of antineoplastic agents (Hickman, 1992). Although breast cancer is most often treated with conventional cytotoxic agents it has proved difficult to induce apoptosis in breast cancer cells using these drugs (Rasbridge et al., 1994). Improved clinical response may be obtained by identifying therapies that are particularly effective in

activating apoptosis and determining how those therapies may be modified to effect maximum apoptosis induction. The cell cycle apparatus and apoptosis have recently attracted the attention of researchers intent on developing new types of anticancer therapy (Lundberg & Weinberg, 1999; Qin & Ng, 2002). On the other hand, the MCF-7 human breast cancer cell line has been used as an excellent experimental model to improve the efficacy of different therapies before its use in patients (Matsuo et al., 2000; Trouet et al., 2001). We will concentrate in this review on the evolution of the chemical structures and the biological properties whilst, in general, the chemical syntheses will be referred to through the corresponding original references.

2. Benzo-fused seven-membered derivatives linked to pyrimidines

Having previously reported the synthesis and anticancer activities of acyclic 5-fluorouracil (5-FU) *O,N*-acetalic compounds **1**-**2** (Campos et al., 1996), cyclic *O,N*-acetalic compounds was synthesized with the objective of increasing the lipophilicity of the target molecules. In this way we have reported the synthesis and anticancer activities of compounds **3** (Campos et al., 1997), **4**-**6** (Saniger et al., 2003a), **7** (Marchal et al., 2007), **8**-**9** (Saniger et al., 2003a) and *trans*-**10** (Saniger et al., 2003b) (Figure 1). In all cases, the linkage between the 5-FU moiety and the seven-membered ring was carried out through its *N*-1 atom.

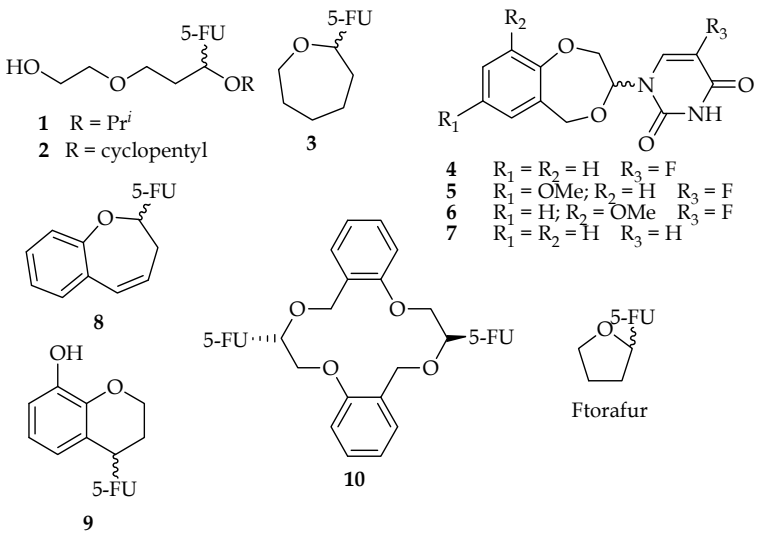


Fig. 1. Several 5-FU derivatives showing interesting antitumour activities.

2.1 Antiproliferative activities of cyclic *O,N*-acetals

The IC_{50} values of the 5-FU cyclic *O,N*-acetals are shown in Table 1 (entries 4-6, 8-10).

The most active 5-FU-derived compounds are **4**, **5** and **10** (entries 5, 6 and 11). Compound **3** (entry 4) shows the least antiproliferative activity (IC_{50} = 23 ± 0.88 μM). The lipophilicity in this structure has been increased by means of a fused benzene ring, and an unsaturation has been introduced to give **8**. An increase has been obtained in its antiproliferative activity (IC_{50} = 14 ± 1.02 μM, entry 9). On comparing structures **8** and **4**, it is worth emphasizing that the

Entry	Compound	clog *P*[a]	IC_{50} (μM)[b]	Cell Cycle (48 h)[c]			Apoptosis[d]	
				G_0/G_1	G_2/M	S	**24 h**	**48 h**
1	**Control**			68.39	12.04	19.57	1.24	1.24
2	5-FU	**-0.89**[e]	2.75	58.07	2.10	39.38	56.75	52.81
3	Ftorafur	**-0.18**[f]	3 ± 0.11	45.62	0.00	54.38	52.20	58.06
4	3	**0.86**	23 ± 0.88	50.99	18.51	30.49	46.63	53.92
5	4	**1.09**	7 ± 0.61	74.41	15.77	9.82	8.45	12.17
6	5	**1.16**	4.5 ± 0.33	73.41	13.15	13.44	1.50	3.50
7	6	**1.16**	22 ± 0.93	71.76	10.08	18.16	40.08	46.73
8	7	**0.87**	5 ± 0.25	69.01	11.38	19.61	5.30	5.22
9	8	**2.54**	14 ± 1.02	86.14	1.60	12.26	57.33	51.37
10	9	**0.70**	69 ± 2.31	68.61	9.60	21.79	54.33	35.49
11	**10**	**2.18**	5.5 ± 0.58	82.48	5.13	12.40	14.37	19.05

[a] Calculated by a fragmental system based on Rekker's method (Rekker & de Kort,1979), the CDR option of the PALLAS 2.0 programme (PALLAS FRAME MODULE, a prediction tool of physicochemical parameters, is supplied by CompuDrug Chemistry Ltd, PO Box 23196, Rochester, NY 14696, USA). [b]See ref. (Campos et al., 2000). [c]Determined by flow cytometry: see ref. (Saniger et al., 2003a). [d]Apoptosis was determined using an annexin V-based assay (Chadderton et al., 2000). The data indicate the percentage of cells undergoing apoptosis in each sample. All experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations. [e]The experimental log *P* value is –0.83 (Buur et al., 1985). [f]The experimental log *P* value, measured by the octanol-buffer partitioning technique at pH 7.4 is –0.36 ± 0.5 (Gulyaeva et al., 2003).

Table 1. Antiproliferative activities, cell cycle dysregulation, and apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 24 and 48 h for the compounds.

bioisosteric change of carbon for oxygen and the saturation of the double bond in compound **4** increases the antiproliferative activity twice (IC_{50} = 7 ± 0.61 μM, entry 5). The introduction of a methoxy group into the benzene ring of **4** provokes different influences on the antiproliferative activities. Thus, the C-7 substitution produces an increase of the antiproliferative activity (**5**, IC_{50} = 4.5 ± 0.33 μM, entry 6), whilst if C-9 is the substituted position it gives rise to a decrease in the antiproliferative activity (**6**, IC_{50} = 22 ± 0.93 μM, entry 7). The structural nature of **9** (entry 10, Table 1) implies that this compound cannot be considered as a 5-FU prodrug and we suspected that the remaining compounds (entries 4-7, 9-11, Table 1) would not be 5-FU prodrugs. To start with and to confirm it we decided to change the 5-FU moiety of **4** for the naturally occurring pyrimidine base uracil to produce **7**, with the prospect of finding an antiproliferative agent endowed with a new mechanism of action (Marchal et al., 2007).

2.2 Apoptosis induction of cyclic *O,N*-acetals

Apoptosis has been studied in terms of cancer development and treatment with attempts made to identify its role in chemotherapeutic agent-induced cytotoxicity. Cytotoxic agents often induce only a fraction of the cells to become apoptotic. To fully exploit apoptosis as a mechanism of antineoplastic agent response, a larger proportion of cells need to be

recruited into apoptosis. Paclitaxel (Taxol®), cyclophosphamide and cytosine arabinoside are the only commonly used cytotoxic agents shown to elicit apoptosis in breast cancer cells (Meyn et al., 1995; Milas et al., 1995). Quantitation of apoptotic cells was done by monitoring the binding of fluorescein isothiocyanate (FITC)-labelled annexin V (a phosphatidylserine-binding protein) to cells in response to our title compounds as described (Chadderton et al., 2000). The apoptosis study shows that compounds **3**, **6**, **8** and **9**, at their IC_{50} concentrations, provoke early apoptosis in the cells treated for 24 and 48 h. It is worth pointing out that compound **6** (entry 7) induces greater apoptosis at 48 h (46.73%) than at 24 h (40.08%) and so does compound **3** [48 h (53.92%) and 24 h (46.63%), entry 4]. The compounds that show the most important apoptotic indexes at 24 h are **8** (57.33%, entry 9) and **9** (54.33%, entry 10), whereas at 48 h are **3** (53.92%, entry 4) and **8** (51.37%, entry 9). These compounds are more potent as apoptosis inductors against the MCF-7 human breast cancer cells than paclitaxel (Taxol®), which induced programmed cell death of up to 43% of the cell population (Saunders et al., 1997). Accordingly, the early apoptotic inductions and the low IC_{50} values give rise to a significant antitumor activity.

2.3 Cell cycle distribution of cyclic 5-FU *O,N*-acetals

Cell cycle regulation has attracted a great deal of attention as a promising target for cancer research and treatment (Sampath & Plunkett, 2001; Gali-Muhtasib & Bakkar, 2002). The use of cell-cycle-specific treatments in cancer therapy has greatly benefited from the major advances that have been recently made in the identification of the molecular actors regulating the cell cycle and from the better understanding of the connections between cell cycle and apoptosis. As more and more "cell cycle drugs" are being discovered, their use as anticancer drugs is being extensively investigated (Gali-Muhtasib & Bakkar, 2002). To study the mechanisms of the antitumour and antiproliferative activities of the compounds, the effects on the cell cycle distribution were analyzed by flow cytometry. Control DMSO-treated cell cultures contained 68.39% G_0/G_1-phase cells, 12.04% G_2/M-phase cells and 19.57% S-phase cells. Cyclic *O,N*-acetals **4-10** (entries 5-11) provoke a G_0/G_1-phase cell cycle arrest whereas ftorafur [1-(2-tetrahydrofuranyl)-5-fluorouracil], a known prodrug of 5-FU, induces a S-phase cell cycle arrest.

In fact, a correlation between treated cells with compounds **4-10** recruited in the G_0/G_1 phase when treated at their IC_{50} concentrations, and their calculated lipophilicities by the CDR option of the PALLAS 2.0 programme:

$$\log (\%\ G_0/G_1) = 1.801\ (\pm 0.009) + 0.055\ (\pm 0.003)\ \text{clog}\ P \qquad (1)$$

$$n = 7,\ r^2 = 0.976,\ s = 0.006,\ F_{1,5} = 206.10,\ \alpha < 0.001$$

where n is the number of compounds, r^2 is correlation coefficient, s is the standard deviation, F is the F ratio between the variances of observed and calculated activities, and data within parentheses are standard errors of estimate. This equation means that the more lipophilic the compound is the more cells are recruited in the G_0/G_1 phase.

2.4 Modification of the molecular markers caused by the cyclic 5-FU *O,N*-acetals

Due to the fact that the cyclic *O,N*-acetals accumulate the cells in the G_1-phase the expression pattern of cyclin D1 was studied. This cyclin is one of the cyclin-dependent

kinase (CDK) activator subunits, specifically to CDK4, being responsible of the progression of the cell through the G_1-phase. Compounds **4** and **10** gave rise to a spectacular inhibition of cyclin D1 up to its total disappearance. This fact did not take place with 5-FU because the cyclin D1 level increased in relation to those of the parental MCF-7 cells. On one hand, this would explain why these compounds accumulate the cells in the G_1-phase (on inhibiting cyclin D1 the cell cannot progress to the S phase) and on the other, they show a different mechanism of action from the one shown by 5-FU: in short, they are not prodrugs. 5-FU Increases the cyclin D1 production so that cells pass in most cases toward the S phase where they are held back. It has been reported (Stacey, 2003) that cyclin D1 works as an active "switch" in the progression of the cellular cycle and that elevated levels of cyclin D1 promote the entry of the cell into the S phase. Moreover, compound **10** increases the expression of proteins p21 or p27 even up to double of the control. These proteins belong to the family INK2 of the CDK inhibiting proteins that work by hindering the association and activation of cyclins with their complexes (Sherr, 2000) and hence they halt cells in the G_1 and G_2/M phases. Compounds also affect the cdc2 activity that, regulated by their corresponding cyclins A or B, is essential for the entrance into mitosis during the cellular cycle (Sherr, 1993). Compounds **4**, **10** and **5**, with the exception of **8** and 5-FU, significantly decrease the cdc2 activity. Cdc2 is needed during the cellular cycle in the final phase of G_1, in the control point named "start" to be committed to the mitotic cycle. This is because at the end of G_2 (at the beginning of the mitosis) (Lees & Harlow, 1993), the inhibition of cdc2 by the *O,N*-acetals implies the halting of the cycle in G_1 and the non-entrance of the tumor cells in mitosis. The increase of the cdc2 expression caused by 5-FU is due to the fact that this higher activity is necessary for the cells to pass rapidly to the S phase, where cells are stopped by this fluoropyrimidine. Finally, compound **8** also significantly increases the cdc2 levels, which may be because its premature activation is one of the requirements for apoptosis (Shi et al., 1994); in fact this compound is the one that induces a higher proportion of programmed cellular death in the MCF-7 treated cells.

2.5 Apoptosis markers

Since the synthesized compounds induce very important apoptosis, we have carried out studies of the expression of some of the genes that intervene in this phenomenon, among which p53 and the family bcl-2 are outstanding. The tumour suppressor gene p53 protects the integrity of the genome so that if the DNA of the cell is damaged by an agent, an overexpression of it is produced inducing the stopping in G_1 for the repair of the damage, or if this is not possible, enter in apoptosis (Agarwal et al., 1995). On the other hand, the members of the family of proteins Bcl-2 work as regulators of apoptosis, Bcl-2 and Bcl-XL protecting against apoptosis. Bax, Bak and Bad induce such a phenomenon (Reed, 1997).

The treatment of the MCF-7 cells (wild-type p53) with these compounds provoked in general an increase in the protein expression of p53, mainly for 5-FU and **8**, and a marked decrease of the levels of bcl-2 for all of them. These data show that p53 activity is restored with the compounds, allowing the entrance of the tumour cells in apoptosis, which permits their elimination by this mechanism. In the same way bcl-2 inhibition facilitates the entrance of cells into the programmed cell death.

3. Benzo-fused seven-membered derivatives linked to purines

Later on, we substituted the pyrimidine bases for the purine one (with several substituents at its position 6), with the objective of increasing both the lipophilicity and the structural diversity of the target molecules (Figure 2). Their syntheses (using conventional heating and microwave irradiation) and biological activities have been recently published (Conejo-García et al., 2008).

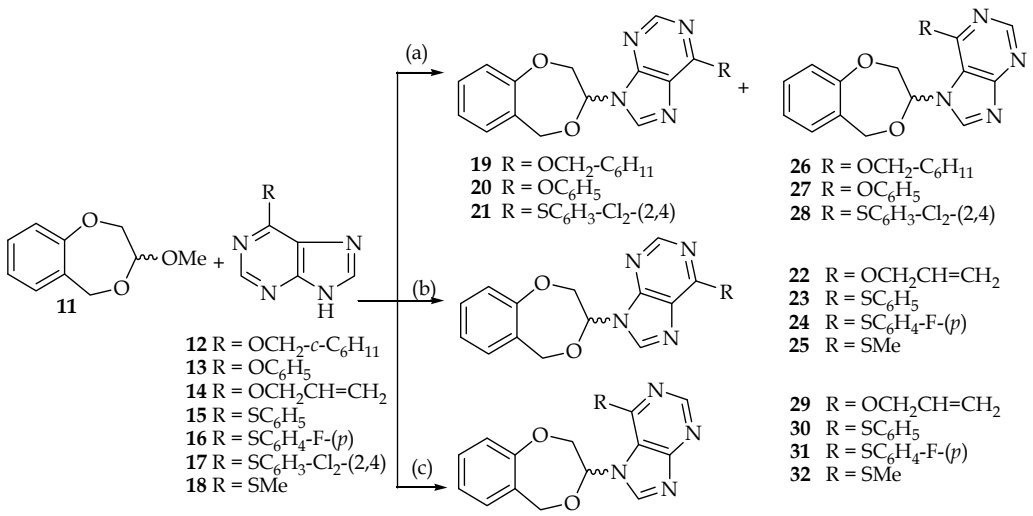


Fig. 2. *Reagents and conditions:* Purine bases (**12-18**), TCS, HMDS, $SnCl_4$, anhydrous MeCN. Method (a), (b) or (c): 45 °C, 24-72 °C; (b) microwave, 130 °C, 5 min; (c) microwave, 100 °C, 5 min.

3.1 Biological activities

The antitumour potential of the target molecules is reported against the MCF-7 human breast cancer cell line including 5-FU as reference drug (Table 2) (Conejo-García et al., 2008). The purine *O,N*-acetals **19-32** are more active than their corresponding purine bases **12-18**. The differences in the antiproliferative effect of the *N*-7′ and *N*-9′ regioisomers are not significant with the exception of the allyloxy derivatives **22** and **29**. The biological effect is dependent on the substituent present on position 6 of the purine ring although a clear structure-activity relationship between the size of this moiety and the antiproliferative effect of the MCF-7 human breast cancer cell line is not observed. The most active compound (**22**), that presents an allyloxy group as substituent at position 6 of the purine ring, shows an IC_{50} = 5.04 ± 1.68 μM nearly equipotent as 5-FU. The following two more active compounds, **23** and **28**, present bulky substituents as the phenylthio and 2,4-diclorophenylthio ones, respectively.

Compound	IC_{50} (µM)	Compound	IC_{50} (µM)	Compound	IC_{50} (µM)
5-FU	4.32 ± 0.02	**19**	11.6 ± 0.93	**27**	21.1 ± 2.93
12	32.3 ± 1.42	**20**	24.7 ± 3.82	**28**	8.4 ± 0.91
13	28.4 ± 0.45	**21**	12.0 ± 0.59	**29**	20.9 ± 1.24
14	44.7 ± 0.74	**22**	5.04 ± 1.68	**30**	15.6 ± 3.74
15	31.0 ± 0.16	**23**	7.12 ± 0.46	**31**	14.1 ± 0.67
16	31.6 ± 0.54	**24**	11.2 ± 1.32	**32**	31.8 ± 5.46
17	21.4 ± 0.42	**25**	24.0 ± 1.95		
18	23.6 ± 4.11	**26**	13.4 ± 1.94		

Table 2. Antiproliferative activities against the MCF-7 cell line for 5-FU (Núñez et al., 2007), for the purine bases (**12-18**), and for the seven-membered alkylated purine derivatives (*N*-9′ isomers: **19-25**; *N*-7′ isomers: **26-32**).

To study the mechanisms of the antitumour and antiproliferative activities of the most active compounds (**22**, **23** and **28**), the effects on the cell cycle distribution were analyzed by flow cytometry (Table 3). DMSO-treated cell cultures contained a 58.62 ± 0.74 of the G_0/G_1-phase cells, a 33.82 ± 0.72 of the S-phase cells and a 7.55 ± 1.34 of the G_2/M-phase cells. In contrast, MCF-7 cells treated during 48 h with the IC_{50} concentrations of **22**, **23** and **28** showed important differences in cell cycle progression compared with DMSO-treated control cells. The cell cycle regulatory activities can be divided into the following two groups: (a) the breast cancer cells showed an accumulation in the S-phase, up to 37.00 ± 2.00 of the cells, mainly at the expense of the G_0/G_1-phase population that decreased to a percentage of 55.63 ± 1.57 of the cells; (b) compounds **23** and **28** accumulated the cancerous cells in the G_2/M-phase (11.08 ± 1.01 and 19.16 ± 0.56, respectively) at the expense of the S-phase cells (26.82 ± 1.26 and 22.73 ± 0.37, respectively).
In response to **23** (and **28**), the percentage of apoptotic cells increased, from 0.22 ± 0.31 in control cells to a maximum of 73.37 ± 0.12 (and 65.28 ± 1.92) apoptotic cells at a concentration equal to their IC_{50} against the MCF-7 cell line. This is a remarkable property because the demonstration of apoptosis in MCF-7 breast cancer cells by known apoptosis-inducing agents has proved to be difficult.

Compound	Cell cycle[a]			Apoptosis[b]
	G_0/G_1	S	G_2/M	
Control	58.62 ± 0.74	33.82 ± 0.72	7.55 ± 1.34	0.22 ± 0.16
22	55.63 ± 1.57	37.00 ± 2.00	7.37 ± 0.43	44.47 ± 2.98
23	59.10 ± 1.28	26.82 ± 1.26	11.08 ± 0.01	73.37 ± 0.12
28	58.10 ± 0.19	22.73 ± 0.37	19.16 ± 0.56	65.28 ± 1.92

[a] Determined by flow cytometry (Boulaiz et al., 2003). [b]Apoptosis (48 h) was determined using an Annexin V-based assay (Boulaiz et al., 2003). The data indicate the percentage of cells undergoing apoptosis in each sample. All experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations.

Table 3. Cell cycle distribution and apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 48 h for the three most active compounds.

4. Anticancer and SAR studies on (1,2,3,5-tetrahydro-4,1-benzoxazepine-3-yl)-pyrimidine and -purine derivatives

The synthesis and the mechanistic aspects of bioisosteres containing a 4,1-benzoxazepine *N*-alkylated pyrimidine (**33-45**) or purine (**46-58**) (Figure 3) have been thoroughly discussed (Díaz-Gavilán et al., 2006; Díaz-Gavilán et al., 2007).

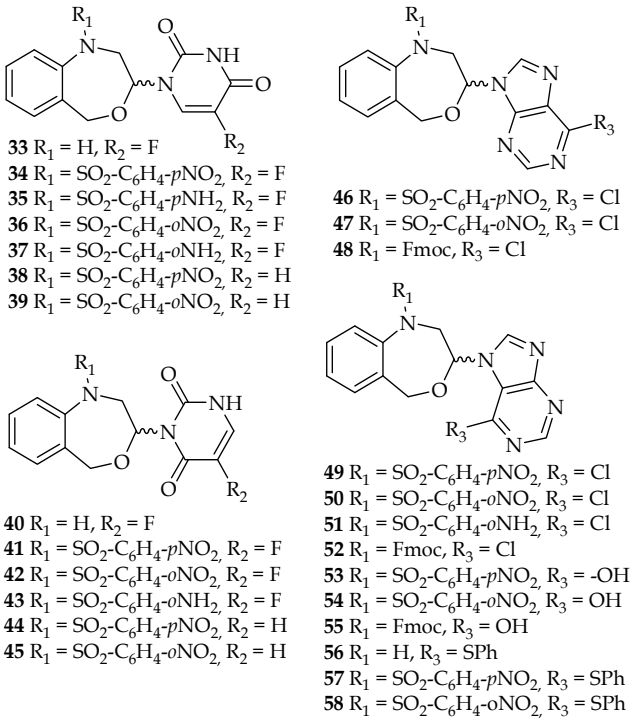


Fig. 3. New benzoxazepine *O,N*-acetals containing pyrimidine and purine rings.

4.1 Antiproliferative activities

Table 4 show the antiproliferative activity (IC_{50} values) on MCF-7 human breast cancer cells found for the pyrimidine and purine derivatives **33-58** (Díaz-Gavilán et al., 2008a). The most potent molecules were the purine derivatives. Compounds **48**, **50**, **52** and **57** presented IC_{50} values below 1 μM. Between the pyrimidine derivatives **33-45**, those containing 5-fluorouracil (R_2 = F) showed improved activities than those derived of uracil (R_2 = H). Bonding of the pyrimidine ring through *N*-1″ or *N*-3″ affected the activity though only slightly, when R_1 = H, **33** *versus* **40**. The substitution on *C*-6″ of the purine ring is essential for the activity[1]. Bulky, lipophilic groups afforded the best values of IC_{50} [R_3 = Cl (**46-52**), -SPh (**57, 58**)] while purinone compounds [R_3 = OH (**53-55**)] were comparable to the pyrimidine analogues in terms of activity. Bonding of the purine ring through *N*-7″ or *N*-9″

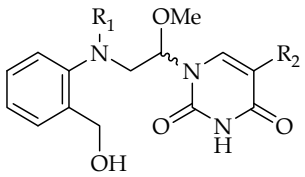
[1] For the numbering of the compounds, the atoms of the benzoxazepine are tagged with numbers without primes, the atoms of the R_1 group are numbered with primes (′), while the pyrimidine and purine bases are numbered with double primes (″).

affected the activity to a lesser extent, and the positive or negative character of this effect depended on the nature of R_1 (see **46/49** R_1 = *p*Ns, **47/50** R_1 = *o*Ns, **48/52** R_1 = Fmoc).
Both, pyrimidine and purine derivatives, were more potent when R_1 is not hydrogen. The lipophilic character of R_1 increased the activity and no limit of volume had been observed for the studied groups. The electron-withdrawing character of R_1 could help to increase the activity (Díaz-Gavilán et al., 2004). Carbonyl derivatives were more potent than the sulfonyl ones.

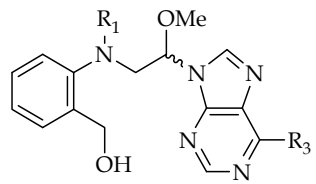
Compds	IC_{50} (μM)	Compds	IC_{50} (μM)	Compds	IC_{50} (μM)	Compds	IC_{50} (μM)
33	>100	**40**	72.40 ± 11.3	**47**	2.10 ± 0.69	**54**	19.66 ± 5.27
34	19.33 ± 1.04	**41**	19.81 ± 0.08	**48**	0.67 ± 0.18	**55**	53.57 ± 13.1
35	14.37 ± 0.69	**42**	22.63 ± 0.11	**49**	1.22 ± 0.12	**56**	48.92 ± 9.89
36	19.70 ± 0.15	**43**	43.70 ± 0.09	**50**	0.92 ± 0.01	**57**	0.86 ± 0.12
37	54.82 ± 1.04	**44**	44.28 ± 4.65	**51**	9.14 ± 1.24	**58**	2.59 ± 0.57
38	39.78 ± 2.60	**45**	50.90 ± 3.87	**52**	0.84 ± 0.09		
39	45.17 ± 0.48	**46**	2.73 ± 0.17	**53**	>100		

Table 4. Antiproliferative activities against the MCF-7 cells for *N*-1″- (**33-39**) and *N*-3″- (**40-45**) pyrimidines and for *N*-9″- (**46-48**) and *N*-7″-purines (**49-58**).

When R_1 = benzensulfonamido, it can be observed for pyrimidine and purine derivatives that the *ortho*-substitution on R_1 is preferred to *para*, in terms of potency, and the nitro group renders better results than the amino one. As an exception, compound **57** (R_1 = *p*Ns, R_3 = SPh), is more potent than **58** (R_1 = *o*Ns, R_3 = SPh). The new related acyclic *O,N*-acetals **59-69** (Figure 4) were obtained as minor products in the condensation reaction between the *O,O*-acetals and pyrimidine (Díaz-Gavilán et al., 2006) or purine (Díaz-Gavilán et al., 2007) bases. Their antiproliferative activities have also been studied on MCF-7 human breast cancer cells and the IC_{50} values obtained are shown in Table 5. Acyclic purine *O,N*-acetals (**67-69**) show higher potency than the pyrimidine acyclic derivatives (**59-66**). The *N*-7″-alkylated purine **68** presented an excellent value of IC_{50}. In contrast to the cyclic analogues, the presence of an *o*-NO_2 or *p*-NO_2 group does not modify the activity of the *N*-9″-isomers (**67** and **69**).



59 R_1 = H, R_2 = F
60 R_1 = CO-C_6H_5, R_2 = F
61 R_1 = SO_2-C_6H_4-*p*NO_2, R_2 = F
62 R_1 = SO_2-C_6H_4-*o*NO_2, R_2 = F
63 R_1 = SO_2-C_6H_4-*o*NH_2, R_2 = F
64 R_1 = SO_2-C_6H_4-*p*NO_2, R_2 = H
65 R_1 = SO_2-C_6H_4-*p*NO_2, R_2 = H (*N*-3")
66 R_1 = SO_2-C_6H_4-*o*NO_2, R_2 = H



67 R_1 = SO_2-C_6H_4-*p*NO_2, R_3 = Cl
68 R_1 = SO_2-C_6H_4-*o*NO_2, R_3 = Cl (*N*-7")
69 R_1 = SO_2-C_6H_4-*o*NO_2, R_3 = Cl

Fig. 4. New acyclic *O,N*-acetals containing pyrimidine and purine moieties.

Compds	IC_{50} (μM)	Compds	IC_{50} (μM)
59	35.97 ± 0.40	**65**	55.22 ± 12.14
60	16.14 ± 0.77	**66**	64.81 ± 0.05
61	55.22 ± 12.14	**67**	18.70 ± 0.08
62	90.99 ± 6.06	**68**	3.25 ± 0.23
63	>100	**69**	11.30 ± 1.27
64	45.76 ± 2.45		

Table 5. Antiproliferative activities against the MCF-7 cell line for acyclic *N*-1″- and *N*-3″-pyrimidines (**59-66**) and *N*-9″- and *N*-7″ purines (**67-69**).

Compounds **48** and **57** were selected to identify the molecular key targets of its anti-cancer activity (Díaz-Gavilán et al., 2008a). Completion of the human genome sequence and the advent of DNA microarrays using cDNAs enhanced the detection and identification of hundreds of differentially expressed genes in response to anticancer drugs. In this way gene-expression patterns of treated human breast cancer cells in comparison with parental MCF-7 cells were obtained. For this purpose, the expression of about 22,000 different human genes was analyzed using the Agilent 60-mer oligo microarray platform and the Human 1A Oligo Microarray Kit (V2) (Agilent Technologies, CA, USA).

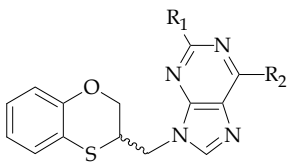
The up-regulated and the down-regulated genes include genes that encode for different metabolic pathways, cellular development process, signal molecules, response to stress, regulation of the cell cycle and apoptosis, etc. Analysis of the mRNAs, which are deregulated (up-regulated or down-regulated) at least 2-fold in treated cells, revealed the following results: 26 genes up-regulated and 59 genes down-regulated in **48**-MCF-7 treated cells; and 26 genes up-regulated and 17 genes down-regulated in **57**-treated human breast cancer cells. Each compound revealed a somewhat unique expression pattern together with the up-regulation of significant genes involved in different cellular functions and a significant down-regulation of genes for **48**. One of the more important results in the current study was the ability of **48** to modulate the expression of genes involved in apoptosis or its delay of mitosis. This effect can be explained by the accumulation of cells in the G_2/M checkpoint of cell cycle, particularly GP132, the receptor for an unknown ligand, which activates a G2 alpha protein (Díaz-Gavilán et al., 2008a). This is transcriptionally up-regulated by stress-inducing and cell-damaging agents and that is involved in caspase-mediated apoptosis (Lin & Ye, 2003). Similarly, the ERN1 gene that belongs to the Ser/Thr protein kinase family is a potent unfolded-protein response transcriptional activator and acts by triggering growth arrest and apoptosis (Yoneda et al., 2001). However, **57** induced the down-regulation of a gene involved in the metastatic progression of cancer such as RAC1, a Ras-like protein member of the Rho family of the GTPase key downstream target in Ras signalling (Baugher et al., 2005).

The studies by microarray technology showed that the main molecular targets of some of these compounds (**48** and **57**) are pro-apoptotic genes with protein kinase activity such as GP132, ERN1 or RAC1, which prevent the metastatic progression (Díaz-Gavilán et al., 2008a).

5. Synthesis and anticancer activity of (*RS*)-9-(2,3-dihydro-1,4-benzoxathiin-3-ylmethyl)-9*H*-purines

The 2,3-dihydro-1,4-benzodioxin ring system is present in a large number of structures of therapeutic agents possessing important biological activities (Guillaumet, 1996). Some of them

are antagonists of α-adrenergic receptors, giving them antihypertensive properties (Quaglia et al., 1999; Pallavicini et al., 2006). Others have affinities with serotonin receptors which are involved in nervous breakdown and schizophrenia (Birch et al., 1999). Sixteen years ago, 2,3-dihydro-1,4-benzodioxins were developed as inhibitors of 5-lipoxygenase, an enzyme involved in the oxygenation of arachidonic acid to the leukotriens; they are also useful for the treatment of inflammatory diseases such as asthma and arthritis (Satoh et al., 1995). The occurrence of the 2,3-dihydro-1,4-benzodioxin structure in various naturally abundant compounds has been also reported (Fukuyama et al., 1992). Paradoxically, despite the considerable development of biologically active compounds with the 2,3-dihydro-1,4-benzodioxin moiety, the 2,3-dihydro-1,4-benzoxathiin skeleton has still remained inaccessible.
The importance of 5-FU as the first-choice drug in carcinomas of the gastrointestinal tract is well known despite its side-effects. With the aim of diminishing the toxicity and obtaining biologically active derivatives of 5-FU suitable for oral administration great effort has been made in the preparation of 5-FU prodrug derivatives. A review of the literature on the various prodrugs of 5-FU has been published (Malet-Martino et al., 2002). Various 5-FU prodrugs are active against certain malignant cell lines due to an inhibition of thymidilate synthase by the formation of 5-fluorodeoxyuridine monophosphate or by the incorporation of 5-fluorouridine triphosphate into RNA. During various synthetic studies on masked 5-FU derivatives, it has been found that the bond strength between the *N*-1 atom in the 5-FU ring and its *N*-1 substituent is an important factor influencing the antitumour activity and the toxicity of the compounds. The previous results indicated that the weaker the bond strength, the stronger are the antitumour activity and the toxicity of the masked compounds (Ozaki, 1996). In the case of *N*-alkyl-5-FU derivatives, the strong $N\text{-}1_{5\text{-FU}}\text{-}C_{exocyclic}$ bond conversely prevented these derivatives from being easily hydrolyzed *in vivo* and showed no antitumour activity against L1210 leukaemia (Ozaki, 1996). When oxygen was introduced at the α-position to the alkyl group, the N-C bond became labile under hydrolytic conditions and the resulting derivatives showed antitumour activity.
As it has been demonstrated before, compounds **4-6, 8-10** may be considered as drugs with their own entity and antitumour activity independent of that of 5-FU. If the previously described compounds are not prodrugs, it is not necessary to maintain the *O,N*-acetalic characteristic with the corresponding weakness of the *O,N*-acetalic bond. Therefore, molecules are being designed in which both structural entities (such as the benzoheterocyclic ring and the purine base) are linked by a heteroatom-C-C-base-N-atom bond. Very recently the design, synthesis and biological evaluation of a series of 2- and 6-disubstituted (*RS*)-9-(2,3-dihydro-1,4-benzoxathiin-3-ylmethyl)-9*H*-purine derivatives **70-80** were described [Figure 5, Table 6] (Díaz-Gavilán et al., 2008b).



70 R_1 = H, R_2 = Cl
71 R_1 = H; R_2 = Br
72 R_1 = H; R_2 = SMe
73 R_1 = H; R_2 = OPh
74 R_1 = H; R_2 = SPh
75 R_1 = H; R_2 = NHPh
76 R_1 = H; R_2 = $OCH_2CH{=}CH_2$
77 R_1 = H; R_2 = OCH_2Ph
78 R_1 = H; R_2 = SCH_2Ph
79 R_1 = H; R_2 = $OCH_2C_6H_{11}$
80 R_1 = Cl; R_2 = Cl

Fig. 5. The 1,4-benzoxathiin system linked to several purines.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
5-FU	4.32 ± 0.02	**73**	20.5 ± 1.11	**77**	23.2 ± 1.26
70	10.6 ± 0.66	**74**	10.5 ± 1.06	**78**	16.7 ± 3.03
71	6.18 ± 1.70	**75**	11.2 ± 2.73	**79**	17.4 ± 1.60
72	20.5 ± 1.81	**76**	17.5 ± 0.25	**80**	8.97 ± 0.83

Table 6. Antiproliferative activities against the MCF-7 cell line for 5-FU (Villalobos et al., 1995), and the six-membered alkylated purine derivatives.

The three most potent compounds (**70**, **71** and **80**) were subjected to cell cycle and apoptosis studies on the MCF-7 human breast cancer cell line (Table 7). The following two consequences can be stated: (a) in contrast to 5-FU, the six-membered compounds **70**, **71** and **80,** provoked a G_0/G_1-phase cell cycle arrest when the MCF-7 cells were treated during 48 h with the IC_{50} of the compounds, mainly at the expense of the S-phase populations. The fact that at similar doses the novel derivatives exhibit different sequences of cell cycle perturbations in comparison with 5-FU indicates that these compounds act by different pathways (Marchal et al., 2004). In the case of **71** it is worth pointing out that, moreover, there is an increase in the G_2/M-phase of the cancerous cells; and (b) the apoptotic indices of the target compounds are very important, especially for **80** (58.29% for **70**, 63.05% for **71**, and 76.22% for **80**). Up to now and according to our knowledge, compound **80** is the most important apoptotic inducer against the MCF-7 human breast cancer cell line so far reported.

Compound	Cell cycle[a]			Apoptosis[b]
	G_0/G_1	S	G_2/M	
Control	58.62 ± 0.74	33.82 ± 0.72	7.55 ± 1.34	0.22 ± 0.16
5-FU[c]	58.07 ± 0.11	39.38 ± 0.98	2.10 ± 0.12	52.81 ± 1.05
70	69.71 ± 1.50	23.73 ± 1.65	6.56 ± 0.17	58.29 ± 0.75
71	62.85 ± 0.87	26.71 ± 1.25	10.43 ± 0.38	63.05 ± 0.26
80	70.30 ± 0.32	23.67 ± 2.40	6.06 ± 2.72	76.22 ± 2.02

[a] Determined by flow cytometry (Marchal et al., 2004).
[b] Apoptosis was determined using an Annexin V-based assay (Marchal et al., 2004). The data indicate the percentage of cells undergoing apoptosis in each sample.
[c] Data were taken from ref. (Campos et al., 2005). All experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations.

Table 7. Cell cycle distribution and apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 48 h for the three most active compounds as antiproliferative agents.

6. Conclusion

Breast cancer is the commonest malignancy in women and comprises 18% of all cancers in women. Normal breast development is controlled by a balance between cell proliferation and apoptosis, and there is strong evidence that tumour growth is not just a result of uncontrolled proliferation but also of reduced apoptosis. The balance between proliferation

and apoptosis is crucial in determining the overall growth or regression of the tumour in response to chemotherapy, radiotherapy and more recently, hormonal treatments. All of these approaches act in part by inducing apoptosis. Understanding these relationships could allow individually tailored treatments to maximize tumour regression and efficacy of treatment. It could also help to answer why some tumours fail to respond and thereby indicate new routes of drug development.

Starting from Ftorafur, a known 5-FU prodrug, which shows an 58% apoptosis induction in the MCF-7 human breast cancer cell line after treatment for 48 h, the seven-membered cyclohomologue 5-FU *O,N*-acetal **3** and the benzo-fused dihydro oxepine *O,N*-acetal **8** present apoptosis inductions higher than 50%. By using molecular modification strategies widely used in medicinal chemistry, lately compounds **23** and **28**, having in common the benzo-fused 2,3-dihidro-5*H*-1,4-dioxepin and a 6-substituted purine moieties, show 73% and 65% apoptosis inductions. Finally and following our Drug Anticancer Programme, the benzo-fused 1,4-oxathiane moiety linked to the *N*-9 atom of a 2,6-dichloropurine **80** was designed and synthesized. According to our knowledge this is the most important apoptotic inducer against the MCF-7 human breast cancer cell line so far reported. This compound is a more potent apoptosis inductor than the clinically-used drug paclitaxel (Taxol®), which induced programmed cell death up to 43% of cell population. Their mechanisms of action at molecular level are being studied at present.

7. Acknowledgements

This study was supported by the Instituto de Salud Carlos III (Fondo de Investigación Sanitaria) through project no. PI10/00592.

8. References

Agarwal, M. L.; Agarwal, A.; Taylor, W. R. & Stark, G. R. (1995). p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.92, No.18, (August 1995), pp. 8493-8497, ISSN 0027-8424.

Baugher, P. J.; Krishnamoorthy, L.; Price, J. E. & Dharmawardhane, S. F. (2005). Rac1 and Rac3 isoform activation is involved in the invasive and metastatic phenotype of human breast cancer cells. *Breast Cancer Research*, Vol.7, No.6, (September 2005), pp. R965-R974, ISSN 1465-542X.

Birch, A. M.; Bradley, P. A.; Gill, J. C.; Kerrigan, F. & Needham, P. L. (1999). N-Substituted (2,3-dihydro-1,4-benzodioxin-2-yl)methylamine derivatives as D(2) antagonists/5-HT(1A) partial agonists with potential as atypical antipsychotic agents. *Journal of Medicinal Chemistr*, Vol.42, No.17, (January 1999), pp. 3342-3355, ISSN 0022-2623.

Boulaiz, H.; Prados, J.; Melguizo, C.; García, A. M.; Marchal, J. A.; Ramos, J. L.; Carrillo, E. & Aránega, A. (2003). Inhibition of growth and induction of apoptosis in human breast cancer by transfection of gef gene. *British Journal of Cancer,* Vol.89, (July 2003), pp. 192-198, ISSN 0007 – 0920/03.

Buur. A.; Bundgaard, H. & Falch, E. (1985). Prodrugs as drug delivery systems. Part 37. Prodrugs of 5-fluorouracil. IV. Hydrolysis kinetics, bioactivation and physicochemical properties of various *N*-acyloxymethyl derivatives of 5-

fluorouracil. *International Journal of Pharmaceutics*, Vol.24, No.1, (April 1985), pp. 43-60, ISSN 0378-5173.

Campos, J.; Pineda, M. J.; Gómez, J. A.; Entrena, A.; Trujillo, M. A.; Gallo, M. A. & Espinosa, A. (1996). 5-Fluorouracil Derivatives. 1. Acyclonucleosides through a Tin (IV) Chloride-Mediated Regiospecific Ring Opening of Alkoxy-1,4-Diheteroepanes. *Tetrahedron,* Vol.52, No.26, (June 1996), pp. 8907-8924, ISSN 0040-4020.

Campos, J.; Gómez, J. A.; Trujillo, M. A.; Gallo, M. A. & Espinosa, A. (1997). Diheterocyclanes as Synthons for the Preparation of Novel Series of Nucleoside and Acyclonucleoside Analogues. *Il Farmaco*, Vol.52, No.5, (May 1997), pp. 263-269, ISSN 0014-827X.

Campos, J.; Núñez, M. C.; Rodríguez, V.; Gallo, M. A. & Espinosa, A. (2000). QSAR of 1,1'-(1,2-Ethylenebisbenzyl)bis(4-substitutedpyridinium) Dibromides as Choline Kinase Inhibitors: a Different Approach for Antiproliferative Drug Design. *Bioorganic & Medicinal Chemistry Letters*, Vol.10, No.8, (April 2000), pp. 767-770, ISSN 0960-894X.

Campos, J.; Núñez, M. C.; Conejo-García, A.; Sánchez-Martín, R. M.; Hernández-Alcoceba, R.; Rodríguez-González, A.; Lacal, J. C.; Gallo, M. A. & Espinosa, A. (2003). QSAR-derived choline kinase inhibitors: How rational can antiproliferative drug design be? *Current Medicinal Chemistry*, Vol.10, No13, (July 2003), pp. 1095-1112, ISSN 0929-8673.

Campos, J.; Saniger, E.; Marchal, J. A.; Aiello, S.; Suárez, I.; Boulaiz, H.; Aránega, A.; Gallo, M. A. & Espinosa, A. (2005). New Medium Oxacyclic O,N-Acetals and Related Open Analogues: Biological Activities. *Current Medicinal Chemistry*, Vol.12, No12, (June 2005), pp. 1423-1438, ISSN 0929-8673.

Chadderton, A.; Villeneuve, D. J.; Gluck, S.; Kirwan-Rhude, A. F.; Gannon, B. R.; Blais, D. E. & Parissenti, A. M. (2000). Role of specific apoptotic pathways in the restoration of paclitaxel-induced apoptosis by valspodar in doxorubicin-resistant MCF-7 breast cancer cells. *Breast Cancer Research and Treatment*, Vol.59, No.3, (February 2000), pp. 231-244, ISSN 0167-6806.

Conejo-García, A.; Núñez, M. C.; Marchal, J. A.; Rodríguez-Serrano, F.; Aránega, A.; Gallo, M. A.; Espinosa, A. & Campos, J. M. (2008). Regioespecific Microwave-Assisted Synthesis and Cytotoxic Activity against Human Breast Cancer Cells of (*RS*)-6-Substituted-7- or 9-(2,3-Dihydro-5*H*-1,4-Benzodioxepin-3-yl)-7*H*- or -9*H*-Purines. *European Journal of Medicinal Chemistry*, Vol.43, No.8, (October 2007), pp. 1742-1748, ISSN 0223-5234.

Díaz-Gavilán, M.; Rodríguez-Serrano, F.; Gómez-Vidal, J. A.; Marchal, J. A.; Aránega, A.; Gallo, M. A.; Espinosa, A. & Campos, J. M. (2004). Synthesis of Tetrahydrobenzoxazepine Acetals with Electron-Withdrawing Groups on the Nitrogen Atom. Novel Scaffolds Endowed with Anticancer Activity against Breast Cancer Cells. *Tetrahedron*, Vol.60, No.50, (December 2004), pp. 11547-11557, ISSN 0040-4020.

Díaz-Gavilán, M.; Gómez-Vidal, J. A.; Entrena, A.; Gallo, M. A.; Espinosa, A. & Campos, J. M. (2006). Study of the factors that control the ratio of the products between 5-fluorouracil, uracil, and tetrahydrobenzoxazepine *O,O*-acetals bearing electron-

withdrawing groups on the nitrogen atom. *Journal of Organic Chemistry*, Vol.71, No.3, (October 2005), pp. 1043-1054, ISSN 0022-3263.

Díaz-Gavilán, M.; Choquesillo-Lazarte, D.; González-Pérez, J. M.; Gallo, M. A.; Espinosa, A. & Campos, J. M. (2007). Synthesis and Reactivity of (*RS*)-6-Chloro-7- or 9-(1,2,3,5-Tetrahydro-4,1-Benzoxazepin-3-yl)-7*H*- or 9*H*-Purines Bearing a Nitrobenzene-sulfonyl Group on the Nitrogen Atom. *Tetrahedron*, vol.63, No.24, (June 2007), pp. 5274-5286, ISSN 0040-4020.

Díaz-Gavilán, M.; Gómez-Vidal, J. A.; Rodríguez-Serrano, F.; Marchal, J. A.; Caba, O.; Aránega, A.; Gallo, M. A.; Espinosa, A. & Campos, J. M. (2008a). Anticancer Activity of (1,2,3,5-Tetrahydro-4,1-Benzoxazepine-3-yl)-Pyrimidines and -Purines against the MCF-7 Cell Line: Preliminary cDNA Microarray Studies. *Bioorganic & Medicinal Chemistry Letters*, Vol.18, No.4, (February 2008), pp. 1457-1460, ISSN 0960-894X.

Díaz-Gavilán, M.; Conejo-García, A.; Cruz-López, O.; Núñez, M. C.; Choquesillo-Lazarte, D.; González-Pérez, J. M.; Rodríguez-Serrano, F.; Marchal, J. A.; Aránega, A.; Gallo, M. A.; Espinosa, A. & Campos, J. M. (2008b). Synthesis and Anticancer Activity of (*RS*)-9-(2,3-Dihydro-1,4-Benzoxathiin-3-ylmethyl)-9*H*-Purines. *ChemMedChem*, Vol.3, No.1, (January 2008), pp. 127-135, ISSN 1860-7179.

Fukuyama, Y.; Hasegawa, T.; Toda, M.; Kodama, M. & Okazaki, H. (1992). Structures of americanol A and isoamericanol A having a neurotrophic property from the seeds of Phytolacca americana. *Chemical & Pharmaceutical Bulletin*, Vol.40, No.1, (January 1992), pp. 252-254, ISSN 0009-2363.

Gali-Muhtasib, H. & Bakkar, N. (2002). Modulating cell cycle: current applications and prospects for future drug development. *Current Cancer Drug Targets*, Vol.2, No.4, (December 2002), pp. 309-336, ISSN 1568-0096.

Guillaumet, G. (1996). 1,4-Dioxins, oxathiins, dithiins and their benzo derivatives. In : *Comprehensive Heterocyclic Chemistry II*, (first edition) Katritzky, A.R. ; Rees, C. W. & Scriven, E. F. V., Vol.6, pp. 447-481, Elsevier, ISBN: 978-0-08-096518-5, Oxford, UK.

Gulyaeva, N.; Zaslavsky, A.; Lechner, P.; Chlenov, M.; McConnell, O.; Chait, A.; Kipnis, V. & Zaslavsky, B. (2003). Relative hydrophobicity and lipophilicity of drugs measured by aqueous two-phase partitioning, octanol-buffer partitioning and HPLC. A simple model for predicting blood-brain distribution. *European Journal of Medicinal Chemistry*, Vol.38, No.4, (April 2003), pp. 391-396, ISSN 0223-5234.

Harris, J. R.; Lippman, M. E.; Morrow, M. & Hellman, S. (1996). *Diseases of the Breast* (first edition), Lippincott-Raven, ISBN 0-397-51470-0, New York.

Hickman, J. A. (1992). Apoptosis induced by anticancer drugs. *Cancer and Metastasis Reviews*, Vol.11, No.2, (April 1992), pp. 121-139, ISSN 0167-7659.

Lees, E. M. & Harlow, E. (1993). Sequences within the conserved cyclin box of human cyclin A are sufficient for binding to and activation of cdc2 kinase. *Molecular and Cellular Biology*, Vol.13, No.2, (January 1993), pp. 1194-1201, ISSN:0270-7306.

Lin, P. & Ye, R.D. (2003). The Lysophospholipid Receptor G2A Activates a Specific Combination of G Proteins and Promotes Apoptosis. *Journal of Biological Chemistry*, Vol.278, No.16, (February 2003), pp. 14379-14386, ISSN 0021-9258.

Lundberg, A. S. & Weinberg, R. A. (1999). Control of the cell cycle and apoptosis. *European Journal of Cancer*, Vol.35, No.4, (December 1998), pp. 531, ISSN 0959-8049.

Malet-Martino, M.; Jolimaitre, P. & Martino, R. (2002). The prodrugs of 5-fluorouracil. *Current Medicinal Chemistry: Anti-Cancer Agents*, Vol.2, No2, (March 2002), pp. 267-310, ISSN 1568-0118.

Marchal, J. A.; Boulaiz, H.; Suárez, I.; Saniger, E.; Campos, J.; Carrillo, E.; Prados, J.; Gallo, M. A.; Espinosa, A. & Aránega, A. (2004). Growth inhibition, G1-arrest, and apoptosis in MCF-7 human breast cancer cells by novel highly lipophilic 5-fluorouracil derivatives. *Investigational New Drugs*, Vol.22, No.4, (November 2004), pp. 379-389, ISSN 0167-6997.

Marchal, J. A.; Nuñez, M. C.; Suarez, I.; Diaz-Gavilan, M.; Gomez-Vidal, J. A.; Boulaiz, H.; Rodriguez-Serrano, F.; Gallo, M. A.; Espinosa, A.; Aranega, A. & Campos, J. M. (2007). A synthetic uracil derivative with antitumor activity through decreasing cyclin D1 and Cdk1, and increasing p21 and p27 in MCF-7 cells. *Breast Cancer Research and Treatment,* Vol.105, No.3, (November 2006), pp. 237-246, ISSN:0167-6806.

Matsuo, S.; Tanako, S.; Yamashita, J. & Ogawa, M. (2000). Synergistic cytotoxic effects of tumor necrosis factor, interferon-γ and tamoxifen on breast cancer cell lines. *Anticancer Research*, Vol.12, No.5, (September 2000), pp. 1575-1579, ISSN 0250-7005.

Meyn, R. E.; Stephens, L. C.; Hunter, N. R. & Milas, L. (1995). Kinetics of cisplatin-induced apoptosis in murine mammary and ovarian adenocarcinomas. *International Journal of Cancer*, Vol.60, No.5, (July 2006), pp. 725-729, ISSN 0020-7136.

Milas, L.; Hunter, N. R.; Kurdoglu, B.; Mason, K. A.; Meyn, R. E.; Stephens, L. C. & Peters, L. (1995). Kinetics of mitotic arrest and apoptosis in murine mammary and ovarian tumors treated with taxol. *Cancer Chemotherapy and Pharmacology*, Vol.35, No.4, (April 1995), pp. 297-303, ISSN 0344-5704.

Núñez, M. C.; Rodríguez-Serrano, F.; Marchal, J. A.; Caba, O.; Aránega, A.; Gallo, M. A.; Espinosa, A. & Campos, J. M. (2007). 6´-Chloro-7- or 9-(2,3-dihydro-5*H*-4,1-benzoxathiepin-3-yl)-7*H*- or 9*H*-purines and their corresponding sulfones as a new family of cytotoxic drugs. *Tetrahedron*, Vol.63, No.1, (January 2007), pp. 183–190, ISSN 0040-4020.

Ozaki, S. (1996). Synthesis and antitumor activity of 5-fluorouracil derivatives. *Medicinal Research Reviews*, Vol.16, No.1, (January 1996), pp. 51-86, ISSN 0198-6325.

Pallavicini, M.; Fumagalli, L.; Gobbi, M.; Bolchi, C.; Colleoni, S.; Moroni, B.; Pedretti, A.; Rusconi, C.; Vistoli, G. & Valotti, E. (2006). QSAR study for a novel series of ortho disubstituted phenoxy analogues of α1-adrenoceptor antagonist WB4101. *European Journal of Medicinal Chemistry*, Vol.41, No.9, (September 2006), pp. 1025–1040, ISSN 0223-5234.

Qin, L. F. & Ng, I. O. (2002). Induction of apoptosis by cisplatin and its effect on cell cycle-related proteins and cell cycle changes in hepatoma cells. *Cancer Letters*, Vol.175, No.1, (January 2002), pp. 27-38, ISSN 0304-3835.

Quaglia, W.; Pigini, M.; Piergentili, A.; Giannella, M.; Marucci, G.; Poggesi, E.; Leonardi, A. & Melchiorre, C. (1999). Structure-Activity Relationships in 1,4-Benzodioxan-Related Compounds. 6. Role of the Dioxane Unit on Selectivity for □1-

Adrenoreceptor Subtypes. *Journal of Medicinal Chemistry*, Vol.42, No.15, (March 1999), pp. 6359-6370, ISSN 0022-2623.

Rasbridge, S. A.; Gillet, C. E.; Seymour, A. M.; Patel, K.; Richards, M. A.; Rubens, R. D. & Millis, R. R. R. (1994). The effects of chemotherapy on morphology, cellular proliferation, apoptosis and oncoprotein expression in primary breast carcinoma. *British journal of cancer*, Vol.70, No.2, (August 1994), pp. 335-341, ISSN 0007-0920.

Reed, J. C. Double identity for proteins of the Bcl-2 family. (1997). *Nature*, Vol.387, No.6635, (June 1997), pp. 773-776, ISSN 0028-0836.

Rekker, R. F. & de Kort, H. M.(1979). The hydrophobic fragmental constant; an extension to a 1000 data point set. *European Journal of Medicinal Chemistry*, Vol.14, No.6, (July 1979), pp. 479-488, ISSN 0009-4374.

Sampath, D. & Plunkett, W. (2001). Design of new anticancer therapies targeting cell cycle checkpoint pathways. *Current Opinion in Oncology*, Vol.13, No.6, (November 2001), pp. 484-490, ISSN 1040-8746.

Saniger, E.; Campos, J. M.; Entrena, A.; Marchal, J. A.; Boulaiz, H.; Aranega, A.; Gallo, M. A. & Espinosa, A. (2003a). Medium Benzene-fused Oxacycles with the 5-Fluorouracil Moiety: Synthesis, Antiproliferative Activities and Apoptosis Induction in Breast Cancer Cells. *Tetrahedron.* Vol.59, No.29, (July 2003), pp. 5457-5467, ISSN 0040-4020.

Saniger, E.; Campos, J. M.; Entrena, A.; Marchal, J. A.; Boulaiz, H.; Aránega, A.; Gallo, M. A. & Espinosa, A. (2003b). Neighbouring Group Participation as the Key Step in the Reactivity of Acyclic and Cyclic Salicyl-Derived *O,O*-Acetals with 5-Fluorouracil. Antiproliferative Activity, Cell Cycle Dysregulation and Apoptotic Induction of New *O,N*-Acetals against Breast Cancer Cells. *Tetrahedron.* Vol.59, No.40, (September 2003), pp. 8017-8026, ISSN 0040-4020.

Satoh, Y.; Powers, C.; Toledo, L. M.; Kowalski, T. J.; Peters, P. A. & Kimble, E. F.(1995). Derivatives of 2-[[*N*-(Aminocarbonyl)-*N*-hydroxyamino]methyl]-1,4-benzodioxan as Orally Active 5-Lipoxygenase InhibitorsJournal of Medicinal Chemistry, Vol.38, No.1, (August 1994), pp. 68-75, ISSN 0022-262.

Saunders, D. E.; Lawrence, W. D.; Christensen, C.; Wappler, N. L.; Ruan, H. & Deppe, G. (1997). Paclitaxel-induced apoptosis in MCF-7 breast-cancer cells. *International Journal of Cancer*, Vol.70, No.2, (January 1997), pp. 214-220, ISSN 0020-7136.

Sherr, C. J. (1993). Mammalian G1 cyclins. *Cell*, Vol.73, No.6, (June 1993), pp. 1059-1065, ISSN 0092-8674.

Sherr, C. J. (2000). The Pezcoller lecture: cancer cell cycles revisited. *Cancer Research. Vol.*60, No.14, (July 2000), pp. 3689-3695, ISSN 0008-5472.

Shi, L; Nishioka, W. K.; Th'ng, J.; Bradbury, E. M.; Litchfield, D. W. & Greenberg, A. H. (1994). Premature p34cdc2 activation required for apoptosis. *Science*, Vol.263, No.5150, (February 1994), pp. 1143-1145, ISSN 0036-8075.

Stacey, D. W. (2003). Cyclin D1 serves as a cell cycle regulatory switch in actively proliferating cells. *Current Opinion in Cell Biology, Vol.*15, No.2, (April 2003), pp. 158-163, ISSN 0955-0674.

Trouet, A.; Passioukov, A.; Derpooten, K. V.; Fernández, A. M.; Abarca-Quiñones, J.; Baurain, R.; Lobl, T. J.; Olilla, C. & Dubois, V. (2001). Extracellulary tumour-activated prodrugs for the selective chemotherapy of cancer: Application to

doxorubicin and preliminary in vitro and in vivo studies. *Cancer Research*, Vol.61, No.7, (April 2001), pp. 2843-2846, ISSN 0008-5472.

Villalobos, M.; Olea, N.; Brotons, J. A.; Olea-Serrano, M. F.; Ruiz de Almodovar, J. M. & Pedraza, V. (1995). The E-screen assay: a comparison of different MCF-7 cell stocks. *Environmental Health Perspectives*, Vol.103, No.9, (September 1995), pp. 844-850, ISSN 0091-6765.

Yoneda T.; Imaizumi, K.; Oono, K.; Yui, D.; Gomi, F.; Katayama, T. & Tohyama, M. (2001). Activation of caspase-12, an endoplasmic reticulum (ER) resident caspase, through tumor necrosis factor receptor-associated factor 2-dependent mechanism in response to the ER stress. *Journal of Biological Chemistry*, Vol.276, No.17, (January 2001), pp. 13935-13940, ISSN 0021-9258.

7

The Analogues of DNA Minor-Groove Binders as Antineoplastic Compounds

Danuta Drozdowska
Medical University in Białystok, Poland

1. Introduction

Chemotherapy and hormonal therapy play important role in the treatment of breast cancer, a leading cause of cancer death in women. Although there are a lot of effective medicines applied, a significant number of patients do not respond to these therapeutic agents. Drug resistance, in addition to side effects of chemotherapy and hormonal therapy, necessitates the search for new specific tumor targeting compounds. Breast cancer cell lines have been widely used not only to investigate breast cancer pathobiology, but also to screen and characterize new therapeutics. Especially MCF-7 and MDA-MB-231 breast cancer cell lines often serve as *in vitro* models in cancer research. In this chapter, some of new compounds which showed antiproliferative and cytotoxic effects against MCF-7 and/or MBA-MB-231 breast cancer cell lines are presented.

2. Minor groove binders (MGB)

The minor groove of double helical B-DNA is becoming a site of a great interest for developing new drugs since it is the site of non-covalent high sequence specific interactions for a large number of small molecules. Minor groove binders are one of the most widely studied class of agents characterized by a high level of sequence specificity and possessing varied biological activities. Most of them exhibit antiviral, antibacterial and antiprotozoal properties. Furthermore, some of these have shown antitumor activity.
The focus of this chapter will be on anti-cancer compounds, active against MCF-7 and/or MBA-MB-231 breast cancer cell lines, which are derived from the non-covalent minor groove binders such as netropsin, distamycin A and related compounds, the Hoechst 33258, DAPI, berenil or pentamidine (Fig.1). These compounds interact only physically with DNA and cause only reversible inhibition of DNA-dependent functions. They possess an inherent curvature that matches approximately the helical curve of the minor groove of B-DNA.
The DNA binding process in the minor groove can be described by two steps (Bailly & Chaires, 1998). In the first, electrostatic and hydrophobic interactions transfer the ligand from solution into the DNA minor groove. In the case of positively charged compounds, such as distamycin, this results in DNA counter-ion exchange. In the second step, various specific interactions are established between the bound ligand and the functional groups of the base pairs of the DNA. The interactions usually include a combination of hydrogen

bonds, hydrophobic and van deer Waals contacts, and electrostatic interactions (Gallmeier & König, 2003).

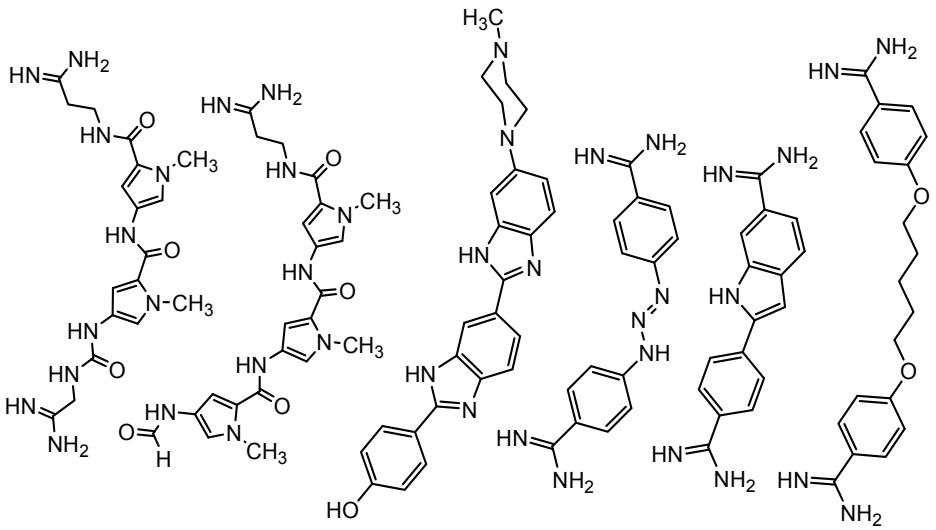


Fig. 1. Structures of minor groove binders.

Although DNA minor groove binding drugs have been extensively reviewed in the last years, defining the chemical and biological aspects of the newly synthesized compounds, only few of them have shown antitumor activity and reached clinical trials.

2.1 Carbocyclic analogues of distamycin and netropsin

Distamycin and netropsin have been shown to be highly DNA sequence-specific and bind preferentially to AT-rich regions of DNA. These oligoamides are highly polar compounds that nevertheless show significant cytotoxic properties.

DNA-binding model of netropsin and distamycin become the inspiration to searches of new compounds with similar interaction to DNA. The class of synthetic heteroaromatic oligopeptides, projected after the models the netropsin and distamycin, received the name lexitropsins (Kopka et al., 1985). Lexitropsins connected with molecules of different known drug, e.g. alkylating agents, are called combilexins (Sondhi et al., 1997). Until now thousands of MGB analogues have been synthesized and some reviews about recent results about analogues of netropsin, distamycin and of some lexitropsins and combilexins or related hybrid molecules with sequence reading, intercalating or alkylating activity were described and evaluated for prospective applications (Bailly & Chaires, 1998; Baraldi et al., 2004; Nelson et al. 2007; Pindur et al, 2005; Reddy et al. 2001). Here the carbocyclic lexitropsins are presented.

The derivatives containing benzene in place of N-methylpyrrole rings, with a minor modification of cationic heads, bind to AT sequences less strongly than the extensively studied MGB, however these compounds show sequence selectivity. It is worth noting that carbocyclic analogues of netropsin and distamycin are readily available, can be modified easily, and are stable under most experimental conditions.

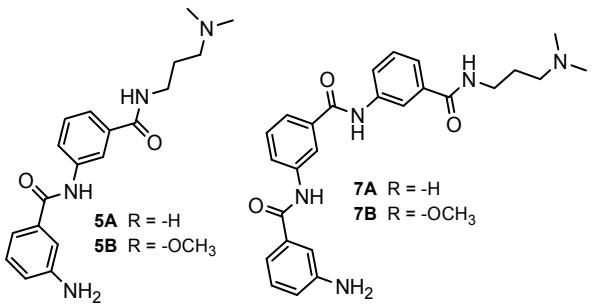


Fig. 2. Structures of carbocyclic netropsin and distamycin analogues.

The showed compounds **5A** and **5B** possess a dimethylamino group in place of the amidinium moiety normally present in netropsin. The synthesis of such C-terminus-modified analogues provides a number of advantages. First, the compounds containing a modified terminus are chemically stable, and thus the synthetic methodology is readily adaptable to preparation of further analogues. Second, they are not hygroscopic and are easy to handle. Third, the dimethylamino group is uncharged, and thus column chromatography or recrystallization can readily purify products and intermediates. Finally, with a pK_a about 9.3, this moiety would be protonated at physiological pH of 7.4 to provide favorable electrostatic attraction to the negative electrostatic charge of DNA. The compounds **7A** and **7B** have the requisite charged end groups and number of potential hydrogen-bonding loci equal to that of distamycin. To obtain information on the DNA binding modes of these types of compounds, additional derivatives, **5B** and **7B**, substituted in *ortho* position to amide moieties of each phenyl ring with a methoxy group were synthesized and designed to provide improved distinction between minor-groove and intercalation binding modes. The methoxy group protrudes from the plane of the aryl ring and would unfavourable clash with the aromatic rings of the base pairs in the intercalation cavity of DNA.

Compounds of this type have the potential for development as carriers for the groove-specific delivery of functionalized groups to DNA and as template inhibitors of transcription. Described compounds were tested for their antitumour activity in the standard cell line of the mammalian tumour MCF-7. The compounds concentration, which inhibits 50% of colony formation, is in the range 24.43 – 105.35 μM, whereas IC_{50} for netropsin studied in the same cell line, is 5.40 μM and for distamycin is 56.95 μM.

During the past years, studies have indicated that antitumour activity of DNA-binding drugs is, at least in part, the result of the inhibition of enzymes that regulate DNA topology: the topoisomerases. Compounds **7A** and **7B** inhibited topoisomerases activity contrary to **5A** and **5B**. This fact indicates that topoisomerase inhibition is selective and sensitive to the number of repeating benzene carboxamide units – a minimum of three benzene carboxamide units are necessary for the inhibition of topoisomerases.

On the basis of molecular modelling it seems that the structure of benzene oligopeptides might be a useful starting framework for synthesis of selective DNA minor groove binding molecules. Molecular modelling of their interaction with $d(CGCGAATTCGCG)_2$ showed that their structure are effectively isohelical with DNA minor groove however with decreased affinity for the minor groove of AT-rich regions in comparison to netropsin and distamycin. From the energetic analysis it appeared that van der Waals and electrostatic interactions are more important than specific hydrogen bonds in stabilizing the ligand-

duplex complexes. Compounds **5A** and **5B** are effectively isohelical with the DNA minor groove. The superior DNA-binding afforded by **5A** and **5B** in comparison to **7A** and **7B** results from their more effective penetration into minor groove and smaller perturbation of molecular structure upon complex formation (Bielawski et al., 2000).
In continuation of rational drug design program aiming to develop distamycin analogues, potential minor-groove binders, and inhibitors of topoisomerases, compounds **1C - 8C** and **1D - 6D** (Fig. 3) were synthesized and examined. This compound skeleton was combined the structural features of distamycin and furamidine.

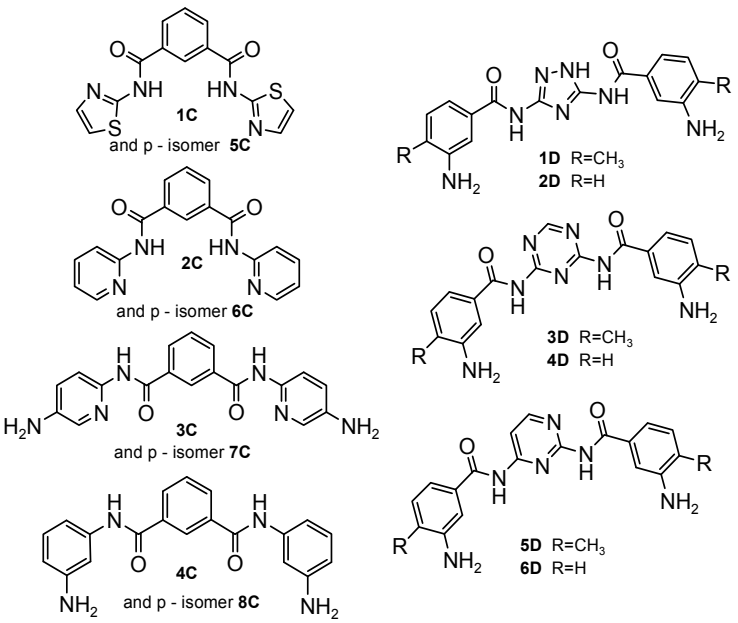


Fig. 3. Structures of distamycin analogues.

Distamycin analogues **1C - 8C** were tested for *in vitro* cytotoxicity towards human breast cancer cells MCF-7 and MDA-MB-231. All of these compounds showed antiproliferative and cytotoxic effects against both cell lines in the range 3.47 – 12.53 μM for MDA-MB-231 and 4.35 – 12.66 μM for MCF-7. All of compounds demonstrated activity against DNA topoisomerases I and II at the concentration 50 μM. Ethidium bromide assay showed that these compounds bind to plasmid pBR322 but weaker than distamycin. The most interesting seems compound **1C** with a time-dependent reduction in proliferation observed in both cell lines at concentrations: 6.38 μM for MCF-7 and 8.79 μM for MDA-MB-231. Compound **5C** with IC_{50} respectively 10.99 μM for MCF-7 and 3.47 μM for MDA-MB-231 cells is also interesting. All of investigated compounds are more potent than chlorambucil, which MCF-7 IC_{50} averages 24,6 μM. The most active analogues due to possession a free amino group can serve as potential carriers of strong acting elements, e.g. alkylating groups.
All of new oligopeptides **1D - 6D** exhibit tumour cell cytotoxicity towards the standard cell line of the mammalian tumour MCF-7 and IC_{50} of examined compounds is in the range 183.53 – 232.50 μM. It is similar value as other minor groove binder DAPI (IC_{50}=176 μM) but weaker than distamycin with IC_{50}=56.95 μM or Hoechst 33258 (IC_{50}=55 μM) and presented earlier analogue of distamycin **7A** (40.73 μM).

Both presented groups of compounds demonstrated activity against DNA topoisomerase I and II. Ethidium bromide assay showed that these compounds bind to plasmid pBR322 but weaker than distamycin.

2.2 Other carbocyclic potential MGB

As a part of ongoing rational drug design programme aiming at development of carbocyclic minor groove binders six other compounds were synthesized and evaluated (Fig.4).

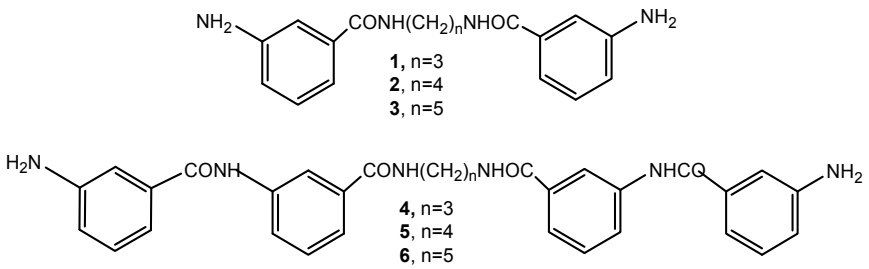


Fig. 4. Structures of carbocyclic potential MGB.

All of the tested compounds showed concentration-dependent activity. Against MDA-MB-231 cells, compounds are more cytotoxic than pentamidine with IC_{50} = 17.74 μM and netropsin with IC_{50} = 228.80 μM. The compound concentration that inhibited 50% of colony formation is in the range 8.10 to 17.52 μM. IC_{50} against MCF-7 cell line were in the range 209.8 to 406.62 μM, while IC_{50} of pentamidine was 14.31 μM and netropsin 5.40 μM. From these data we can see that the compounds **1-6** are nearly twenty times more active against MDA-MB-231 than against MCF-7 cells.

Data from relaxation assays of topoisomerase I and II demonstrated that compounds **1-6** have topoisomerase I inhibitory activity in the range from 10 to 40 μM and topoisomerase II inhibitory activity in the range from 30 to 100 μM.

The influence of compounds **1-6** on the amidolytic activity of urokinase, thrombin, plasmin and trypsin was also investigated. Compounds **1, 2** and **3** are ineffective as amidolytic activity inhibitors. None of investigated compounds inhibited activity of thrombin. Compounds **4-6** are inhibitors of plasmin meanwhile amidolytic activity of urokinase inhibit **5** and **6.** Trypsin activity is inhibited only by compound **6**.

The investigation compounds showed interesting spectrum of their activity. We can see that they bind to minor groove B-DNA and inhibit topo I and topo II activity. Some of them are also inhibitors of plasmin and urokinase. The differences in antiproliferative and cytotoxic effect against MCF-7 and MBA-MD-231 breast cancer cell lines demonstrate that mechanism of action of our compounds is not dependent only from DNA-binding mode but can be partially connected with the fact that in the case of MDA-MB-231 cells higher uPA/uPAR (urokinase plasminogen activator system) expression and high plasminogen-binding was observed than in MCF-7 cell line (Dass et al., 2008).

2.3 Bisamidine derivatives

The aromatic bisamidines, such as DAPI, berenil or pentamidine (Fig.1.), exhibit a wide spectrum of antimicrobial, antiviral, and antitumour properties (Baraldi et al., 2004). A number of natural and synthetic bisamidines are known to bind to B-DNA (Bailly &

Chaires, 1998). However, the precise genomic targets and modes of action these ligands are not known. Most studies have focused on the abilities of bisamidines to inhibit the binding of regulatory proteins to oligonucleotide length recognition sequences that are rich in A and T base pairs. The lack of quantitative correlation between DNA binding and antimicrobial and antitumour activity for these molecules in all of the organisms studied can be attributed to the idea that DNA binding is only the first step in a multistep process.

To investigate DNA binding properties of bisamidines derivatives, novel extended diphenylfuran analogues **KB1-KB4** (Fig.5) possessing different dicationic terminal side chains were synthesized (Bielawski et al., 2001b). In the topoisomerase II assay, the relaxation of DNA was inhibited with all four drugs and the extent of inhibition was directly proportional to the drug concentration. Compounds **KB2-KB4** did not inhibit the topoisomerase I mediated relaxation of supercoiled DNA, compound **KB1** showed inhibiting activity at 80 μM.

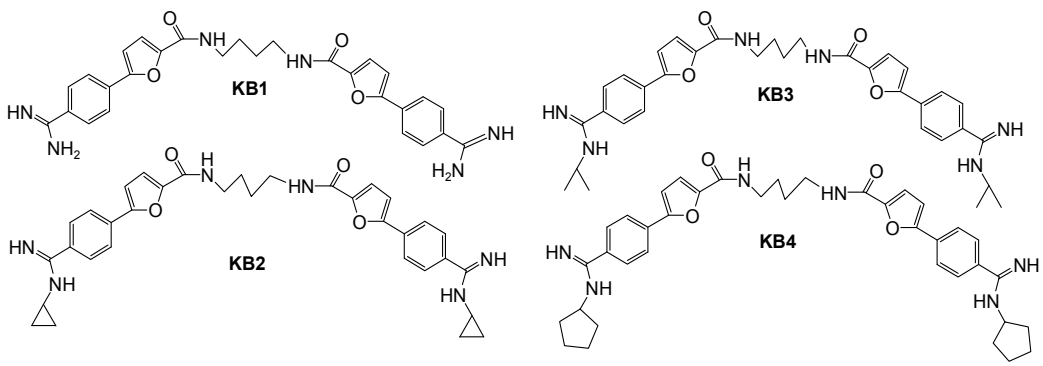


Fig. 5. Structures of novel bisamidines.

The ultrafiltration assay showed that examined compounds have significant affinity for DNA. The DNA-binding data using homopolymers poly(dA-dT) · poly(dA-dT) and poly(dG-dC) · poly(dG-dC) indicated that these compounds show moderate specificity for AT base pairs. The cytotoxicity effects of **KB1-KB4** were studied in cultured breast cancer MCF-7 cells and found to be 63 μM, 85 μM, 77 μM and 97 μM, respectively. The novel bisamidines showed comparable antitumour activity to Hoechst 33258, but were substantially more cytotoxic compared to DAPI. These data showed that in broad terms the cytotoxic potency of bisamidines **KB1-KB4** in cultured breast cancers MCF-7 cells decreases with the size of the alkyl group substituent (cyclopropyl > isopropyl > cyclopentyl), in accord with their increases in DNA affinity (Bielawski et al., 2001a). This suggests that DNA-binding may be implicated in the cytotoxicity of these bisamidines, possibly by inhibiting interactions between cellular proteins and their DNA targets.

3. Synthetic minor groove binders as carriers for alkylating moieties

DNA alkylating agents are a major class of anticancer drugs for the treatment of various cancers including breast cancer. The first nitrogen mustard used in therapy was mechlorethamine, and the related compounds chlorambucil, melphalan, and cyclophosphamide remain in use today. A drawback common to all DNA alkylating agents is their high chemical reactivity. This can result in loss of drug by reaction with other

cellular nucleophiles, particularly proteins, and low-molecular weight thiols. This makes them vulnerable to cellular resistance mechanisms such as increased levels of glutathione. Other limitations, discussed particularly for mustards, are a lack of intrinsic DNA binding affinity of the core *N,N*-bis(2-chloroethyl)amine pharmacophore, and a requirement for bifunctional cross-linking of DNA to be fully cytotoxic. These characteristic lower their potency and the observed high ratio of genotoxic monoadducts to cross-links (up to 20:1) contributes to their known carcinogenicity. There is also evidence that the major guanine N7 adduct formed by mustards and other alkylators is readily repaired, which may also result in lower cytotoxicity (Osborne et al., 1995). For these reasons there has been much interest in the concept of specially targeting alkylating agents to DNA by attaching them to DNA affine carrier molecules, as this could in principle address these limitations. Increasing the concentration of drug in the vicinity of DNA would mean less chance of losing active drug by reaction with other cell components. Additionally, the use of DNA-affine carriers with their own defined binding geometry makes it possible to alter both the region and sequence specificity of alkylation compared with that of the alkylators

3.1 Distamycin related alkylating agents

Work on the targeting of nitrogen mustard alkylating agents to DNA by the use of DNA minor groove-binding ligands has shown that this strategy can greatly enhance both the *in vitro* cytotoxicity and the *in vivo* antitumour activity of the mustard moiety, when compared with untargeted mustards of similar reactivity. The main representative of this class that was clinically tested is tallimustine (Fig. 6), a benzoic acid nitrogen mustard derivative of distamycin (Cozzi, 2003).

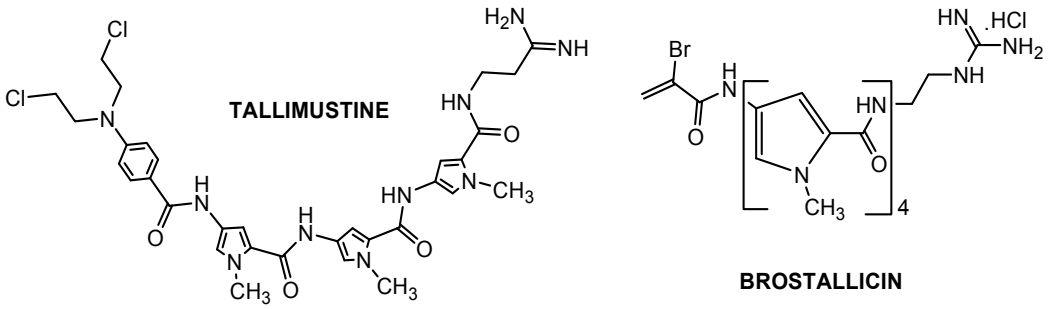


Fig. 6. Structures of tallimustine and brostallicin.

Tallimustine (TAM) showed cytotoxicity against L1210 murine leukemia more than two orders of magnitude higher then distamycin and more than one order of magnitude higher then classical nitrogen mustard melphalan. This compound is a very sequence and regiospecific alkylator, reacting only by monoalkylation at the N3 position of the 3′-adenine in the sequence 5′-TTTTGA-3′.

Whereas the cytotoxicity of TAM is related to the ability to form interstrand cross-links in DNA with consequent inhibition of DNA replication and transcription, the mechanism of antitumour action of tallimustine, although it is not yet fully elucidated may be due to its ability to inhibit the binding of some transcription factors to their consensus sequences in DNA. The cell cycle phase perturbations caused by tallimustine and melphalan were different and can be related to the different DNA damage done by these two drug.

Tallimustine showed excellent antitumor activity in preclinical tests, but also a severe myelotoxicity (Cozzi, 2003).

A second generation DNA minor groove binder, structurally related to distamycin is brostallicin (PNU-166196), alpha-bromo-acrylamido tetra-pyrrole derivative ending with a guanidino moiety (Fig.6). This compound showed broad antitumour activity in preclinical models and dramatically reduced *in vitro* myelotoxicity in human hematopoietic progenitor cells compared with that of TAM and other MGB. Brostallicin showed a 3-fold higher activity in melphalan-resistant L1210 murine leukemia cells than in the parental line (IC_{50}-0.46 and 1.45 ng/ml, respectively) under conditions in which the cytotoxicity of conventional antitumor agents was either unaffected or reduced. This melphalan-resistant cell line has increased levels of glutathione (GSH) in comparison with the parental cells. Conversely, GSH depletion by buthionine sulfoximine in a human ovarian carcinoma cell line (A2780) significantly decreased both the cytotoxic and the proapoptotic effects of brostallicin. A 2–3-fold increase in GST-levels resulted in a 2–3-fold increase in cytotoxic activity of brostallicin. Similar results were obtained for GST-transfected human breast carcinoma cells (MCF-7).

In an *in vivo* experiment, A2780 clones were implanted into nude mice. The antitumor activity of brostallicin was higher in the GST-overexpressing tumors without increased toxicity. Regarding the mechanism of action, brostallicin interacts reversibly with the DNA minor groove TA-rich sequences but appears unreactive in classical *in vitro* DNA alkylation assays. Evidence of both covalent interaction of brostallicin with plasmidic DNA in the presence of GSH and of enhanced cytotoxicity in cancer cells characterized by high levels of GSH was obtained (Geroni et al., 2002). Brostallicin was selected for clinical development and is presently in clinical trials in Europe and the United States (Fedier et al. 2003). The phase II of studies of brostallicin in combination with cisplatin for metastatic breast cancer is currently in the stage of testing.

3.2 Carbocyclic lexitropsins with chlorambucil moiety

The carbocyclic lexitropsins investigated so far were not such active to be used as agent in breast cancer therapy but the application of them as potential carriers of strong acting elements was also examined. For example, derivatives with N-terminal chlorambucil group have been synthesized (Fig.7.).

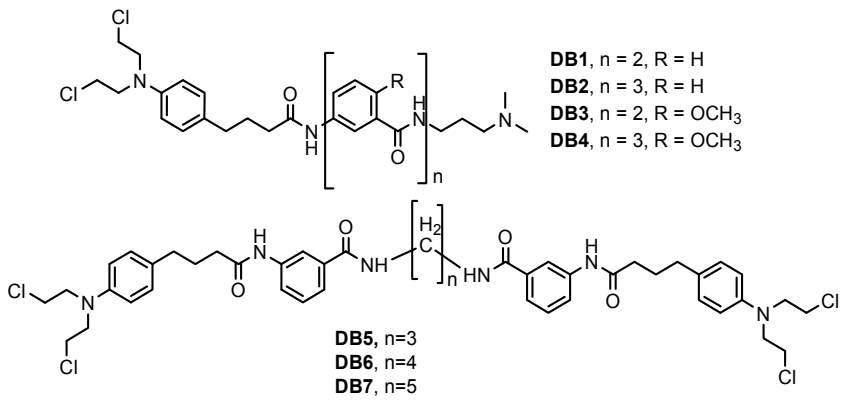


Fig. 7. Structures of carbocyclic lexitropsins with chlorambucil moiety.

After the molecular mechanics refinement calculations, energetically favoured complexes of compounds **DB1** and **DB3** with $d(CGCGAATTCGCG)_2$ were obtained (Fig.8.)

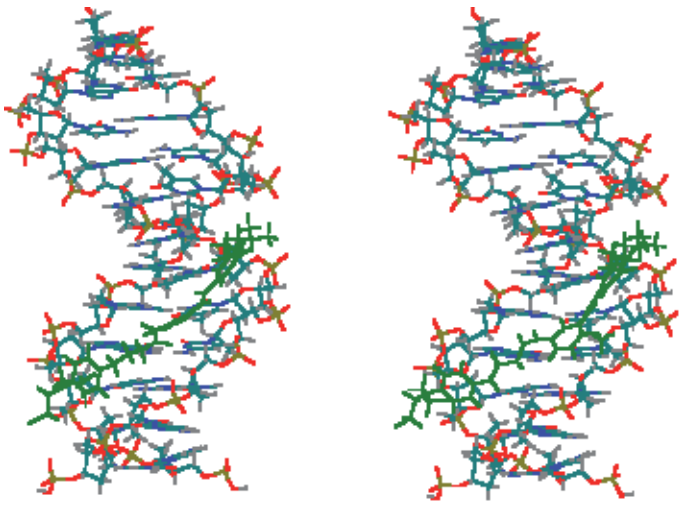


Fig. 8. Views of the low-energy complexes formed between the $d(CGCGAATTCGCG)_2$ and the carbocyclic analogues of distamycin after MD refinement. Left - **DB1**; right - **DB3**. Ligands molecules are shown in green.

Compounds **DB1** and **DB3** form centrosymetric 4 bp complexes with the ligands displaced towards the 5′ end of the 5′-AATT binding site. This displacement facilitates increased Waals contacts with the walls of the minor groove. In addition to the decreasing affinity for the 5′-AATT-3′ match site, there are weaker contacts with the O2 atom of C21 indicating that the binding-site size requirement for **DB1** and **DB3** extends over slightly more than the four central AT base pairs. The energy wells for these ligands within this AT tract are narrow and the data indicate that specific interactions with flanking sequences strongly inhibit ligand translation along the minor groove. The benzene rings **DB1** and **DB3** are positioned roughly in the plane of the bases and the amide groups are located between base pairs. No regular pattern of bifurcated hydrogen bonds then exists. From the analysis of these complexes it appears that van deer Waals and electrostatic interactions are more important in stabilizing the complexes than specific hydrogen bonds formation. This is consistent with the observed reduced affinity to AT pairs and increased affinity towards GC sequences of the carbocyclic lexitropsins with chlorambucil moieties in comparison with distamycin and netropsin. The protonated terminal dimethylamine nitrogen of the (dimethylamino)propyl tail is adjacent to a negatively charged phosphodiester linkage. The hydrophobic methoxy groups of **DB3** are situated outside the minor groove; therefore, the binding energies for **DB1** and **DB3** are almost the same. Compounds **DB1** and **DB3** produce an increase in groove width of ca. 1.5 Å compared with the netropsin-DNA complex (Kopka et al., 1985). Because of flexibility of the aliphatic tether of chlorambucil moiety, there is probably a limited distribution of alkylation sites derived from an individual binding complex rather than a unique alkylation site for each individual bound compound. An accurate definition by molecular modelling of the optimal binding site for the compounds studied alone has been hampered by the fact that the DNA fragment used in the model contains a limited number of binding sites.

The **DB1-DB4** compounds concentration, which inhibits 50% of breast cancer MCF-7colony formation, is in the range 85 - 104 μM. In the case of **DB5-DB7** compounds, this cconcentration is in the range 66 to 124 μM. All of them induced cancer cell death by apoptosis and necrosis.

3.3 Amidine analogues of chlorambucil

Also a number of novel cyclic amidine analogs of chlorambucil (Fig.9) were synthesized and examined for cytotoxicity in breast cancer cell cultures.

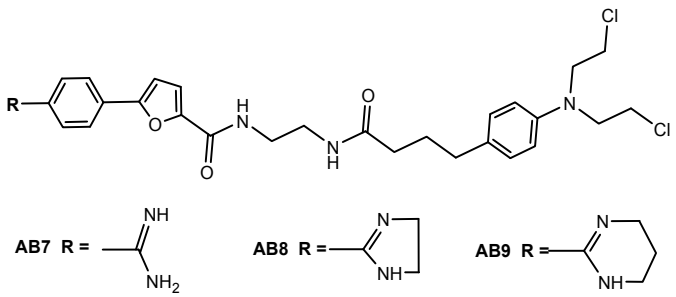


Fig. 9. Structures of amidine analogues of chlorambucil **AB7-AB9**.

In terms of reduction in cell viability, the compounds rank in both MCF-7 and MDA-MB-231 cells in the order **AB9 > AB7 > AB8 >** chlorambucil. The values of IC_{50} were relatively higher for **AB 9** and **AB 7** which possess a cationic 4,5-dihydro-1*H*imidazol and amidine function, respectively. Among the derivatives, compound **AB 8** in both MDA-MB-231 and MCF-7 proved to be only slightly more potent than chlorambucil, with IC_{50} values of 70 and 76 μM, respectively, compared to 92 and 97 μM for chlorambucil. In contrast, compound **AB9,** which contains the 4,5-dihydro-1*H*imidazol moiety is clearly much more active and showed a high level of cytotoxic potency, IC_{50} 22 and 18 μM in MCF-7 and MDA-MB-231, respectively. Compound **AB9,** the most active of the series, is approximately five times more potent than chlorambucil.

The degree to which these compounds inhibited cell growth breast cancer cells was directly correlated to DNA-binding affinity.

The ability of compounds **AB7–9** to inhibit topoisomerases I and II activity was quantified by measuring the action on supercoiled pBR322 DNA substrate as a function of increasing concentration of the ligands by the use of agarose gel electrophoresis. Chlorambucil as a control was, as expected, ineffective in this assay. The investigation indicate that cyclic amidine analogs of chlorambucil are a potent catalytic inhibitor of topoisomerase II but not topoisomerase I. Compound **AB9** was the most potent topoisomerase II inhibitors, with 50% inhibitory concentration (IC_{50}) 5 μM. (Bielawska et al., 2004).

Compound **AB7** and chlorambucil were compared for their effects on collagen and DNA synthesis in breast cancer MDA-MB-231 cells. IC_{50} values for chlorambucil and its amidine analogue for collagen synthesis were found to be about 44 and 19 μM, respectively. Increased ability of **AB7** to suppress the protein synthesis, compared to chlorambucil, was found to be related to an inhibition of prolidase activity and expression. The phenomena were probably a result of disruption of β_1-integrin and the insulin-like growth factor-I (IGF-I) receptor mediated signaling caused by this compound. Expression of β_1-integrin receptor, as well as focal adhesion kinase $pp125^{FAK}$ (FAK), growth-factor receptor-bound protein 2

(GRB2), son of sevenless protein 1 (Sos1) and phosphorylated mitogen activated protein kinases (MAPK), extracellular-signal-regulated kinase 1 (ERK1) and kinase 2 (ERK2) but not Src and Shc proteins was significantly decreased in cells incubated for 24 h with 10 μM **AB7**, compared to controls. Chlorambucil in the same conditions did not evoke any changes in expression of all these signaling proteins, as shown by Western immunoblot analysis. In addition, **AB7** revealed a higher antiproliferative activity than chlorambucil, accompanied by a stronger down-regulation of IGF-I receptor expression. The results were confirmed by [3H]thymidine incorporation assay. Incubation of the cells with 10 μM **AB7** for 12 and 24 h contributed to a decrease in DNA synthesis by about 33 and 46% of the control values, respectively, while in case of chlorambucil by about 23 and 29%, respectively. These data suggest that the amidine analogue of chlorambucil (**AB7**) disturbs MDA-MB 231 cell metabolism more potently than does the parent drug, chlorambucil.
The mechanism of this phenomenon may be due to its stronger suppression of β_1-integrin and IGF-I receptor signaling. (Sienkiewicz et al., 2005).

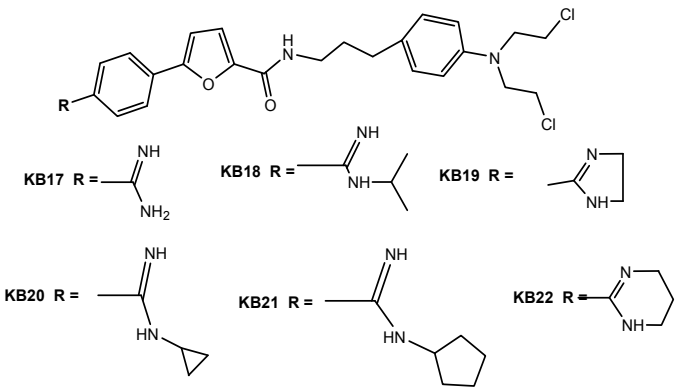


Fig. 10. Structures of amidine analogues of chlorambucil **KB17-KB22**.

As continuation of chlorambucil analogues of amidines investigations, novel nitrogen mustard agents **KB17–KB22** (Fig.10) involving 4-(N,N-bis(2-chloroethyl)-aminophenyl)propylamine linked to a 5-(4-N-alkylamidinophenyl)-2-furancarboxylic acid moiety by the formation of an amide bond have been synthesized, characterized, and evaluated for their *in vitro* cytotoxic activity against MDA-MB-231 and MCF-7 human breast cancer cells. Evaluation of the cytotoxicity of **KB17–KB22** employing a MTT assay and inhibition of [3H]thymidine incorporation into DNA demonstrated that these compounds exhibit remarkable cytotoxic effects in comparison with 4-[bis(2-chloroethyl)amino]benzenebutanoic acid. Compounds **KB17** and **KB19**, which possess a cationic amidine and 4,5-dihydro-1H-imidazol function moiety are approximately ten times more potent than 4-[bis(2-chloroethyl)amino]benzenebutanoic acid. The new compounds were evaluated as DNA topoisomerase II inhibitors. The cytotoxicity of the compounds **KB17–KB22** correlates with their DNA binding affinities and their relative potency as topoisomerase II inhibitors (Bielawski et al., 2009).

3.4 Amidine analogues of melphalan

The amidine analogues of melphalan **KB6-KB10** (Fig.11) differing by the nature of terminal basic side were synthesized and examined (Bielawska et al., 2007). Evaluation of the

cytotoxicity of these compounds was employing a MTT assay in both MDFA-MB-231 and MCF-7 human breast cancer cells. Although growth inhibition was concentration-dependent in either cell line, it was more pronounced at shorter times, in MCF-7 than MDA-MB-231. In terms of reduction in cell viability, the compounds rank in both MCF-7 than MDA-MB-231 cells in the order **KB7 > KB6 > KB8 > KB9 > KB10 >** melphalan.

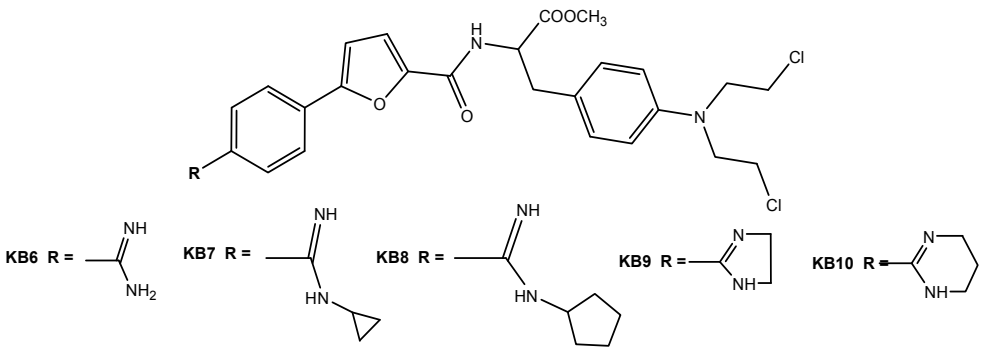


Fig. 11. Structures of amidine analogues of melphalan.

The values of IC_{50} were relatively higher for **KB7** which possess a cationic N-cyclopropylamidine function. Among the derivatives, compound **KB10** in both MDA-MB-231 and MCF-7 proved to be only slight more potent than melphalan, with IC_{50} values of 117 and 100 μM, respectively, and compared to 130 and 125 μM for melphalan. In contrast, compound **KB7** is clearly much more active and showed a high level of cytotoxic potency, IC_{50} 55 and 77 μM in MCF-7 and MDA-MB-231, respectively. Compound **KB7**, the most active of the series, is approximately 2 times more potent than melphalan.

An attempt has also been made correlate the observed biological activity with topoisomerases inhibitory properties and DNA-binding properties of selected compounds. The cytotoxicity of the amidine analogues of melphalan towards cultured human breast cancer cells correlate with topoisomerase II inhibitory properties but not with DNA-binding properties.

A molecular mechanics and molecular dynamics approach was used to examine the structure of complex formed between the $d(CGCGAATTCGCG)_2$ duplex and compound **KB7**. It is predicted that this compound should have a decreased affinity for the minor groove of AT-rich regions in comparison to netropsin and furamidine. From the energetic analysis it appears that van der Waals and electrostatic interactions are more important than specific hydrogen bonds in stabilizing the ligand duplex, similarly like described earlier chlorambucil derivatives of carbocyclic lexitropsins.

These experimental studies suggest that amidine analogues of melphalan **KB6-KB10** may have other consequences for the metabolism of breast cancer cells. There were found that compound **KB7** is a more potent inhibitor of collagen biosynthesis than a parent drug, melphalan (Bielawski et al., 2006).

Melphalan foe 24 h did not affect the expression of proteins involved in the signaling cascade activated by β-integrin receptor. In contrast, compound **KB7** inhibited expression of Shc and MAP-kinases in both estrogen receptor-positive and estrogen receptor-negative breast cancer cells. Decreased expression of FAK-kinase was found only in MDA-MB-231 cells. Another important benefit evoked by the compound **KB7** seems to be inhibition of

phospho-ERK's activation (Bielawski et al., 2006). Upregulation of those kinases was found in various breast cancers (Santen et al., 2002). Blocking these kinases was found to have proapoptotic and antiproliferative effects on MDA-MB-231, that indicates a new target in the treatment of breast malignancies (Fukazawa et al., 2002). Induction of apoptosis by **KB7** in both MDA-MB-231 and MCF-7 breast cancer cells was stronger than by the parent drug and run by activating caspase-3 (Sosnowska et al., 2009).
These results and other recent studies indicate that the amidine analogues of melphalan represent multifunctional inhibitors of breast cancer cells growth and metabolism.

3.5 Alkylating analogues of Hoechst 33258

A series of carbamate derivatives of Hoechst 33258 was prepared as potential anticancer agents (Fig. 12).

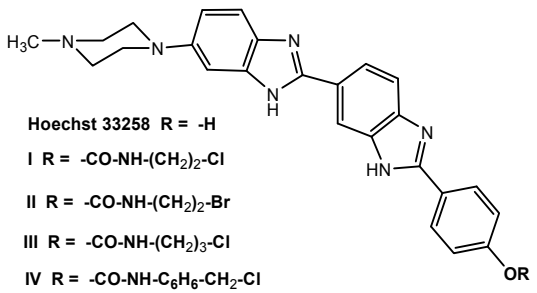


Fig. 12. Structures of alkylating analogues of Hoechst 33258.

These new compounds (**I—IV**) were readily prepared in good yields by addition of chloroethyl, bromoethyl, chloropropyl or 4-(chloromethyl)phenyl isocyanates to Hoechst 33258. Their cytotoxic activity was evaluated on human breast cancer MCF-7. Compounds **I-IV** were more cytotoxic than Hoechst 33258. In particular derivative **IV**, the most active of the series, is up to 3 times more potent than Hoechst 33258. The DNA-binding ability of these compounds was evaluated by an ultrafiltration method using calf thymus DNA. These data show that in broad terms the cytotoxic potency of **I-IV** in cultured breast cancer MCF-7 cells increases, in accord with their increases in DNA affinity, as shown by the binding constant values (Bielawski at al., 2002).

4. Conclusion

An understanding of the mechanism, by which minor groove binding agents interact with DNA has led to the design of agents that can reversibly bind with high selectivity to extended DNA target sequences. Until now thousands of MGB analogues have been synthesized – here has been presented only small part of all investigations.
The described results in the field of distamycin and netropsin, as well as other minor groove binders, and modifications of their structures give the expectation of obtaining a compound with required activity; which will be able to be applied as medical agent in anticancer therapy. Targeting alkylating moieties to DNA by attachment of DNA minor groove binding carrier, such as distamycin, netropsin, or Hoechst 33258 reduces the loss of active drug, due to reaction with other cell components and makes it possible to direct the alkylation both sequence specifically and regiospecifically. These compounds are able to

compete with natural substrates, such as specific transcription factors, and alter gene expression.

An overall conclusion from this review is the increasing molecular-level knowledge about how the simpler minor groove binding agents bind to DNA. This in turn has fed into the design of agents that can reversibly bind with high selectivity to longer sequences of virtually any composition, which likely occur very seldom in the genome. Such compounds are highly effective tools, which are being explored in more and more complex biological systems.

Although the biomedical sciences have recently been in intensive progress, it is difficult to find selective targets for caner chemotherapy. Still many of drugs used today for treating cancer patients, also patients with breast cancer, are in fact practically nonselective and exhibit severe toxicity to normal tissues. Hence, each new synthesized compound gives the chance to obtain a better result than previously.

5. Acknowledgment

I wish to thank my many co-workers, past and present, for the results presented in this chapter. This work is supported by Medical University in Białystok.

6. References

Bailly, C. & Chaires, J.B. (1998) Sequence-Specific DNA Minor Groove Binders. Design and Synthesis of Netropsin and Distamycin Analogues. *Bioconjugate Chem.,* vol.9, No.5, (June 1998), pp. 513 -538.

Baraldi, P.G., Bovero, A., Fruttarolo, F., Preti, D., Tabrizi, M.A., Pavani, M.G. & Romagnoli, R. (2004) DNA Minor Groove Binders as Potential Antitumor and Antimicrobial Agents. *Medicinal Research Reviews,* vol.24, No.4, (July 2004), pp. 475-528.

Bielawska, A., Bielawski, K. & Muszyńska, A. (2004) Synthesis and Biological Evaluation of New Cyclic Amidine Analogs of Chlorambucil. *Il Farmaco,* vol.59, No.2, (February 2004), pp.111-117.

Bielawska, A., Bielawski, K. & Anchim, T. (2007) Amidine Analogues of Melphalan: synthesis, Cytotoxic Activity, and DNA Binding Properties. *Archiv der Pharmazie - Chemistry in Life Sciences,* vol.340, No.5, (May 2007), pp. 251-257.

Bielawski, K., Bielawska, A., Bartulewicz, D. & Różański, A. (2000) Molecular modelling of the interaction of carbocyclic analogues of netropsin and distamycin with $d(CGCGAATTCGCG)_2$. *Acta Biochimica Polonica,* vol.47, No.3, (July 2000), pp. 855-866.

Bielawski, K., Wołczyński, S. & Bielawska, A. (2001a) DNA-Binding Activity and Cytotoxicity of the Extended Diphenylfuran Bisamidines in Breast Cancer MCF-7 Cells. *Biological & Pharmaceutical Bulletin,* vol.24, No.6, (April 2001), pp. 704-706.

Bielawski, K., Bielawska, A. & Wołczyński, S. (2001b) Aromatic Extended Bisamidines: Inhibition of Topoisomerases, and Anticancer Cytotoxicity in Vitro. *Archiv der Pharmazie – Pharmaceutical and Medicinal Chemistry,* vol.334, No.7, (July 2001), pp.235-240.

Bielawski, K., Bielawska, A. & Wołczyński, S. (2002) Synthesis, DNA-Binding Activity and Cytotoxicity of Carbamate Derivatives of Hoechst 33258 in Breast Cancer MCF-7

Cells. *Archiv der Pharmazie – Pharmaceutical and Medicinal Chemistry,* vol.25, No.7, (July 2002), pp. 916—919.

Bielawski, K., Bielawska, A., Sosonowska, K., Miltyk, W., Winnicka, K. & Pałka, J. (2006) Novel Amidine Analogue of Melphalan As a Specific Multifunctional Inhibitor of Growth and Metabolism of Human Breast Cancer Cells. *Biochemical Pharmacology,* vol.72, No.3, (July 2006), pp. 320-331.

Bielawski, K., Bielawska, A. & Popławska, B. (2009) Synthesis and Cytotoxic Activity of Novel Amidine Analogues of Bis(2-chloroethyl)amine. *Archiv der Pharmazie - Chemistry in Life Sciences,* vol.342, No.8, (August 2009), pp. 484 – 490.

Cozzi, P. (2003) The Discovery of a New Potential Anticancer Drug: A Case History. *Farmaco*, vol. 58, No. 3, (March 2003), pp. 213-220.

Dass, K., Ahmad, A., Azmi, A.S., Sarkar, S.H. & Sarkar, F.H. (2008) Evolving Role of uPA/uPAR System in Human Cancers, *Cancer Treatment Reviews*, vol.34, No.2, (April 2008), pp. 122-136.

Fedier, A., Fowst, C., Tursi, J., Geroni, C., Haller, U., Marchini, S. & Fink, D. (2003) Brostallicin (PNU-166196) – a new DNA minor groove binder that retains sensitivity in DNA mismatch repair-deficient tumour cells. *British Journal of Cancer,* vol.89, No.8, (October 2003), pp. 1559-1565.

Fukazawa, H., Noguchi, K., Murakami, Y. & Uehara, Y. (2002) Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Kinase (MEK) Inhibitors Restore Anoikis Sensitivity In Human Breast Cancer Cell Lines With A Constitutively Activated Extracellular-Regulated Kinase (ERK) Pathway. *Molecular Cancer Therapeutics,* vol.1, No.5, (March 2002), pp. 303-309.

Gallmeier, H.C. & König, B. (2003) Heteroaromatic Oligoamides with dDNA Affinity. *European Journal of Organic Chemistry,* vol.2003, No.18, (September 2003), pp. 3473-3483.

Geroni, C., Marchini, S., Cozzi, P., Galliera, E., Ragg, E., Colombo, T., Battaglia, R., Howard, M., D'Incalci, M. & Broggini, M. (2002) Brostallicin, a Novel Anticancer Agent Whose Activity Is Enhanced upon Binding to Glutathione. *Cancer Research,* vol.62, No.8, (April 2002), pp. 2332-2336.

Kopka, M.L., Yoon, C., Goodsel, D., Pjura, P. & Dickerson, R.E. (1985) The Molecular Origin of DNA-Drug Specificity of Netropsin and Distamycin. *The Proceedings of the National Academy of Sciences USA*, vol.82, No.5, (March 1985), pp. 1376-1380.

Nelson, S.M., Ferguson, L.R. & Denny, W.A. (2007) Non-covalent Ligand/DNA Interactions: Minor Groove Binding Agents. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, vol.623, No.1-2, (November 2007), pp. 24-40.

Osborne, M.R., Lawley, P.D., Crofton-Sleigh, C. & Warren, W. (1995) Products From Alkylation of DNA in Cells by Melphalan: Human Soft Tissue Sarcoma Cell Line RD and Escherichia Coli WP2. *Chemico-Biological Interactions*, vol.97, No.3, (August 1995), pp. 287-296.

Pindur, U., Jansen, M. & Lemster, T. (2005) Advances in DNA-Ligands with Groove Binding, Intercalating and/or Alkylating Activity: Chemistry, DNA-Binding and Biology. *Current Medicinal Chemistry*, vol. 12, No. 24, (November 2005), pp. 2805-2847.

Reddy, B.S.P., Sharma, S.K. & Lown, J.W. (2001) Recent Developments in Sequence Selective Minor Groove DNA Effectors. *Current Medicinal Chemistry*, vol.8, No.5, (May 2001), pp. 475-508.

Santen, R.J., Song, R.X., McPherson, R., Kumar, R., Adam, L., Jeng, M.H. & Yue, W. (2002) The role of mitogen-activated protein (MAP) kinase in breast cancer. *The Journal of Steroid Biochemistry and Molecular Biology,* vol.80, No.2, (February 2002), pp. 239-256.

Sienkiwicz, P., Bielawski, K., Bielawska, A. & Pałka, J. (2005) Inhibition of Collagen and DNA Biosynthesis by a Novel Amidine Analogue of Chlorambucil Is Accompanied by Deregulation of β_1-integrin and IGF-I Receptor Signaling in MDA-MB-231 Cells. *Environmental Toxicology and Pharmacology,* vol.20, No.1 (July 2005), pp. 118-124.

Sondhi, S.M., Praveen, B.S. & Lown, J.W. (1997). Lexitropsin Conjugates: Action on DNA targets. *Current Medicinal Chemistry,* vol.4, No.5 (October 1997), pp. 313-358.

Sosnowska, K., Bielawski, K., Winnicka, K., Rusak, M. & Bielawska, A. (2009) Induction of Apoptosis by an Amidine Analogue of Melphalan in Cultured Normal Human Skin Fibroblasts, MDA-MB-231 and MCF-7 Breast Cancer Cells. *Farmaceutyczny Przegląd Naukowy,* vol.3, No.1, (January 2009), pp. 37-43.

8

Fractionation and Characterization of Bioactive Components in Kefir Mother Culture that Inhibit Proliferation of Cultured MCF-7 Human Breast-Cancer Cells

Chujian Chen[1,2], Hing Man Chan[3] and Stan Kubow[1]
[1]*School of Dietetics and Human Nutrition, Macdonald Campus of McGill University, Ste-Anne-de-Bellevue, Quebec*
[2]*School of Public Health, Nantong University, Nantong, Jiangsu*
[3]*Community Health Program, University of Northern British Columbia, Prince George, British Columbia*
[1,3]*Canada*
[2]*People's Republic of China*

1. Introduction

Breast cancer is the most commonly diagnosed cancer in women. Resistance to therapy is the major reason for failure of cancer treatment (Mesner *et al*., 1997). There is a critical need to identify new chemotherapeutic agents that can increase susceptibility to anti-breast cancer drugs or overcome resistance mechanisms, which would improve patient outcomes, prevent relapse, and prolong patient survival. Kefir is an acidic and mildly alcoholic fermented milk that originated in the Caucasian mountains of former Soviet Union, and enjoys a rich tradition of health benefits. Consumption of fermented dairy foods has been associated with lower incidence of breast cancer (Ronco *et al*., 2002). Research on the putative health benefits of fermented milks suggested that by-products of bacterial fermentation of proteins, lipids and carbohydrates present in fermented milks exert health benefits beyond basic nutrition including anti-tumor action, immune system enhancement and antioxidant effects (Parvez *et al*., 2006). Conjugated linoleic acid (Schonberg *et al*., 1995), sphingolipids (Dillehay *et al*., 1994), polysaccharides (Shiomi *et al*., 1982), organic acids (Garrote *et al*., 2000) and some proteins and peptides (Svensson *et al*., 1999) have been shown to have antimutagenic and antitumor effects. Promising results regarding anti-tumor activity of yogurt extracts in cell culture (Biffi *et al*., 1997) and kefir extracts in animal feeding studies (Cervikbas *et al*., 1994; Furukawa *et al*., 2000; Shiomi *et al*., 1982) have been reported. However, the bioactive components in kefir and the mechanisms involved in the various biofunctional effects of kefir are still not well characterized, particularly in terms of anti-cancer activities. In our recent studies, kefir extracts exerted potent anti-proliferative effects on cultured human mammary tumor cells as compared to extracts of yogurt or milk (Chen *et al*., 2007). The aim of the present study was to fractionate and characterize bioactive components in kefir mother culture that exert antiproliferative effects in MCF-7 cells.

2. Materials and methods

Kefir samples were provided by Liberte Inc. (Brossard, Canada). The large-scale production of kefir involves a two-step fermentation process. The first fermentation is achieved by directly adding kefir grains (2-10%) to milk that has been pasteurized and cooled to 20-25°C. After a period of fermentation lasting around 24 h, the grains are removed by filtration. The filtrate (kefir mother culture) is added to milk (1-3%), which is further fermented for 24 h and packaged for the consumer market (final kefir commercial product). Samples from three different batches were used. Upon receipt of the samples, they were immediately well stirred, and centrifuged at 4°C, 32,000 x g, for 60 min (Sorvall RC 5C Centrifuge, rotor ss-34, Sorvall Instruments, Wilmington, USA). The supernatant was filtered with a 0.45 µm membrane filter followed by a 0.2 µm filter. The filtrates were stored at −80°C for future use.

2.1 Macronutrients and minerals

A Flexi-Dry MP lyophilizer (FTS Systems Inc., Stone Ridge, USA) was used for triplicate determination of moisture. Ten grams of homogenized sample was transferred into pre-weighed aluminium weigh boat, frozen at -80°C for approximately 1 h and then freeze dried for 48 h. The boat was weighed again and the moisture was calculated. A LECO FP-428 Nitrogen Determination System (LECO Corporation, St. Joseph, USA) was used to determine nitrogen content in triplicate for freeze-dried samples. Crude protein content was calculated using a conversion factor of 6.25. Protein in solution was determined by using Bio-Rad protein assay kit according to the instruction with the kit (Bio-Rad Laboratories, Hercules, USA). Peptide concentrations were analyzed by the method of Church *et al.* (1983) (opthialdehyde; OPA). Crude fat was analyzed in triplicate with an automatic Soxtec extraction system (Soxtec HT6 Tecator AB, Hoganas, Sweden). Three grams of freeze-dried, well-mixed sample was loaded and analyzed. Proper amount of freeze-dried samples were digested in 70% (w/v) nitric acid (Fisher Scientific, trace metal grade) and minerals (i.e., Ca, Mg, Zn, Fe, Na and K) were determined by using Hitachi Z-8200 Zeeman polarized atomic absorption spectrophotometer (Nissei Sango Ltd., Mississauga, ON, Canada).

2.2 Organic acids

Lactic acid content was analyzed using a Sigma lactate kit assay (Sigma Diagnostics, Cat. No. 735-10, St. Louis, USA). Organic acids were determined by HPLC according to the method of Guzel-Seydim *et al.* (2000). Five mL of each sample was diluted with 25 mL 0.01 M H_2SO_4, vortexed for 1 min followed by centrifugation at 2000 x g for 10 min. Supernatants were collected and filtered through 0.2 µm filter. Volumes of 20 µl of samples and standards were injected into a Beckman Gold HPLC system (Beckman Coulter, Fullerton, USA) equipped with an Aminex HPX-87H (300 mm x 7.8 mm) organic acid column (Bio-Rad Laboratories, Hercules, USA). Degassed 8 mM sulfuric acid (H_2SO_4) was used as the mobile phase. The organic acids oxalate, citrate, malate, succinate, formate and acetate were detected at 215 nm. Organic acids were quantified using external standards (organic acid analysis standard, Bio-Rad Laboratories, Hercules, USA).

2.3 Molecular weight cut-off fractionation (MWCO)

Centriplus centrifugal filter devices were used to get MWCO fractions at 3000 Da (Millipore, Bedford, USA). Ten milliliters whole extract were loaded to the sample reservoir and the

assembled device was centrifuged at 4°C, 3000 x g for 290 min. The filtrates were collected for further analysis.

2.4 Size exclusion HPLC (SEC) separation

One hundred microliters of kefir mother culture extract was injected into a TSK G2000SWXL column (78 mm x 30 mm, SUPELCO, Bellefonte, USA) and separated with a Shimadzu LC-6AD Liquid Chromatograph system (Shimadzu Scientific Instruments, Inc. Columbia, USA) with UV detection at 210 nm. The separation buffer used was a mixture of 45% acetonitrile in 0.1% trifluoroacetic acid (TFA) with a flow rate of 0.4 ml/min for 40 min. Nine fractions were collected for each HPLC run, and fractions from five to ten runs were pooled. The above nine fractions were evaporated with N_2 and then freeze dried, stored at −80°C for cell culture incubations and for further analytical analyses.

2.5 Reverse phase HPLC (RP-HPLC) fractionation

The fraction(s) collected with SEC HPLC that showed antiproliferative effects on MCF-7 cells were further analyzed with a Prosphere 300 C4 column (5µm, 250 mm x 4.6 mm) (Alltech Associate, Inc. Deerfield, USA) using a Beckman Gold HPLC System (Beckman Coulter, Fullerton, USA). After the column was equilibrated with buffer A (0.1% TFA in water) at a flow rate of 1 ml per min, the fractions were eluted with a linear gradient of buffer A (0.1% TFA in water) and buffer B (60% of acetonitrile in 0.1% TFA: 40% of 0.1% TFA in water) as follows: 0 to 60 min, 0 to 90% B; and 61 to 65 min, 90 to 0% B. Dual channel absorbance was monitored at 210 nm (channel A) and 280 nm (channel B).

2.6 Preparative HPLC fractionation

Three batches of extracts of kefir mother culture were pooled and fractionated using the Centriplus centrifugal filter devices to obtain fractions of compounds with molecular weights less than 3000 Da. The fractions of MWCO less than 3000 Da were freeze-dried using a FLEXI-DRY MP Freeze Dryer (FTS Systems, Inc. Stone Ridge, U SA). Five g of the lyophilized MWCO fractions were dissolved in 20 ml of water. Ten milliliters of reconstituted solution were loaded on a C4 preparative column (300 Å, 5 µm, 300 mm x 50 mm) (Vydac Company, Herperia, CA) and separated with a Water Delta Prep 4000 HPLC system (Waters Corporation, Milford, USA). After the column was equilibrated with buffer A (0.6% acetic acid in water) at a flow rate of 13 ml and the fractions were eluted with a linear gradient of buffer A and buffer B (0.6% acetic acid in acetonitrile) as follows: 0 to 70 min, 0 to 60% B; 70 to 80 min, 60 to 70% B; 80 to 100 min, 70 to 80% B; 100 to 105 min, 80 to 0% B. A total of 100 fractions were collected between 0 and 100 min, with samples taken at 1-min intervals. The fractions were then lyophilized and kept at −80°C for further cell culture and analysis. The fractions were reconstituted by adding water and peptide and protein concentrations were determined before cell culture assays.

2.7 Capillary electrophoresis

Capillary zone electrophoresis (CE) was performed using a P/ACE ™ 2200 HPAC instrument controlled by System Gold software (Beckman, Fullerton, CA, USA) coupled to an IBM PC 486 computer (IBM Corp., Portmouth, England) for data acquisition and analysis. A neutral uncoated fused silica capillary column (57 cm × 50 µm, the length from intake to detector was 50 cm) was assembled in the P/ACE cartridge (Polymicro

Technologies, Phoenix, Arizona USA) for capillary separations. Injection volume, buffer concentration and running voltage were optimized to achieve the best resolution with the shortest running time. The capillary was flushed with 1 M sodium hydroxide, followed by nanopure water, 0.1 M sodium hydroxide, nanopure water, 1 M hydrochloric acid, and again water, each for 5 min at a pressure of 40 psi. A solution of 5% polybrene and 2% ethylene glycol was then passed for 10 min at 40 psi. Excessive coating was then removed by flushing with water for 2 min. Further capillary flushing was then performed for additional 10 min using 200 mM formic acid buffer. Before each sample application, the capillary was rinsed with 1 min water, 1 min 0.1 M sodium hydroxide, 1 min water and 3 min separation buffer (200 mM formic acid in water, pH 2.0). After the completion of each run, the capillary was rinsed with nanopure water for 1 min, 0.1 M sodium hydroxide for 1 min and nanopure water for 1 min. Peptide standard and sample injections were carried out at the anode end of the capillary using N_2 pressure (0.5 psi) for 5 sec and was separated at a constant temperature of 20°C with a 200 mM formic acid (pH 2.0) as separation buffer. On-line detection was performed using an UV detector at 200 nm. Twenty kilovolts was applied across the run buffers for best separation. The capillary was re-coated after every 5 runs.

2.8 MALDI-TOF for estimation of molecular weight

Reconstituted SEC-HPLC and RP-HPLC fractions were analyzed using a MALDI-TOF mass spectrometer (Voyager DE-STR; Applied Biosystems, Palo-Alto, CA, USA) with a laser at 337 nm and an acceleration voltage of 20.000 V.

2.9 Mass spectrometry

Mass spectrometric analysis in the positive ion mode was performed on a triple quadupole mass spectrometer (SCIEX API III Biomolecular mass analyzer, Thornhill, Ontario, Canada). Lyophilized reversed phase HPLC fractions having antiproliferative effect on MCF-7 cells were reconstituted in 0.5 mM ammonium acetate in methanol or in 10% acetic acid in 20% aqueous methanol. The resulting solution was then infused into the electrospray ion-source by a syringe pump (Harvard Apparatus Model 22, South Natick, MA) at a flow rate of 1.5 ml/min. The ion-spray voltage was set at 5.5 kV and the orifice potential was set at 50 V. Argon was used as the collision gas at a collision gas thickness (CGT) of 1.5 x 10^{14} for collision-induced fragmentation MS-MS analysis.

2.10 Cell Culture screening for antiproliferative effects

MCF-7 cells were purchased from ATCC (ATCC, Manassas, USA). Cells were routinely propagated as a monolayer culture in Dulbcco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), in a 75-cm^2 plastic dish at 37°C in a humidified atmosphere with 5% CO_2, and passaged every 3-4 days. Normal human mammary epithelial cell lines (HMEC) were graciously provided by Dr. M.R. Stampfer (Lawrence Berkeley National Laboratory, Berkeley, USA). Cells were routinely propagated as a monolayer culture in Mammary Epithelial Growth Media (MEGM, Clonetics, San Diego, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) in 75-cm^2 plastic dish at 37°C in a humidified atmosphere with 5% CO_2, and passaged every week. For the experiments, both

MCF-7 and HMEC cells were harvest from the dish using 0.25% trypsin-EDTA solution (Sigma, St Louis, MO, USA).

2.11 Cell proliferation experiments in 24-well plates

Cells previously harvested were seeded in 24-well plates, i.e., 10,000 cells for MCF-7 per well in DMEM supplemented with 10% FBS and 5,000 cells for HMEC per well in MEGM supplemented with 10% FBS. The cells were allowed to attach for 24 h. After that period, old media were removed and fresh media and extracts were added to each well. To study the dose response, a serial dilution of each the extract using the culture media was made to achieve final concentrations of extracts at 5%, 2.5%, 1.3%, 0.6%, and 0.3% (vol./vol.), respectively. Because the kefir extracts were acidic (approximately pH 4.5), Dulbecco's Phosphate Buffered Saline (PBS) buffer (Gibco BRL, Grand Island, NY) was added to the culture media to adjust the pH between 7.0 and 7.4. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 6 d. Cell nuclei were counted in order to eliminate the difficulty in counting whole cells due to clumping. Furthermore, a more uniform distribution of nuclei over the grids of a hemacytometer was seen compared with whole cells. Media was aspirated from wells and cells were rinsed with 500 µl of PBS. 250 µl of hypotonic buffer (0.01 M HEPES, 1.5 M $MgCl_2$, pH 7.5) was added to each well. After 2 min, 250 µl of cell lysing solution (10% ethyl hexadecyl dimethyl ammonium bromide, 3% glacial acetic acid, in water) was added. The plate was shaken lightly every minute for 5 min. Cell lysis was confirmed microscopically as indicated by a suspension of clean nuclei. The suspension was mixed and the nuclei were counted using a Coulter Counter (Coulter Counter Corporation, Fullerton USA). Each sample was run in quadruplicate. Control cells were incubated with the culture medium with the dosing vehicle (PBS). To standardize results, each plate had its own control wells with cells treated with PBS and cell proliferation of different treatment was expressed as a percent of control.

2.12 Cell proliferation experiments in 96-well plates

Cells previously harvested were seeded in 96-well plates at 1,000 cells per well for MCF-7 in DMEM supplemented with 10% FBS and for HMEC in MEGM supplemented with 10% FBS. The cells were allowed to attach for 24 h. After that period, old media were removed and fresh media and extracts were added to each well. A serial dilution of each test fraction was made to study the dose-response. PBS buffer was added to the culture media to keep the final pH between 7.0 and 7.4. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 6 d and the cell numbers in each well were determined by using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, USA). Each sample was run in quadruplicate. Control cells were incubated with the culture medium with the dosing vehicle (PBS). To standardize results, each plate had its own control wells with cells treated with PBS and cell proliferation of different treatments was expressed as a percent of control.

2.13 Statistics

All statistics test were performed using SAS 8.2 for PC (SAS, Cary, NC USA). Means were compared with Student's t test. Two-way ANOVA was used to analyze the effects of treatments and doses for the cell culture experiments. The differences among doses and treatments were determined by the Student-Newman-Keuls (SNK) multiple comparison test. Statistical significance was considered at $P < 0.05$.

3. Results

Moisture, macronutrients and some minerals in kefir mother culture and final kefir described in Table 1.

Component	Batch 1		Batch 2		Batch 3	
	Mother culture	Kefir	Mother culture	Kefir	Mother culture	Kefir
Moisture (g)	[1]89.78±0.09	90.40±0.06	91.22±0.19	90.03±0.02	89.56±0.07	90.17±0.08
Ash (g)	0.68±0.005	0.67±0.004	0.66±0.006	0.70±0.003	0.68±0.002	0.67±0.003
Protein (g)	3.05±0.16	2.81±0.03	2.78±0.03	3.14±0.04	3.28±0.01	2.77±0.02
Fat (g)	2.32±0.06	1.77±0.04	2.46±0.06	1.46±0.02	2.36±0.04	1.48±0.01
Calcium (mg)	78.20±0.66	77.70±0.71	78.93±0.58	77.40±0.64	81.53±0.73	80.40±0.56
Iron (mg)	0.02±0.001	0.02±0.001	0.02±0.001	0.02±0.001	0.02±0.001	0.02±0.001
Zinc (mg)	0.28±0.02	0.29±0.02	0.28±0.02	0.30±0.02	0.31±0.03	0.29±0.03
Magnesium (mg)	8.42±0.55	8.65±0.35	8.88±0.56	8.40±0.71	8.97±0.48	8.70±0.42
Sodium (mg)	28.51±1.86	29.0±0.71	28.60±2.03	30.05±2.05	30.99±1.08	31.05±0.72
Potassium (mg)	60.28±0.89	60.60±0.42	59.36±1.16	61.30±1.34	60.40±1.08	60.95±0.78

Table 1. Nutrient composition from three different batches of kefir mother culture and final commercial kefir product (per 100 g wet weight) ([1]Mean±SD, n=3).

No significant differences among nutrients and organic acids were found, except that lactic acid in the kefir commercial product was higher than that of kefir mother culture (Table 2).

Component	Mother culture	Kefir
Lactic acid[1]	[3]77.86±8.94	106.87±9.36*
Uric acid [2]	0.014±0.003	0.012±0.002
Pyruvic acid[2]	0.54±0.15	0.52±0.10
Oxalic acid[2]	[4]ND	ND
Citric acid[2]	3.27±0.17	3.22±0.28
Malic acid[2]	54.79±9.25	57.62±11.01
Succinic acid [2]	1.61±0.24	1.71±0.08
Formic acid[2]	Not detectable	Not detectable
Acetic acid[2]	3.81±0.74	3.46±0.47

Table 2. Organic acid concentrations (mmol/l) in mother culture and final kefir commercial product ([1]Determined by lactic acid kit assay method; [2]Determined by HPLC method of Guzel-Seydim *et al.* (2000); [3]Mean±SD, n=3; [4]ND = Not detectable; *$P < 0.05$).

Capillary electrophoresis analyses showed that the profiles of extracts of mother culture and kefir were similar (data not shown). In our earlier study, the extract of kefir mother culture showed a tendency of having stronger antiproliferative effect on MCF-7 cells than other extracts (Chen *et al.*, 2007). Hence, mother culture was chosen for further fractionation and cell culture tests to identify the bioactive component(s). Extracts of kefir mother culture that were fractionated via the 3000 Da MWCO were analyzed for protein and peptide content (Table 3).

Samples	Moisture (g/100g)	Crude protein[1] (g/100g)	Protein[2] (μg/ml)	Peptides[3] (mmol/l)
Whole extract	[4]96.39±0.24	0.27±0.03	152.91±11.53	5.53±0.26
MWCO<3000	[5]ND	ND	5.10±0.07*	4.21±0.32*
MWCO>3000	ND	ND	195.94±4.11*	7.16±0.36*

Table 3. Protein and peptide concentrations in different MWCO fractions of extracts of kefir mother culture ([1]Determined by using Nitrogen Determinator; [2]Determined by Bradford method (1976); [3]Determined by OPA method of Church *et al.* (1983); [4]Mean±SD, n=3; [5]ND: No data is available; *Significant different at $P < 0.05$ when compared to protein or peptide concentration in whole extract).

Both protein and peptide concentrations were significantly lower in the fraction of MWCO less than 3000 Da ($P < 0.05$) relative to the whole kefir mother culture extract. Both the whole extract and the fraction with the MWCO less than 3000 Da were screened by MCF-7 cell culture using 24-well plates. As shown in Figure 1, the filtrate of MWCO less than 3000 Da had a dose dependent antiproliferative effect on MCF-7 cells comparable to that of the whole mother culture extract.

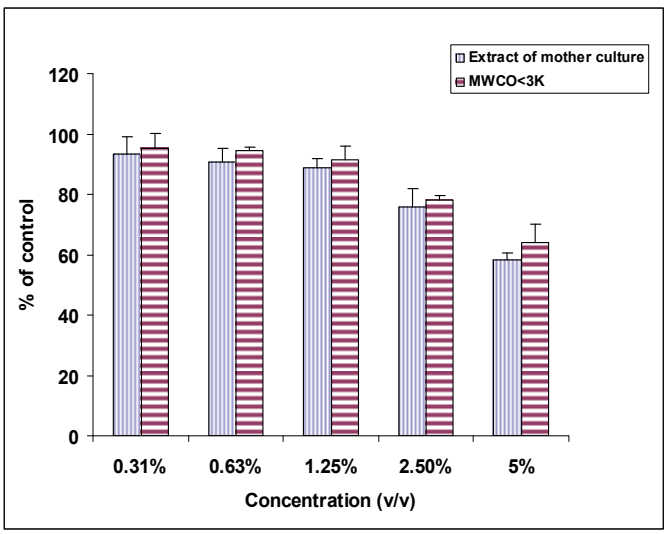


Fig. 1. Antiproliferative effects on MCF-7 cells induced by extracts of: (A) whole kefir mother culture; and (B) fraction of kefir mother culture obtained with MWCO less than 3000 Da. Cell proliferation was analyzed in 24-well plate. Values were graphed as Mean±SD, n=4.

The kefir mother culture filtrate that contained the MWCO less than 3000 Da was further separated with SEC-HPLC. Nine fractions were collected with each run with retention times of 14.6 to 17.0, 17.1 to 19.0, 19.1 to 21.0, 21.1 to 23.7, 23.8 to 25.6, 25.7 to 27.3, 27.4 to 29.1, 29.2 to 31.6, 31.7 to 33.7 min, respectively. Ten runs of SEC-HPLC fractions were pooled, freeze-dried and reconstituted in 2 ml water. The estimated molecular weight, protein and peptide concentrations are shown in Table 4. All the nine fractions were tested with MCF-7 cells in 96-well plates for their antiproliferative effects.

Fraction	Retention time (min)	Molecular Weight (Dalton)	Protein (µg/ml)	Peptides (µM/l)
1	14.4-17.0	13116-29507	145.7±5.12	274.53±2.19
2	17.1-19	7618-12745	118.56±4.89	284.70±5.31
3	19.1-21	4672-7425	43.62±2.84	208.33±2.26
4	21.1-23.7	2588-4565	16.80±1.56	491.14±6.67
5	23.8-25.6	1776-2535	3.23±0.26	422.01±5.17
6	25.7-27.3	1297-1742	2.92±0.2	655.11±4.58
7	27.4-29.1	949-1274	1.38±0.12	1168.33±8.22
8	29.2-31.6	635-934	4.77±0.16	1053.71±5.17
9	31.7-33.7	470-625	3.84±0.22	224.59±1.93

Table 4. Estimated molecular weight, protein and peptide concentration of the nine SEC HPLC fractions, Mean±SD, n=3.

Fractions 7 and 8 were the only fractions to demonstrate a dose-dependent antiproliferative effects on MCF-7 cells with the most potent effect exhibited with fraction 7 (Figure 2).

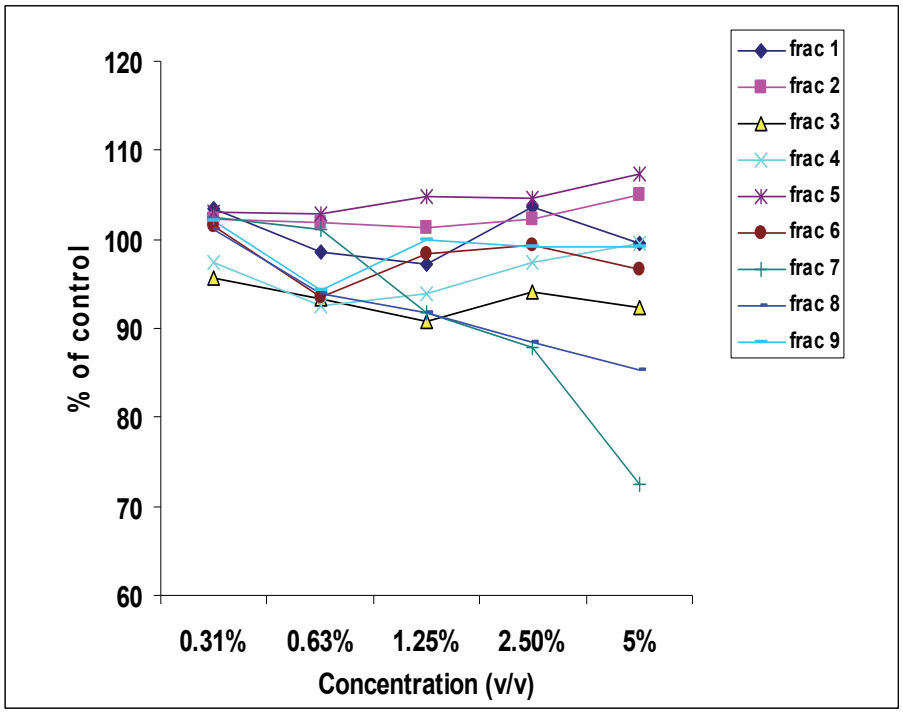


Fig. 2. Antiproliferative effects of 9 SEC-HPLC fractions on MCF-7 cells. Cell proliferation was analyzed in 96-well plate using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Values were graphed as Mean±SD, n=4.

Fraction 7 was also tested with HMEC cells, and no antiproliferative effects were observed (Figure 3). Fraction 7 was further analyzed with RP-HPLC, which showed the presence of about 11 peaks in Fraction 7 (Figure 4). SEC-HPLC Fraction 7 was also analyzed via MALDI-TOF, which indicated that the peak masses ranged from 659 to 2074 Da. (Figure 5).

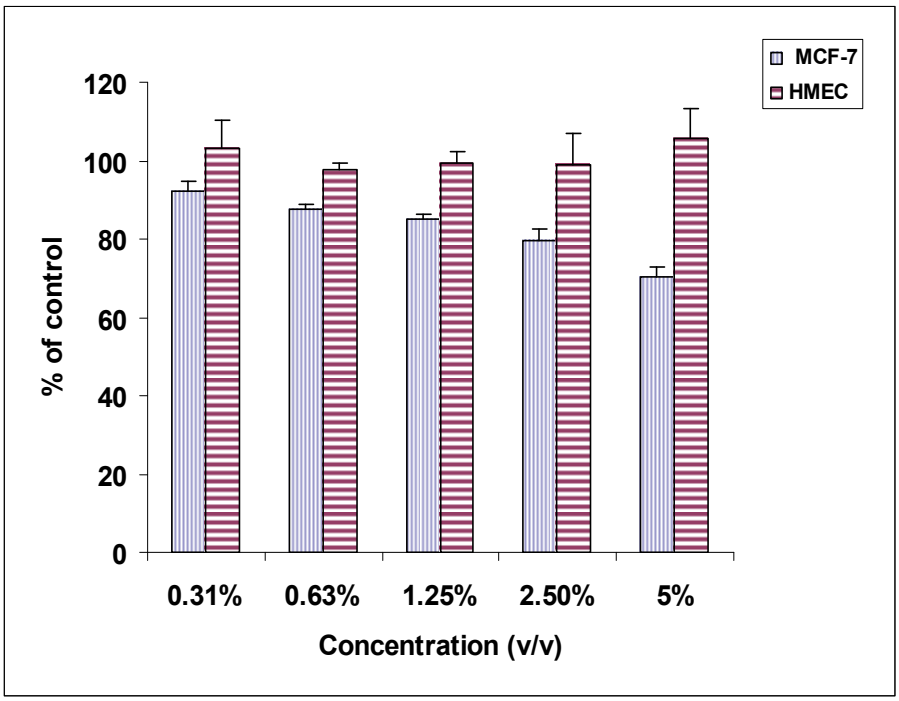


Fig. 3. Antiproliferative effects of SEC-HPLC Fraction 7 on MCF-7 and HMEC cells. Cell proliferation was analyzed in 96-well plate using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Values were graphed as Mean+SD, n=4.

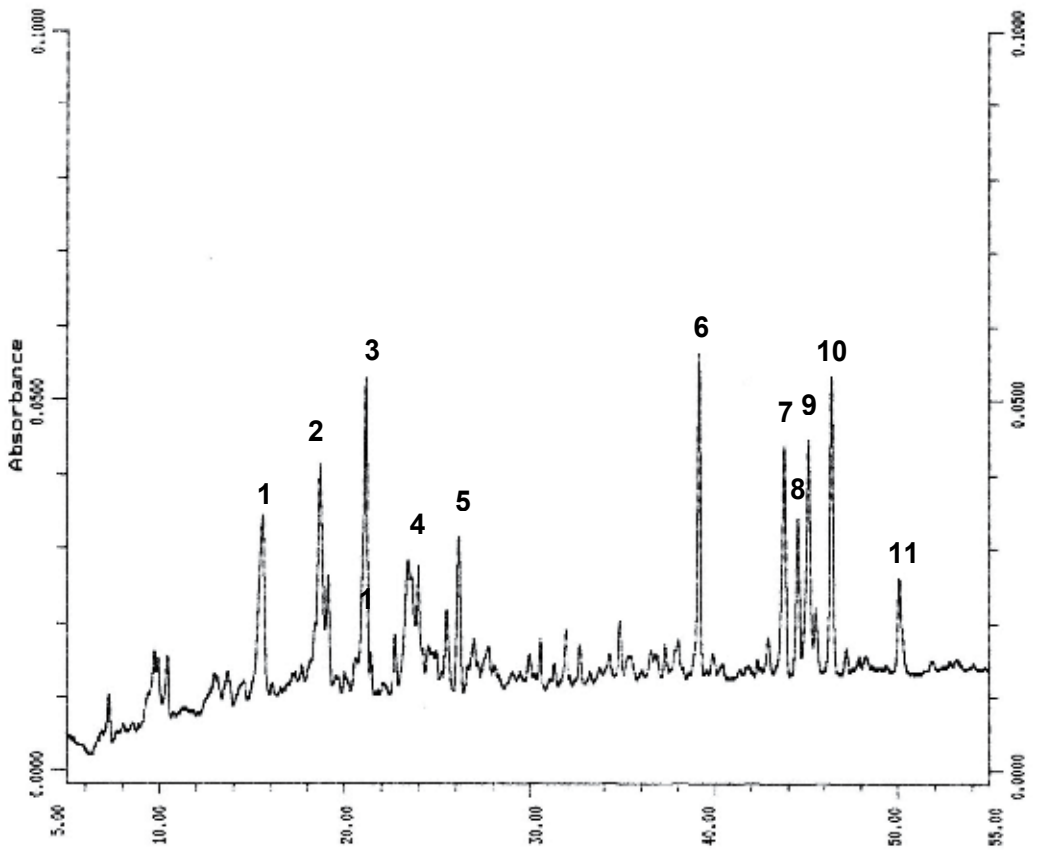


Fig. 4. Elution profile of SEC-HPLC Fraction 7 of kefir mother culture using RP-HPLC. A Prosphere 300 C4, 5 µm, 250 mm x 4.6 mm column was used. After the column was equilibrated with buffer A (0.1% TFA in water) at a flow rate of 1 ml per min, the fractions were eluted with a linear gradient of Buffer A (0.1% TFA in water) and Buffer B (60% of acetonitrile in 0.1% TFA: 40% of 0.1% TFA in water) as follows: 0 to 60 min, 0 to 90% B; and 61 to 65 min, 90 to 0% B. Absorbance was monitored at channel A 210 nm and channel B 280 nm. The retention times of the peaks 1 to 11 are 15.4, 18.6, 21.1, 23.3, 26.1, 39.1, 43.7, 44.5, 45.2, 46.3, and 50.1 min, respectively.

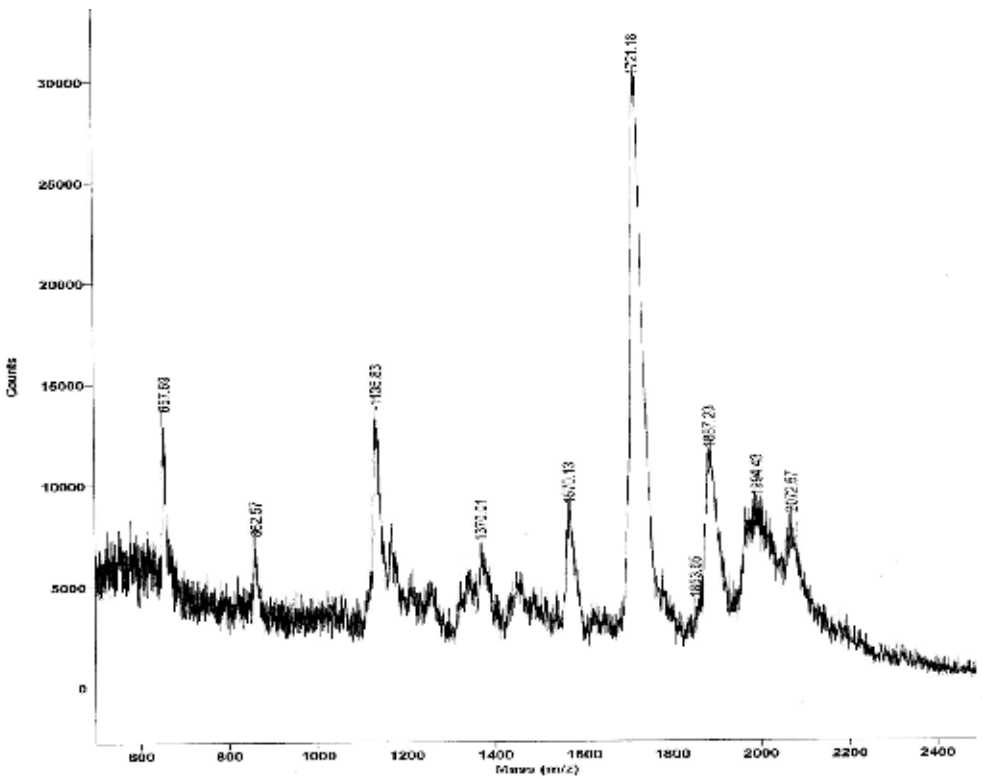


Fig. 5. Mass spectrogram of SEC-HPLC Fraction 7 of kefir mother culture analyzed by a MALDI-TOF mass spectrometer with a laser at 337 nm and an acceleration voltage of 20.000 V.

A preparative C4 column was utilized to obtain sufficient amounts of mother culture kefir fractions of MWCO less than 3000 Da for more extensive structure analysis and cell culture studies. The average nitrogen content of the lyophilized filtrate of MWCO less than 3000 Da was 0.58%. Five g of the lyophilized MWCO less than 3000 Da fractions was dissolved in 20 ml of water. Ten milliliters of reconstituted solution were loaded on the column, and 100 fractions were collected in 1 min intervals (Figure 6).

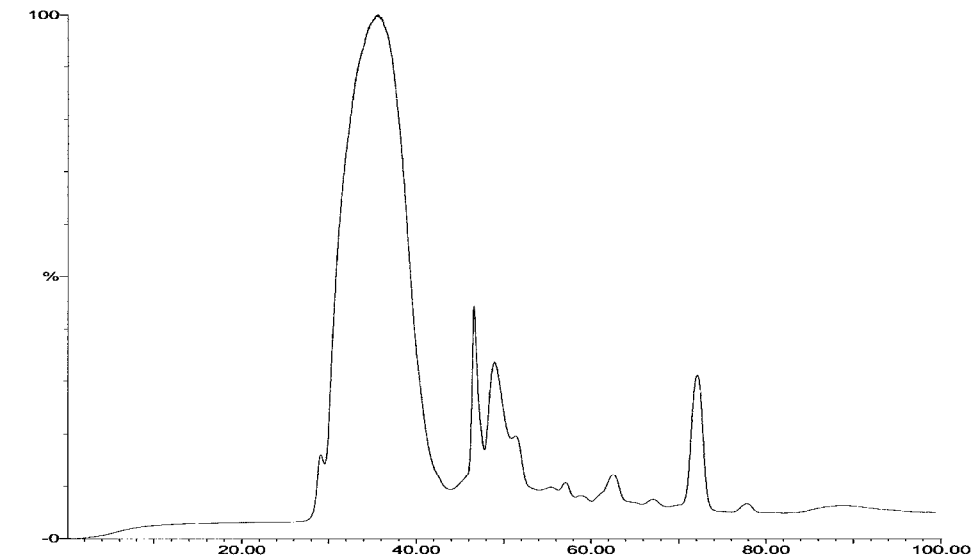


Fig. 6. Elution profile of MWCO less than 3000 Da fraction of kefir mother culture extract analyzed by preparative RP-HPLC. Appropriate amount of samples were loaded on a C4 preparative column (300 Å, 5 µm, 300 mm x 50 mm) (Vydac Company, Herperia, CA) and separated with Waters Delta Prep 4000 HPLC system. After the column was equilibrated with buffer A at a flow rate of 13 ml, the fractions were eluted with a linear gradient of Buffer A (0.6% acetic acid in water) and Buffer B (0.6% acetic acid in acetonitrile) as follows: 0 to 70 min, 0 to 60% B; 70 to 80 min, 60 to 70% B; 80 to 100 min, 70 to 80% B; 100 to 105 min, 80 to 0% B. The absorbance was measured at 210 nm. A total of 100 fractions were collected between 0 and 100 min, with samples taken at 1-min intervals.

The peptide concentration of each preparative HPLC fraction is shown in Figure 7, Fractions 28 to 58, 62 to 64, and 72 to 74 were screened for antiproliferative effects on MCF-7 cells with three concentrations of 0.4%, 2% and 10% (Figure 8).

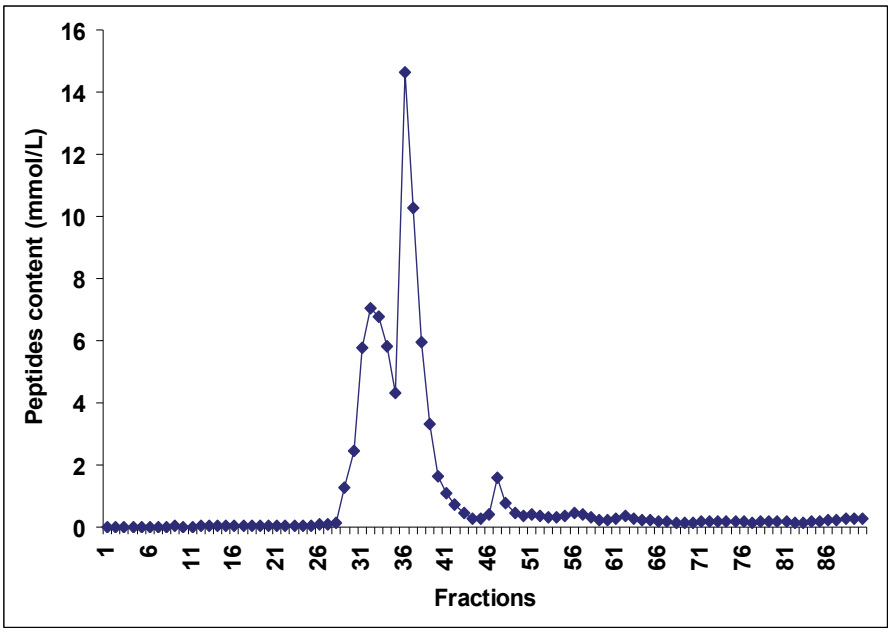


Fig. 7. Peptide concentrations in preparative RP-HPLC fractions. One hundred fractions obtained from preparative RP-HPLC were freeze-dried and then reconstituted with nanopure water to certain concentration. Peptide concentrations were determined in triplicates by OPA method. Mean values were plotted.

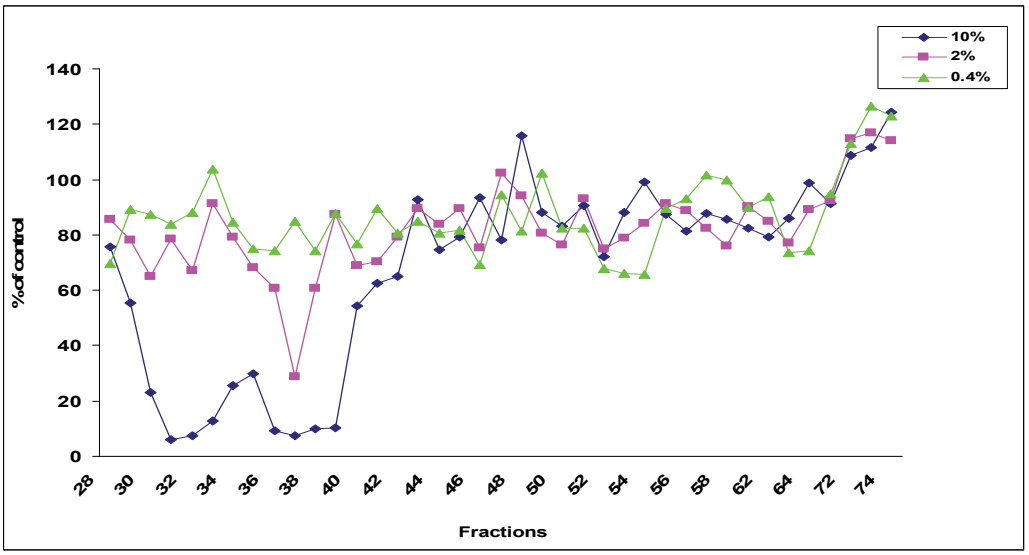


Fig. 8. Antiproliferative effects of fractions of preparative RP-HPLC on MCF-7 cells. Cell proliferation was analyzed in 96-well plate using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Each fraction was diluted at three concentrations and was run in quadruplicate. Mean values were plotted.

Fractions 29 to 41, 62 were found to have antiproliferative effects on MCF-7 cells. These fractions were further screened for antiproliferative effects with serial dilution. A dose dependent antiproliferative effect was observed when MCF-7 cells were treated with fractions 29, 30, 34, and 37, while not on HMEC cells (Figure 9).

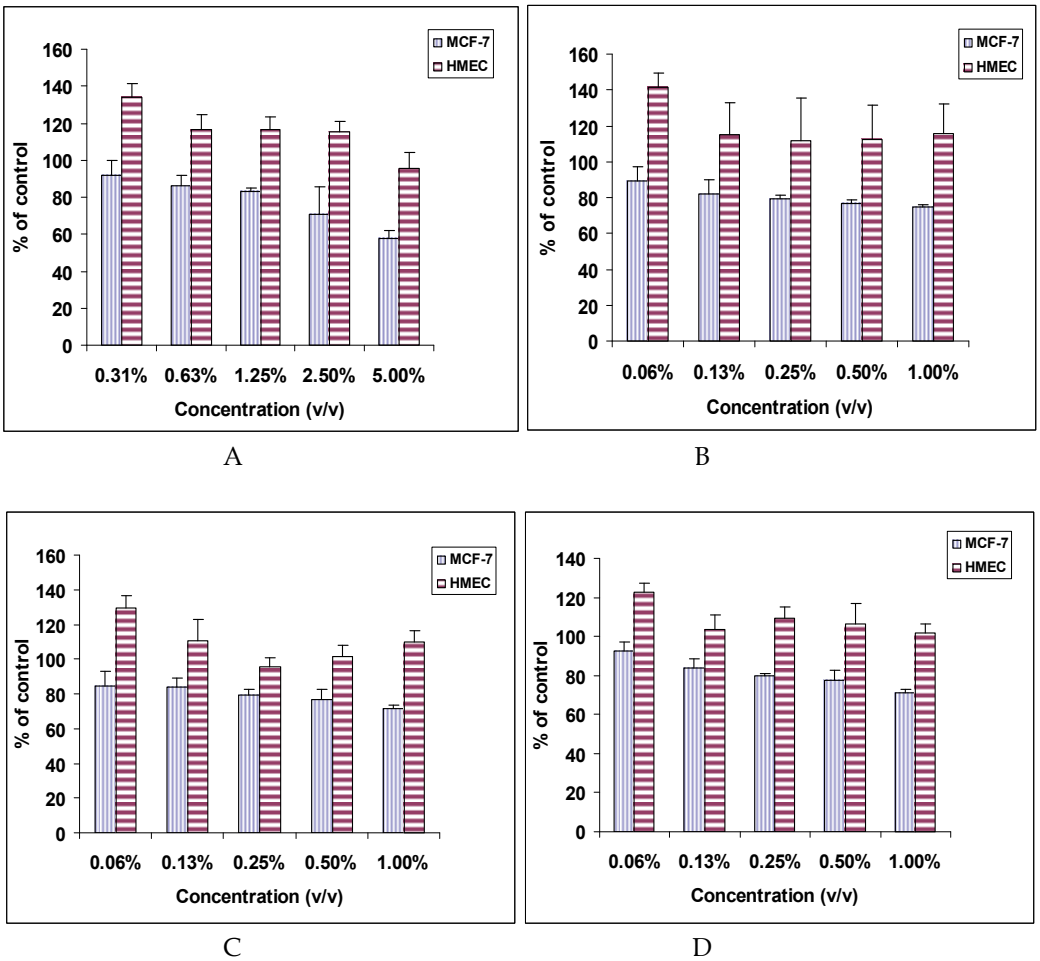


Fig. 9. Dose effect of preparative RP-HPLC Fraction 29 (A), 30 (B), 34 (C), and 37 (D) on MCF-7 and HMEC cells. Cell proliferation was analyzed in 96-well plate using a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. Values were graphed as Mean±SD, n=4.

Tamoxifen is a commonly used medication for breast cancer patients. In this study, only the dosage above 0.2 μM/l showed antiproliferative effects on MCF-7 cells. The effective dose reported here was higher than the value reported by Doisneau-Sixou *et al.* (2003). RP-HPLC Fraction 30 of kefir mother culture extract significantly increased MCF-7 cell susceptibility to tamoxifen (Figure 10). A dose dependent antiproliferative effect was also observed.

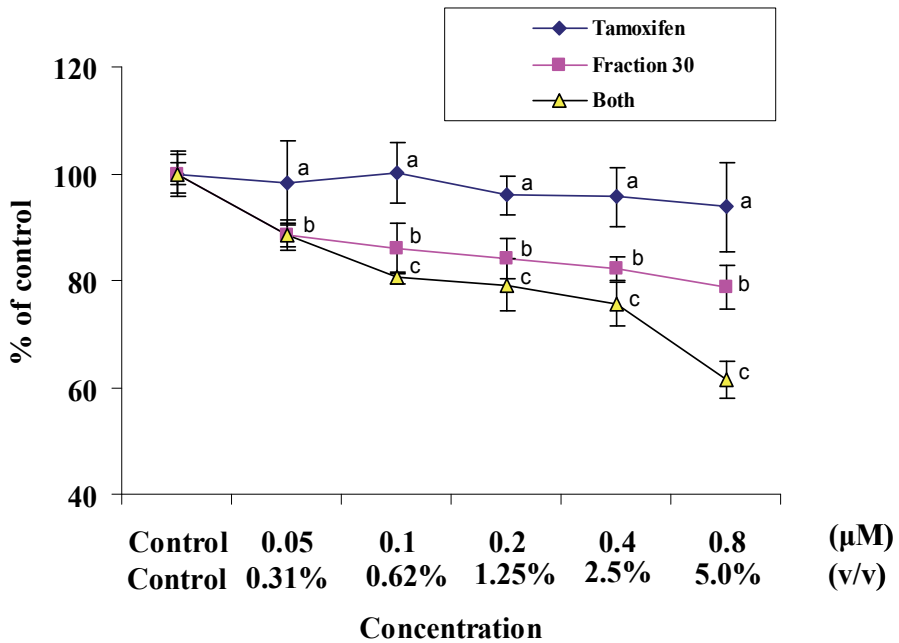


Fig. 10. Antiproliferative effect of RP-HPLC Fraction 30 of kefir alone or in combination with tamoxifen on MCF-7 cells. Means±SD (n=4) with the same letter are not significantly different at $P < 0.05$ for comparison among treatments at the same dose level.

Electrospray mass spectrometry was used to determine the molecular weight profile of the active Fractions 29, 30, 34, and 37. Figure 11 is the ESI-mass spectrum of HPLC fraction 30. The spectrum represents a typical molecular ion profile of the active HPLC fractions (29, 30, 34 and 37). The similarity in the molecular ion profile of the active fractions indicated that the active component(s) is spread over HPLC Fractions 30 to 37. The spectrum showed mainly singly charged molecular ions with m/z ratios ranging from 300 to 900. The peaks appearing below m/z 300 were found to be fragments of the peaks with m/z ranging from 300 to 900.

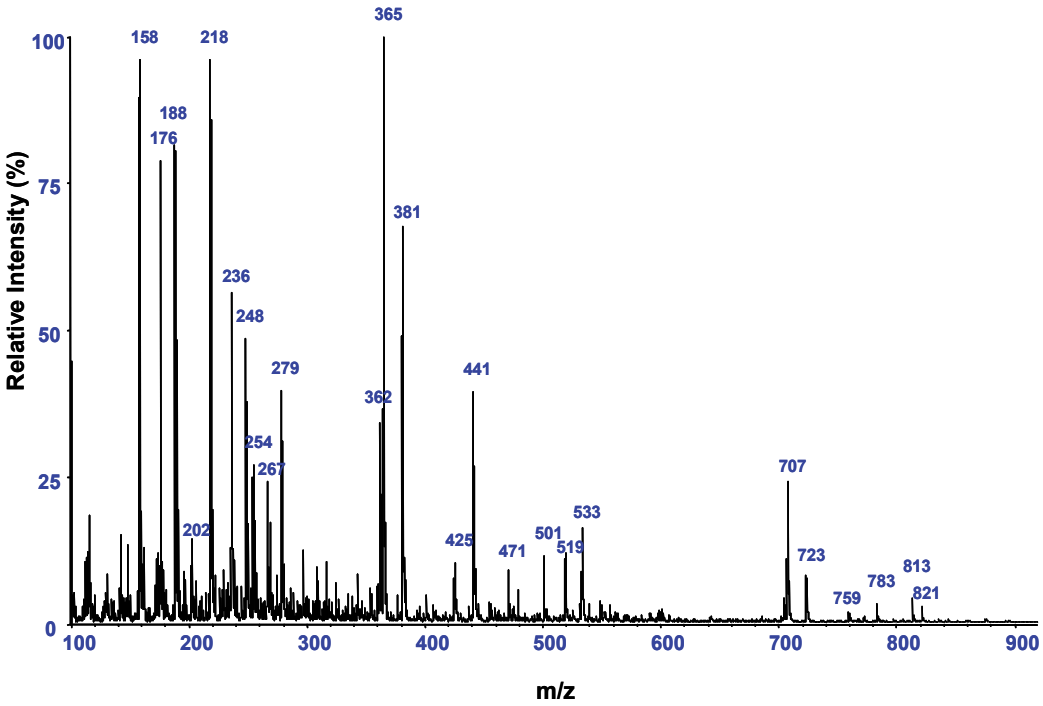
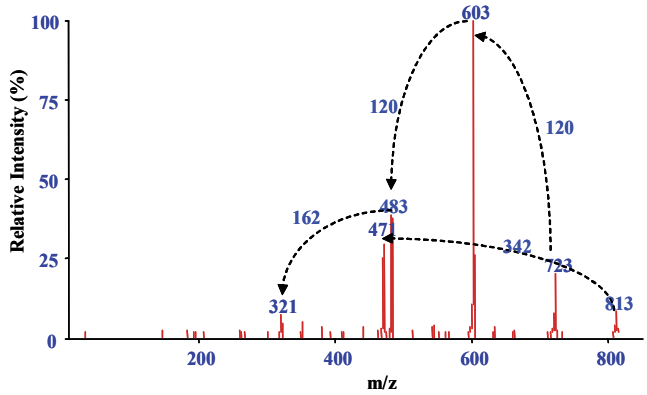


Fig. 11. Mass spectrum of HPLC Fraction 30 done by electrospray mass spectrometry. The mainly singly charged molecular ions with m/z ratios range from 300 to 900. The peaks appearing below m/z 300 were found to be fragments of the peaks with m/z ranging from 300 to 900.

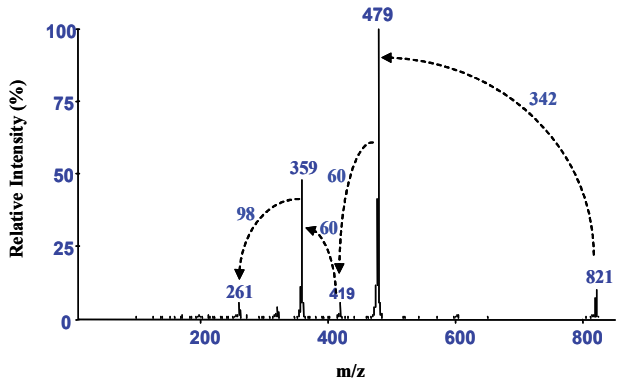
To characterize the composition and chemical structure of the active components in the HPLC fractions, molecular ions ranging from m/z 300 to 900 were subjected to collision ion dissociation (CID) tandem mass spectrometry. Figure 12A, showed the CID spectrum of the molecular ion at m/z 821. Fragmentation of the molecular ion at m/z 821 produced a neutral loss of 342 mass units, which corresponds to the loss of a lactose moiety. It also produced a series of neutral loses of 60 mass units, which correspond to the loss of acetic acid moieties. The neutral loss of 60 atomic mass units may also be derived from sugar cleavage products (Figure 13). The CID spectrum of the molecular ion at m/z 813 (Figure 12B) also produced a neutral loss of 342 (lactose) and neutral losses of 120 atomic mass units, which may be derived from fragmentation of lactose (Figure 13). A neutral loss of 162, which corresponds to the loss of a hexose moiety (glucose or galactose), is also observed. This was also observed at m/z 759. The fragmentation patterns of the molecular ions at m/z 821 and 813 indicate that they have similar structural features. The molecular ions with m/z 707 (Figure 12E) and 723 (Figure 12D) represent sodium and potassium ion dimmers of lactose, respectively. The peaks at m/z at 365 and 381 represent the sodium and potassium ions of lactose respectively (Figure 14).

Another interesting feature about the fragmentation pattern of with m/z ranging from 707 to 823 is that they fragmented into common fragment ions at m/z 261 and 98. Further isolation and fragmentation (MS-MS) of the ion at m/z 261 showed that its major product ion is the ion at m/z 98. Further isolation and fragmentation of the ion at m/z 98 produced

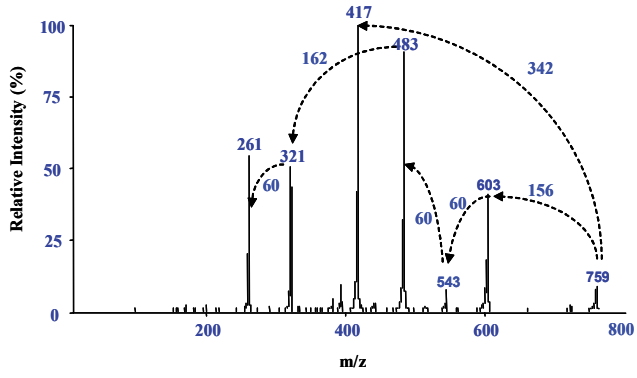
two ions at m/z 54 and 39. The tandem mass spectrometric analysis of the molecular ion of the active HPLC fraction show that most of the peaks produced a stable product ion at m/z 98, but the ion at 98 could not be ascribed to known structure peptide. Analysis of the fragmentation pattern of the molecular ion at m/z 759 (Figure 12C), however, revealed that the molecular ion could be derived from the interaction between serine and lactose.



A

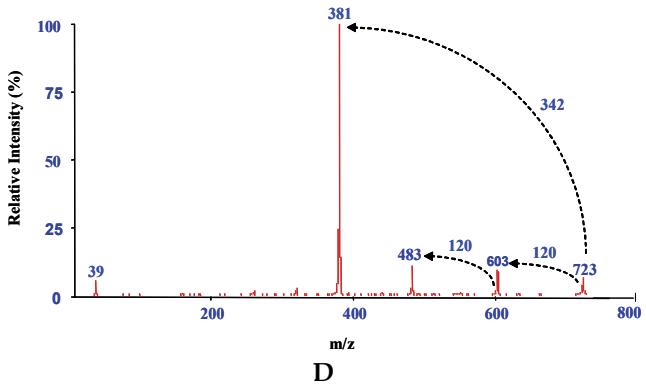


B



C

Fig. 12. (Continued)



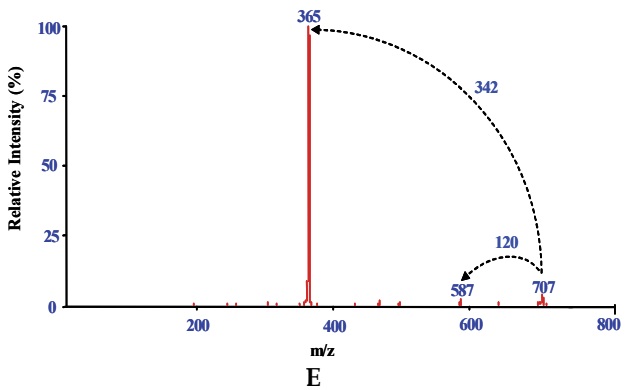


Fig. 12. Spectrum of molecular ion at m/z = 821 (A), m/z=813 (B), m/z= 759 (C), m/z=723 (D), and m/z=707 (E) done by collision ion dissociation (CID) tandem mass spectrometry. As indicated in Figure A, fragmentation of the molecular ion at m/z 821 produced a neutral loss of 342 and 60 mass units, which corresponds to the loss of a lactose moiety and acetic acid moiety respectively. There is also a neutral loss of 98, which could not be ascribed to a known structure. Ion at m/z =261 was also observed. Fragmentation of the molecular ion at m/z 813 produced a neutral loss of 342 and 120 mass units, which might be derived from lactose and hexose moieties (glucose or galactose) respectively. Fragmentation of the molecular ion at m/z 759 produced a neutral loss of 342 and serial loss of 60 mass units. Fragmentation of the molecular ion at m/z=723 produced neutral losses of 342 and 120 atomic mass unit. The main fragment is the ion at m/z 365 with the neutral loss of 342 (lactose) from m/z 707.

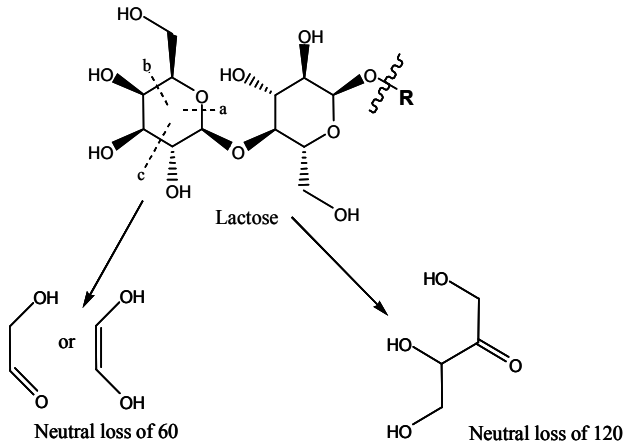


Fig. 13. Scheme 1: Generation of neutral loss of 60 and 120 atomic mass unit from fragmentation of lactose. Fragmentation at "a" and "b" will generate a neutral loss of 60 and fragmentation at "a" and "c" will generate a neutral loss of 120 atomic mass unit. They can be derived from fragmentation of acetic acid and lactose.

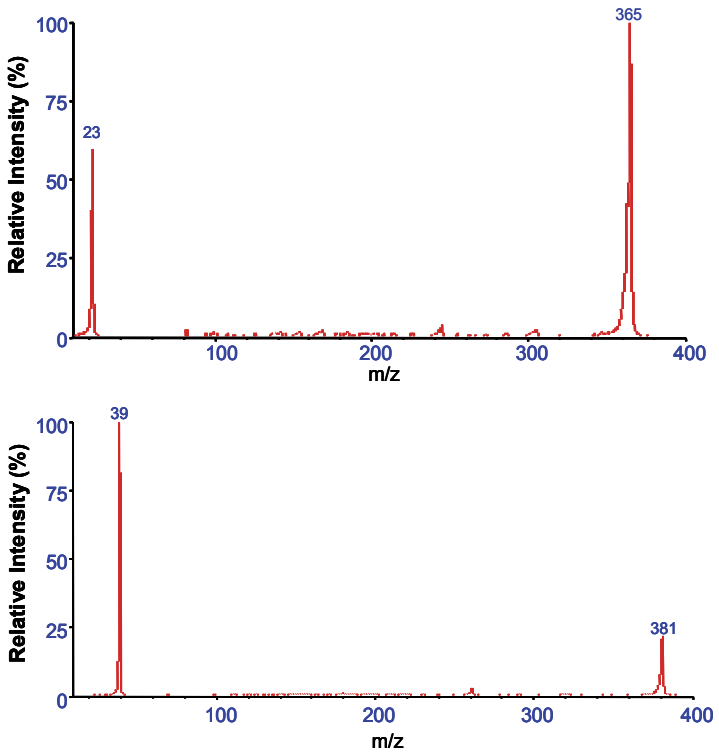


Fig. 14. Spectrum of molecular ion at m/z 365 and 381 done by collision ion dissociation (CID) tandem mass spectrometry. The peaks at m/z at 365 and 381 represent the sodium and potassium ions of lactose respectively.

A possible scheme for the generation of the ion at m/z 759 is presented in Scheme 2 (Figure 15). The proposed structure agrees with the fragmentation pattern. This interaction may be due to chemical reactions. The interaction between the amino group of serine and the reducing end of lactose is likely a chemical reaction similar to the Maillard reaction, and the interaction between the hydroxyl groups of lactose and the carboxyl group of serine is a simple esterification reaction. The reactions, particularly the esterification reaction may also be driven biochemically by esterases. The active fraction likely still contains a mixture of chemicals as suggested by the results of RP-HPLC (Figure 16). The exact molecular structure of these compounds is not known. However, results of the extensive MS analysis suggested that the compounds are possibly complex polymers that have lactose as the backbone structure. The proposed structure shown in Scheme 2 is a product with two lactose units linked by a serine. A more complex molecule with linkage with acetic acid is also possible.

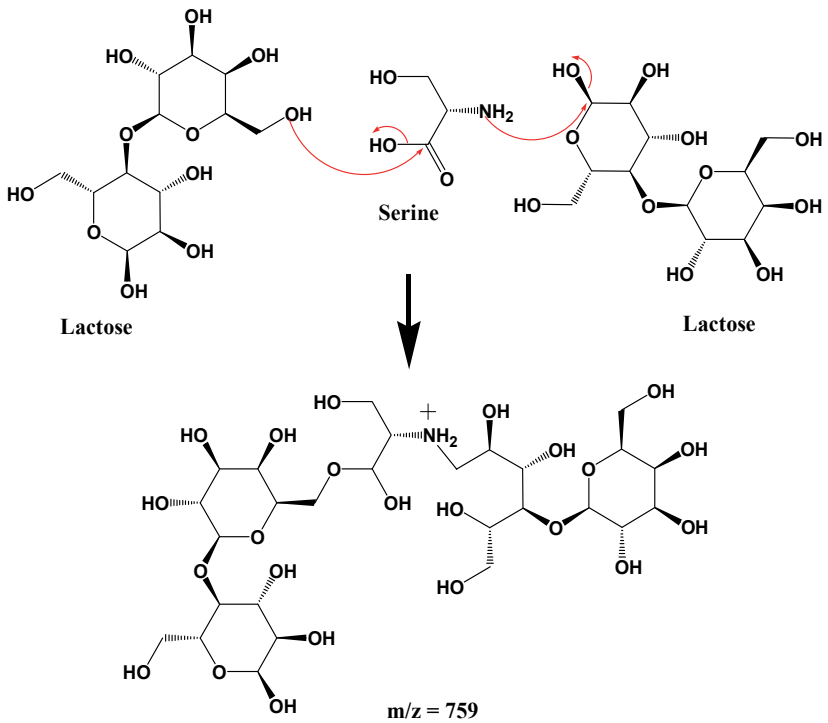


Fig. 15. Scheme 2: A possible interaction for the generation of the ion at m/z 759. As shown in the scheme, the interaction between the amino group of serine and the reducing end of lactose is likely a chemical reaction similar to the Maillard reaction, and the interaction between the hydroxyl groups of lactose and the carboxyl group of serine is a simple esterification reaction. The reactions, particularly the esterification reaction may also be driven biochemically by esterases.

The isolated bioactive fraction showed antiproliferative effects on cancer cells while not on normal cells. As indicated in the RP-HPLC chromatograph, the isolated fractions such as Fraction 30 are likely composed of multiple components that may be acting either singly or synergistically to exert the antiproliferative effects. Although the presence of peptides was suggested in the fractions as determined by the OPA methodology, the MS data did not

support the presence of known peptide structures. The failure to identify any known peptides is likely due to the limitation of the OPA method, which can only detect the presence of primary amines and thus the primary amines in peptides cannot be differentiated from other amine containing compounds.

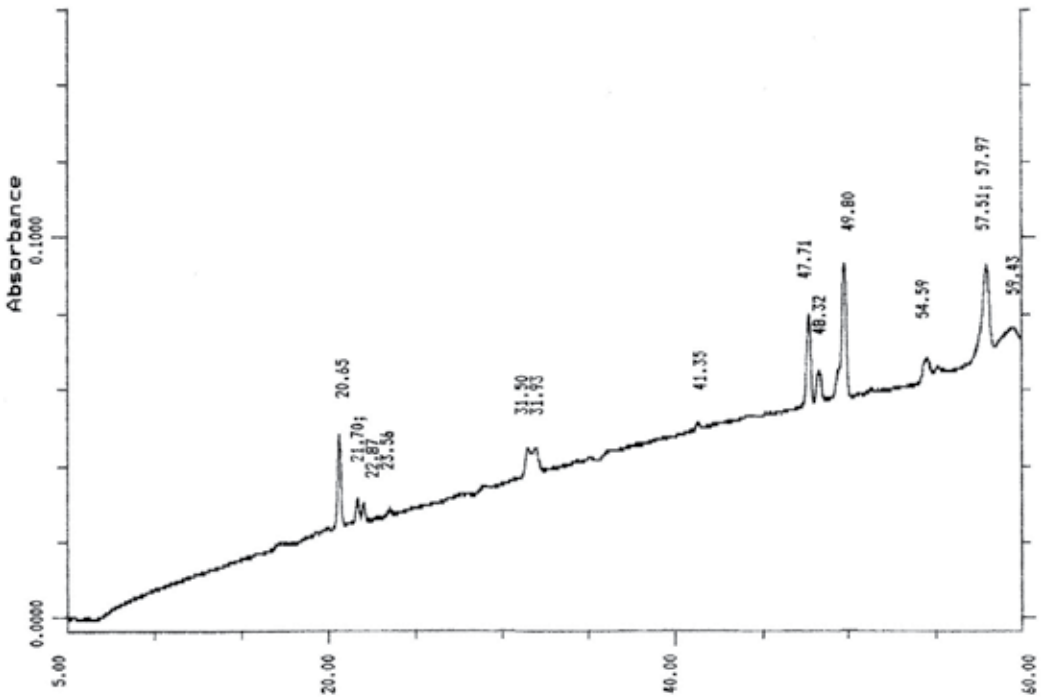


Fig. 16. Chromatograph of preparative HPLC Fraction 30 done by RP-HPLC. 50 µl of reconstituted preparative HPLC Fraction 30 which showed antiproliferative effect on MCF-7 cells was injected into a Prosphere 300 C4 column (5 µm, 250 mm x 4.6 mm) (Alltech Associate, Inc. Deerfield, IL 60015, USA) on Beckman HPLC System and eluted with a linear gradient of Buffer A (0.1% TFA in water) and Buffer B (60% of acetonitrile in 0.1% TFA: 40% of 0.1% TFA in water) as follows: 0 to 60 min, 0 to 90% B; and 61 to 65 min, 90 to 0% B. Absorbance was monitored at channel A 210 nm.

4. Conclusion

Due to the complex mixture of components in the specific fractions and the whole kefir extracts, the antiproliferative role of specific components such as proteins, peptides, organic acids and some small molecules, such as free amino acids, oligosaccharides and their interactions has been unclear. One of the main components of the bioactive antiproliferative kefir fractions is composed of lactose and acetic acid, possibly an end product of fermentation of the milk protein and sugar by the kefir bacteria and yeast. The lactose polymer suggests that the active component may be fragments of previously isolated polysaccharides termed as kefirans (Kooiman, 1968). Kefiran is a water-soluble polysaccharide consisted of approximately equal proportions of D-glucose and D-galactose (Kooiman, 1968; Micheli *et al.*, 1999). Kefiran has been reported to have antitumor activity

(Shiomi *et al*., 1982; Murofushi *et al*., 1983). It was also shown to be one of the substances having few or no side effect(s) in functioning to retard tumor growth in vivo.
It is noteworthy that one of the proposed novel bioactive structures contains serine and glucose or galactose; hence, it is conceivable that this molecule could be a sphingolipid compound. The ceramide component of sphingolipids is derived from serine and sphingolipids such as cerebrosides, which contain either a glucose or galactose. Sphingolipids such as gangliosides are sphingosine compounds that contain several glucose or galactose units. Also, lower molecular weight ceramide moieties have been detected in dairy gangliosides (Colarow *et al*., 2003) and cultured dairy products have been shown to be a rich source of gangliosides (Kathleen *et al*., 2000). Ceramide is derived from sphingomyelin (SpM) and can act as an intracellular second messenger for tumor necrosis factor-alpha, IL-1beta, and other cytokines. Ceramide has also been implicated in the acquired drug resistance that often characterizes breast cancer cells (Liu *et al*., 1999). C2 and C6 ceramides are cell permeable ceramide analogs that have been shown to induce cell apoptosis (Fillet *et al*., 2003). Osada *et al.* (1993) demonstrated that kefir contains an active substance, which enhances IFN-beta secretion of a human osteosarcoma line MG-63 treated with a chemical inducer, poly I: poly C. The active substance in the fermented milk was identified to be SpM. SpM from fermented milk (F-SpM) was a mixture of four molecular species of SpMs having C21-, C22-, C23- and C24-fatty acids. F-SpM enhanced the IFN secretion 14 times; SpMs from other food sources also enhanced IFN secretion but more moderately (2-3 times). We have observed a synergistic effect when MCF-7 cells were treated with ceramide analogs in the presence of extract of mother culture (data not shown). The putative sphingolipid component(s) in kefir that we have detected may have acted synergistically with the ceramide analogs leading to apoptosis of MCF-7 cells. In support of this concept, SpM has been shown to enhance the ceramide formation and ceramide-induced apoptosis in concert with the chemotherapeutic agent, gemcitabine, in human pancreatic cancer cells (Modrak *et al*., 2004). Modification of ceramide metabolism also increases MCF-7 cells sensitivity to cytotoxics (Lucci *et al*., 1999).
In summary, we have isolated the bioactive components from kefir mother culture that show antitumor cell proliferative effects. The MS data indicate that the isolated compounds are likely SpM complexes such as gangliosides that might have anti-tumor activities that are similar to that of ceramide. The biological functions of these ceramide-like compounds warrant further studies. The appearance of multiple components in the isolated bioactive antitumor fractions suggests the possibility that such components may be acting both independently and synergistically to exert the antiproliferative effects. Instead of searching for single compound in kefir, a mixture or a combination of components may be the best approach to have the highest potency for antitumor properties. The potential of using the kefir extract as co-drug for chemotherapy should be explored. In that regard, RP-HPLC Fraction 30 increased significantly the susceptibility of MCF-7 cells to tamoxifen, a commonly used anti-breast cancer drug. These results thus provide the rationale for future research to explore the potential of using kefir extracts as co-drugs for breast cancer chemotherapy and/or a functional food used for prevention of breast cancer.

5. Acknowledgments

This study was supported in part by grants from Liberty Brand Products, Inc. and a Natural Science and Engineering Research of Canada grant to S.K. and H.M.C. The authors thank

the contribution of Dr. Kobi Yeboah for the mass spectrometry analysis. S.K. owns shares in KCLM Research in Nutrition Inc., which holds an exclusive option to license the intellectual property rights to the anticancer properties of kefir and kefir extracts.

6. References

Biffi, A.; Coradini, D., Larsen, R., Riva, L. & Di Fronzo, G. (1997). Antiproliferative Effect of Fermented Milk on the Growth of a Human Breast Cancer Cell Line. *Nutrition and Cancer*, Vol.28, No.1, (January 1997), pp. 93-99, ISSN 0163-5581

Bradford, M.M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry,* Vol.72, No. 1-2, (May 1976), pp. 248–254, ISSN 0003-2697

Cevikbas, A.; Yemni, E., Ezzedenn, F.W. & Yardimici, T. (1994). Antitumoral Antibacterial and Antifungal Activities of Kefir and Kefir Grain. *Phytotherapy Research,* Vol.8, No.2, (March 1993) pp. 78-82, ISSN 0951-418X

Chen, C.; Chan, H.M., & Kubow, S. (2007). Kefir Extracts Suppress In Vitro Proliferation of Estrogen-Dependent Human Breast Cancer Cells But Not Normal Mammary Epithelial Cells. *Journal of Medicinal Food,* Vol.10, No.3, (September 2007), pp. 416-422, ISSN 1096-620X

Church, F.C.; Swaisgood, H.E., Porter, D.H. & Catinani, G.L. (1983). Spectrophotometric Assay Using *O*-Phthaldialdehyde for Determination of Proteolysis in Milk and Isolation Milk Proteins. *Journal of Dairy Science,* Vol.66, No.6, (June 1983), pp. 1219-1227

Colarow, L.; Turini, M., Teneberg, S. & Berger, A. (2003). Characterization and Biological Activity of Gangliosides in Buffalo Milk. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, Vol.1631, No.1, (February 2003), pp. 94-106, ISSN 0006-3002

Dillehay, D.L.; Webb, S.J., Schmelz, E.-M. & Merrill, A.H.Jr. (1994). Dietary Sphingomyelin Inhibits 1,2-Dimethylhydrazine-Induced Colon Cancer in CF1 Mice. *Journal of Nutrition,* Vol.124, No.5, (May 1994), pp. 615–620, ISSN 0022-3166

Doisneau-Sixou, S.F.; Cestac, P., Faye, J.C., Favre, G. & Sutherland, R. (2003). Additive Effects of Tamoxifen and the Farnesyl Transferase Inhibitor FTI-277 on Inhibition of MCF-7 Breast Cancer Cell-Cycle Progression. *International Journal of Cancer,* Vol.106, No.5, (September 2003), pp. 789-798, ISSN 0020-7136

Fillet, M.; Bentires-Alj, M., Deregowski, V., Greimers, R., Gielen, J., Piette, J., Bours, V. & Merville, M.P. (2003). Mechanisms Involved in Exogenous C2- and C6-Ceramide-Induced Cancer Cell Toxicity. *Biochemical Pharmacology,* Vol.65, No.10, (May 2003), pp. 1633-1642, ISSN 0006-2952

Furukawa, N.; Matsuoka, A., Takahashi, T. & Yamanaka, Y. (2000). Anti-Metastatic Effect of Kefir Grain Components on Lewis Lung Carcinoma and Highly Metastatic B16 Melanoma in Mice. *Journal of Agriculture Science Tokyo Nogyo Daigaku,* Vol.45, No.1, (January 2000), pp. 62-70, ISSN 0375-9202

Garrote, G.L.; Abraham, A.G. & Antoni, G.L. (2000). Inhibitory Power of Kefir: The Role of Organic Acids. *Journal of Food Protection,* Vol.63, No.3, (March 2000), pp. 364-369, ISSN 0362-028X

Guzel-Seydim, Z.B.; Seydim, A.C. & Greene, A.K. (2003). Comparison of Amino Acid Profiles of Milk, Yogurt and Turkish Kefir. *Milchwissenschaft,* Vol.58, No.3 (March 2003), pp. 158-160, ISSN 0026-3788

Kathleen, H.; Moore A., Ettinger, C. & Melvin, T.Y. (2000). Variation in Ganglioside Content of Bovine Dairy Products. *Journal of Food Composition and Analysis,* Vol.13, No.5 (March 2000), pp. 783-790, ISSN 0889-1575

Kooiman, P. (1968). The Chemical Structure of Kefiran, the Water-Soluble Polysaccharide of the Kefir Grain. *Carbohydrate Research,* Vol.7, No.2, (June 1968), pp. 200-211

Liu, Y.-Y.; Han, T.-Y., Giuliano, A.E., Ichikawa, S., Hirabayashi, Y. & Cabot, M.C. (1999). Glycosylation of Ceramide Potentiates Cellular Resistance to Tumor Necrosis Factor-Alpha-Induced Apoptosis. *Experimental Cell Research,* Vol.252, No.2 (November 1999), pp. 464-470, ISSN 0014-4827

Lucci, A.; Han, T.Y., Liu, Y.Y., Giuliano, A.E. & Cabot, M.C. (1999). Modification of Ceramide Metabolism Increases Cancer Sensitivity to Cytotoxics. *International Journal of Oncology,* Vol.15, No. 3, (September 1999), pp. 541-546, ISSN 1019-6439

Mesner, P.W. Jr.; Budihardjo, I.I. & Kaufmann, S.H. (1997). Chemotherapy-Induced Apoptosis. *Advances in Pharmacology,* Vol.41, pp. 461-499

Micheli, L.; Uccelletti, D., Paleschi, C. & Crescenzi, V. (1999). Isolation and Characterization of a Lactobacillus Strain Producing the Exopolysacchsride Kefiran. *Applied Microbiology and Biotechnology,* Vol.34, No.1 (December 1999), pp. 137-143, ISSN 0175-7598

Modrak, D.E.; Cardillo, T.M., Newsome, G.A., Goldenberg, D.M. & Gold, D.V. (2004). Synergistic Interaction Between Sphingomyelin and Gemcitabine Potentiates Ceramide-Mediated Apoptosis in Pancreatic Cancer. *Cancer Research,* Vol. 64, No.22, (November 2004), pp. 8405-8410, ISSN 0008-5472

Murofushi, M.; Shiomi, M. & Aibara, K. (1983). Effect of Orally Administered Polysaccharide from Kefir Grain on Delayed-Type Hypersensitivity and Tumor Growth in Mice. *Japanese Journal of Medical Science and Biology,* Vol.36, No.1 (February 1983), pp. 49-53, ISSN 0021-5112

Osada, K.; Nagira, K., Teruya, K., Tachibana, H., Shirahata, S. & Murakami, H. (1993). Enhancement of Interferon-Beta Production with Sphingomyelin from Fermented Milk. *Biotherapy,* Vol.7, No.2, (February 1993), pp. 115-123, ISSN 0921-299X

Parvez, S.; Malik, K.A., Ah, Kang. S. & Kim, H.Y. (2006). Probiotics and Their Fermented Food Products are Beneficial for Health. Journal of Applied Microbiology, Vol.100, No.6, (June 2006), pp. 1171-1185, ISSN 1364-507

Ronco, A.L., Stefani, E. & Dattoli, R. (2002). Dairy Foods and Risk of Breast Cancer: A Case-Control Study in Montevideo, Uruguay. *European Journal of Cancer Prevention*. Vol.11, No.5, (October 2002), pp. 457-463, ISSN 0959-8278

Schonberg, S. & Kordan H.E. (1995). The Inhibitory Effects of Conjugated Dienoic Derivatives (CLA) of Linoleic Acid on the Growth of Human Tumor Cell Lines is in Part Due to Increased Lipid Peroxidation. *Anticancer research,* Vol.15, No.4, (July-August 1995), pp. 1241-1246, ISSN 0250-7005

Shiomi, M.; Sasaki, K., Murofushi, M. & Aibara, K. (1982). Antitumor Activity in Mice of Orally Administered Polysaccharide from Kefir Grain. *Japanese Journal of Medical Science and Biology,* Vol.35, No.2 (April 1982), pp. 75-80, ISSN 0021-5112

Svensson, M.; Sabharwal, H., Mossberg, A.K., Lipniunas, P., Leffler, H., Svanborg, C. & Linse, S. (1999). Molecular characterization of alpha-lactalbumin folding variants that induce apoptosis in tumor cells. *Journal of Biological Chemistry,* Vol.274, No.10 (March 1999), pp. 6388-6396, ISSN 0021-9258

Part 3

Targeting Coagulation Factor VII

9

Factor VII-Targeted Photodynamic Therapy for Breast Cancer and Its Therapeutic Potential for Other Solid Cancers and Leukemia

Zhiwei Hu
Department of Obstetrics, Gynecology & Reproductive Sciences, Yale University School of Medicine, New Haven, U.S.A.

1. Introduction

Photodynamic therapy (PDT) is a treatment (Castano et al., 2006; Gomer, 1989; Oleinick and Evans, 1998) that involves three components: a photoactivatable photosensitizer (PS), laser light, and tissue oxygen. The current applications of non-targeted PDT (ntPDT) involve intravenous injection or topical application of PS followed by irradiation of the diseased lesion with a laser light. Upon activation by laser light, PS converts intracellular oxygen to singlet oxygen ions, which then cause cellular necrosis and/or apoptosis. PDT has clinical indications in the treatment of localized cancers, such as breast cancer, and has therapeutic potential for local recurrence of chemoresistant tumors (Capella and Capella, 2003; Merlin et al., 2003). However, a serious limitation of conventional ntPDT is the toxicity that results from internalization of the PS by normal cells (Taber et al., 1998). To overcome the poor selectivity of PS, antibodies, ligands and peptides have been tested in order to develop targeted PDT (tPDT), which would target PS into tumor cells (Mayo et al., 2003; Sharman et al., 2004) or vascular endothelial cells alone (Bechet et al., 2010). To the best of our knowledge, there are no other published papers reporting the successful development of tPDT that can simultaneously target both the tumor neovasculature and tumor cells except for three recent articles (Duanmu et al., 2011; Hu et al., 2010, 2011) from our laboratory (Figure 1). Here we summarize the development, efficacy and safety tests of dual tumor neovasculature- and tumor cell-targeting PDT by targeting the receptor tissue factor (TF) using its natural ligand factor VII (fVII)-conjugated photoactivable sensitizers for treatment of breast cancer in preclinical studies. We also review the selective expression of TF on angiogenic tumor vasculature and the percentages of TF on cancer cells in several most common solid tumors and leukemia, which we believe could help us to predict what percentage of patients with individual cancer can benefit from these TF-targeting therapies in future clinical trials and applications.

2. Targeting tumor neovasculature, tumor cells or both for development of targeted therapeutics

It is believed that targeting tumor neovasculature is a better strategy for cancer therapy than targeting cancer cells alone for the following reasons (Alessi et al., 2004; Folkman, 1971; Romanque et al., 2008):

i. Accessibility -- Tumor vascular endothelial cells (VECs) are accessible to therapeutic agents in blood circulation.
ii. Effectiveness -- It is estimated that each individual tumor VEC provides nutrition and oxygen to about 50-100 tumor cells.

We believe that targeting both the tumor neovasculature and tumor cells can achieve a better effect than targeting either of these types of cells alone (Hu and Garen, 2000, 2001; Hu and Li, 2010; Hu et al., 2010, 2011; Hu et al., 1999). The key to successful development of therapeutics with this ability to "kill two birds with one stone" is to identify a common but specific target molecule on cancer cells and tumor angiogenic VECs (Duanmu et al., 2011; Hu et al., 2010, 2011).

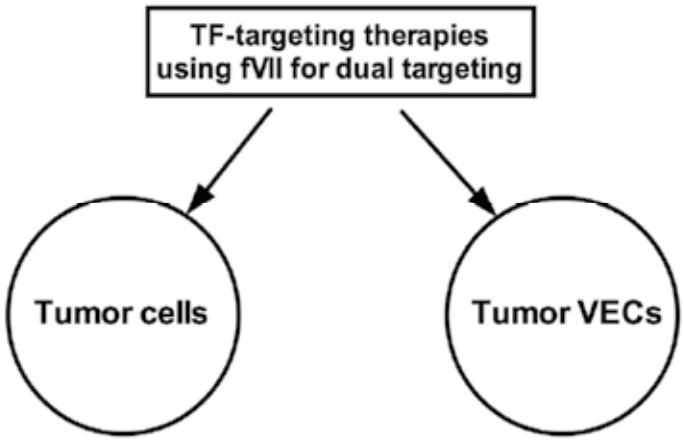


Fig. 1. Tissue factor (TF)-targeting therapies such as factor VII-targeted photodynamic therapy (fVII-tPDT) can simultaneously target both the tumor cells and tumor vascular endothelial cells (VECs) in the treatment of breast cancer with therapeutic potentials for other solid cancers and leukemia.

3. Key points for the successful development of tumor neovasculature- and tumor cell-targeting PDT (tPDT)

The bottleneck for the development of tPDT with the ability to target the tumor neovasculature and tumor cells is to identify a target molecule that is selectively expressed on both angiogenic VECs and tumor cells. Other key requirements are (i) the identification of a targeting vehicle that can specifically bind the target molecule and be internalized by the diseased cells and (ii) the ability to covalently conjugate this vehicle to a photosensitizer without affecting its binding activity or the photoactivity of the photosensitizer. Macromolecules such as antibodies or ligands are frequently used as targeting vehicles (Mayo et al., 2003; Sharman et al., 2004). In this regard, we chose the receptor tissue factor (TF) as the target molecule and its ligand, coagulation factor VII (fVII), as the targeting vehicle for the development of fVII-targeted Sn(IV) chlorine e6 (SnCe6) or verteporfin (VP) PDT for treatment of breast cancer in preclinical studies (Hu et al., 2010, 2011).

4. Tissue factor and vascular endothelial growth factor (VEGF) in tumor angiogenesis

Tumor angiogenesis is the formation of new capillary blood vessels in tumor tissues from existing blood vessels in adjacent normal tissues (Carmeliet and Jain, 2000; McDonald and Choyke, 2003). Tumor angiogenesis is required for the tumor to grow to over microscopic size and to metastasize (Abe, 2008; Blood and Zetter, 1990; Bohle and Kalthoff, 1999; Ellis, 2004; Folkman, 2002; Rouhi et al., 2010; Weidner et al., 1991; Zetter, 1998). Tumor capillary blood vessels are quite different from normal capillary vessels in many aspects, including morphology (typically, they lack tight conjunctions between endothelial cells and even lack endothelial cells on the vessel wall, which forms holes) and permeability. The difference of the morphology of tumor blood vessels is probably due to the expression of novel molecules on the inner endothelial layer of tumor blood vessels, which do not express on the quiescent, resting endothelial cells of normal blood vessels. Identifying these novel molecules on tumor VECs can aid in developing novel therapeutic agents to specifically target tumor blood vessels for novel cancer therapy.

Cell membrane receptor tissue factor (TF) is one such novel protein that is selectively expressed on VECs in the context of pathological angiogenesis in cancer (Contrino et al., 1996; Duanmu et al., 2011; Hu and Garen, 2001; Hu et al., 1999; Takano et al., 2000), wet form macular degeneration (Bora et al., 2003; Tezel et al., 2007) and endometriosis (Krikun et al., 2010). TF is a transmembrane receptor (Fisher et al., 1987; Konigsberg and Nemerson, 1988; Morrissey et al., 1987; Spicer et al., 1987), which forms an exceptionally strong and specific complex with its natural ligand, fVII (Idusogie et al., 1996a; Idusogie et al., 1996b; O'Hara et al., 1987), as the initial step of the coagulation pathway (Spicer et al., 1987). A known molecule connecting TF to tumor angiogenesis is VEGF. VEGF is a key pro-angiogenic factor that is produced by cancer cells (Abe et al., 1999; Lacal et al., 2000). As indicated in its former name vascular permeability factor (VPF) (Watanabe et al., 1997), VEGF results in the formation of immature, leaky and twisted blood vessels in tumors (Carmeliet and Jain, 2000; McDonald and Choyke, 2003). Importantly, VEGF can induce TF expression on VECs *in vitro* and *in vivo* (Armesilla et al., 1999; Hu et al., 2010, 2011; Schabbauer et al., 2007; Shen et al., 2001; Takano et al., 2000; Zucker et al., 1998), which do not normally express TF under physiological conditions (Contrino et al., 1996; Hu et al., 1999; Osterud, 1997; Rao and Pendurthi, 1998; Semeraro and Colucci, 1997).

5. TF is a common but specific target molecule on tumor VECs and tumor cells for development into novel tPDT as a dual-action therapeutic

5.1 TF is selectively expressed by angiogenic VECs in tumor but not by normal resting VECs in normal organs/tissues

Using tumor tissues from patients and mice, Contrino and colleagues (Contrino et al., 1996) and our groups (Duanmu et al., 2011; Hu and Garen, 2001; Hu et al., 1999) separately showed that TF was selectively expressed on angiogenic VECs, such as those in the tumor neovasculature of human breast cancer samples from patients (Contrino et al., 1996) and of human melanoma tumor xenografts in mice (Hu and Garen, 2001; Hu et al., 1999). In contrast, normal quiescent VECs do not express TF (Contrino et al., 1996; Hu and Garen, 2001; Osterud, 1997; Rao and Pendurthi, 1998; Semeraro and Colucci, 1997). Moreover, we very recently show that TF is also expressed by tumor VECs in multidrug resistant human breast tumor xenograft in mice (Duanmu et al., 2011). We conclude that TF is selectively expressed by angiogenic tumor VECs in tumors including multidrug resistant tumors.

Selective expression of TF by angiogenic VECs is similarly observed *in vitro*. Using VECs isolated from human umbilical vein vessels (HUVECs), we (Hu et al., 2010, 2011) and other groups (Camera et al., 1999; Zucker et al., 1998) have shown that TF is selectively expressed on VEGF-stimulated angiogenic HUVECs but not on unstimulated HUVECs.

5.2 TF is also over-expressed by many types of cancer cells including solid cancer and leukemia (Table 1)

Early studies indicated that TF was expressed by cancer cells in solid cancer (Callander et al., 1992) and leukemia (Andoh et al., 1987; Bauer et al., 1989). There are now numerous elegant reviews on the role of TF expression in cancer and tumor angiogenesis (Fernandez and Rickles, 2002; Milsom and Rak, 2008; Rak et al., 2006; Rak et al., 2008; Rickles and Brenner, 2008; Rickles et al., 2003; Ruf and Mueller, 1996; Ruf et al., 2010; Semeraro and Colucci, 1997). We summarize TF expression percentages by cancer cells in several most common solid cancers (Table 1) and leukemia as examples to indicate what percentage of patients with each individual cancer can potentially benefit from TF-targeting therapies in the future clinical trials and applications.

Type of tumor	Case number	% on TC	% on TVEC	References
Breast cancer	115	81%	ND	(Sturm et al., 1992)
	7	100%	100%	(Contrino et al., 1996)
	213	91%	98.6% (stromal cells)	(Ueno et al., 2000)
	Human chemoresistant breast tumor xenograft from mice *	+	+	(Duanmu et al., 2011)
Melanoma	41 primary 42 metastatic	95% 100%	ND	(Kageshita et al., 2001)
	Human melanoma xenograft from mice *	+	+	(Hu et al., 1999)
Lung cancer	25	28%	78% (stromal macrophages, VECs)	(Shoji et al., 1998)
	191 (NSCLC)	43%	ND	(Koomagi and Volm, 1998)
	55	80%	ND	(Sawada et al., 1999)
	50	88%	ND	(Wang et al., 2005)
Hepatocellular carcinoma (HCC)	58	100%	ND	(Poon et al., 2003)
	62	63%	ND	(Kaido et al., 2005)
Pancreatic cancer	55	53%	TF not in normal pancreas	(Kakkar et al., 1995a)
	113	88.4%	ND	(Nitori et al., 2005)
	240 (10 normal pancreas 70 intraductal papillary mucinous neoplasms 40 pancreatic intraepithelial neoplasia, 130 resected or metastatic pancreatic adenocarcinomas)	87.9% overall (77% pancreatic intraepithelial neoplasias 91% intraductal papillary mucinous neoplasms 89% pancreatic cancers)	ND (TF not in normal pancreas)	(Khorana et al., 2007)

Colorectal cancer	67 primary, of which 18 with liver metastasis	46% of primary, 88.9% of liver metastasis	ND	(Shigemori et al., 1998)
	100	57.0%	ND	(Nakasaki et al., 2002)
	50	100%	ND	(Altomare et al., 2007)
Prostate cancer	67	73%	ND	(Abdulkadir et al., 2000)
	73	75.3%	ND	(Akashi et al., 2003)
	32 early stage 22 advanced stage	78% early-stage 60% advanced stage	ND (TF not in benign prostate gland)	(Kaushal et al., 2008)
	Human prostate tumor in mice **	+	+	(Hu and Garen, 2001)
Ovarian cancer	32	84%	ND	(Uno et al., 2007)
Glioma	44 (10 benign gliomas 14 anaplastic astrocytomas 20 glioblastomas)	75% overall (10% in Grade I-II, 86% in grade III 95% in grade IV)	ND	(Hamada et al., 1996)
	68 (23 glioblastomas 13 anaplastic astrocytomas 32 low-grade astrocytomas)	47% overall (91.3% glioblastomas, 46.2% anaplastic astrocytomas, and 15.6% low-grade astrocytomas)	44% overall (73.9% glioblastomas, 53.8% anaplastic astrocytomas, 0% low grade astrocytomas)	(Takano et al., 2000)
	34 gliomas 5 normal brain tissues	58.8% overall (20% of grade I 43% of grade II, 58% of grade III 90% of grade IV)	ND (TF not in normal brain tissues)	(Guan et al., 2002)

Notes:
Abbreviations: ND, not determined; TC, tumor cells; TVEC, tumor vascular endothelial cells; NSCLC, non-small cell lung cancer; HCC: hepatocellular carcinoma.
* Human breast and melanoma tumor xenografts were removed from mice and paraffin or frozen sections were made and immunohistochemically stained for endothelial TF by using a rabbit polyclonal anti-mouse TF antibody (Duanmu et al., 2011) and murine fVII/human IgG1 Fc protein (mouse Icon) (Hu et al., 1999), respectively.
** Mouse Icon protein was intravenously injected into the SCID mice carrying subcutaneous human prostate tumor xenografts and the bio-distribution of mouse Icon protein was studied by immunofluorescence staining for the human IgG1 Fc of the mouse Icon protein using an FITC-conjugated anti-human IgG antibody (Hu and Garen, 2001).
+ TF expression was positively detected.

Table 1. TF expression in breast cancer and other solid cancers.

5.2.1 TF in 80-100% of breast cancer

TF is expressed by breast cancer cell lines and breast cancer cells in tumor tissues (Contrino et al., 1996; Duanmu et al., 2011; Hu et al., 2010, 2011; Sturm et al., 1992; Ueno et al., 2000). Sturm et al. (Sturm et al., 1992) reported that 81% (93 out of 115) breast cancer tissues were TF expression positive on tumor cells. Contrino et al. (Contrino et al., 1996) further showed

that TF was localized on vascular endothelial cells (VEC) and tumor cells within tumors from all 7 (100%) invasive breast cancer cases but not in the VEC or tumor cells of benign tumors from 10 patients with fibrocystic disease of the breast. Ueno et al. (Ueno et al., 2000) showed that 91% (193 cases) of 213 breast cancer tissues were positive for TF expression, which was detected on tumor cells, fibroblasts, monocytes and vascular endothelial cells in the tumor tissues. Taking together, TF expression was indeed detected on tumor cells and tumor vascular endothelial cells in 80-100% of breast tumors.

5.2.2 TF in 95-100% of melanoma

TF is over-expressed by melanoma cancer cells (Bromberg et al., 1995; Hu and Garen, 2000; Hu et al., 1999). Particularly Kageshita et al. reported that TF expression was detected in 95% of primary and 100% of metastatic melanoma tumor tissues from 41 and 24 patients, respectively (Kageshita et al., 2001).

5.2.3 TF in 40-80% of lung cancer

TF is expressed in human lung cancer cell lines (Keller et al., 2001; Minamiya et al., 2004; Rauch et al., 2005; Salge et al., 2001; Zacharski et al., 1983) as well as in about 40-80% of human lung tumor tissues from patients worldwide (Koomagi and Volm, 1998; Sawada et al., 1999; Shoji et al., 1998; Wang et al., 2005). Shoji et al. (Shoji et al., 1998) reported that TF expression on stromal macrophage and vascular endothelial cells was detected in 76% (19 out of 25) of lung cancer patients, while lung cancer TF expression was detected in only 28% of these patients. Koomagi and Volm (Koomagi and Volm, 1998) reported that 75 of 175 cases (42.8%) with non-small-cell lung carcinomas (NSCLC) in Germany were TF positive and a significant association was found between TF expression and microvessel density. Sawada et al (Sawada et al., 1999) reported that TF was detected in NSCLC cell lines and in 46 of 55 specimens (80%) of surgically resected NSCLC tumors in Japan. Wang et al (Wang et al., 2005) reported that 88% of 50 human NSCLC tumors overexpressed TF in the United States.

5.2.4 TF in 63-100% of hepatocellular carcinoma (HCC)

Our unpublished study showed that TF is expressed by HCC SNU-423 line and human hepatoblastoma Hep G2 line. SNU-423 is a human HCC line with chemoresistance from a 40 years old male patient (Park et al., 1995). Hep G2 was isolated from a child (Aden et al., 1979; Knowles et al., 1980) and was recently re-characterized as hepatoblastoma (Lopez-Terrada et al., 2009). TF is also over-expressed by the cancer cells in HCC tumor tissues (Kaido et al., 2005; Poon et al., 2003). Poon et al reported in 2003 that TF expression was detected in 58 of 58 patients (100%) with HCC in Hong Kong, China and that TF expression level correlated significantly with tumor microvessel density ($p=0.002$), an indicator for tumor angiogenesis (Poon et al., 2003). Kaido et al reported in a retrospective study in 2005 that 39 of 62 Japanese patients with HCC, who survived for more than 5 years after surgery, were TF positive (62.9%) and had higher recurrence rate (Kaido et al., 2005). They concluded that TF expression was closely associated with tumor invasion and metastasis, and might serve as a prognostic factor of recurrence in HCC (Kaido et al., 2005). These two reports showed that TF was expressed in ~63% or even 100% of Japanese and Chinese patients with HCC, respectively. However, both papers did not investigate the TF expression on tumor VECs. So far there are no published papers reporting the percentage of TF expression in the US patients with HCC.

5.2.5 TF in 53-90% of pancreatic cancer

Several groups showed that TF expression was found on pancreatic cancer tissues or cell lines (Cohen and Burtness, 2006; Haas et al., 2006; Hobbs et al., 2007; Iijima et al., 1991; Kakkar et al., 1995a; Kakkar et al., 1995b; Khorana et al., 2007; Khorana and Fine, 2004; Lindahl et al., 1993; Lindahl et al., 1992; Nitori et al., 2005; Silberberg et al., 1989; Taniguchi et al., 1998; Tesselaar et al., 2007; Ueda et al., 2001), but not in normal pancreatic cells (Cohen and Burtness, 2006; Kakkar et al., 1995a). Kakkar et al. in 1995 reported that TF expression was detected in 29 of 55 (53%) pancreatic tumors (Kakkar et al., 1995a). They found that TF expression was significantly correlated with the histological grade of the tumors, i.e., the poorer the differentiation of the tumor, the more expression of TF. Moreover, Kakkar et al. reported that TF was not detected in all of 18 normal human pancreas tissues (Kakkar et al., 1995a), indicating that TF was specifically expressed on pancreatic tumor cells in the pancreas. Nitori et al. in 2005 confirmed the observation of TF expression on pancreatic tumors (Nitori et al., 2005). They reported that 88.4% of 113 Japanese patients with pancreatic ductal adenocarcinoma were TF positive on cancer cells, and that increased TF expression was significantly correlated with the extent of the primary tumor, lymph node metastasis, lymphatic distant metastasis, advanced tumor-node-metastasis stage, and high tumor grade. Therefore, they concluded that TF expression on tumor cells was a predictor for survival. Recently, Khorana et al. reported that TF was expressed in 77% pancreatic intraepithelial neoplasias, 91% intraductal papillary mucinous neoplasms, and 89% pancreatic cancers, but not in normal pancreas (Khorana et al., 2007). High TF expression was associated with high level of VEGF and increased microvessel density. Therefore they stated that "TF represents an attractive and novel therapeutic target in pancreatic cancer, and anti-TF approaches deserve further study in this setting" (Khorana et al., 2007). All of these previous studies indicated that TF indeed could serve as a therapeutic target on pancreatic cancer cells (and probably on tumor neovasculature) in pancreatic cancer for TF-targeting therapies in the treatment of this intractable cancer.

5.2.6 TF in 57-100% of colorectal cancer

Shigemori et al. (Shigemori et al., 1998) reported that TF was detected in 57% of colorectal cancer from 79 patients, and its expression was significantly increased ($p=0.01$) in metastatic tumors (88%) from 17 patients. Later, Nakasaki et al. reported the same percentage (57%) of TF expression in 100 colorectal cancer tissues (Nakasaki et al., 2002). Using ELISA, Altomare et al. (Altomare et al., 2007) detected higher TF expression (100%) in 50 surgical colorectal cancer specimens.

5.2.7 TF in 60-78% of prostate cancer

TF expression was detected in urological cancers including renal cell carcinoma, bladder cancer and prostate cancer (Akashi et al., 2003; Forster et al., 2003; Kaushal et al., 2008; Langer et al., 2007; Lwaleed et al., 2000; Nieva, 2007; Ohta et al., 2002). Abdulkadir et al. (Abdulkadir et al., 2000) reported that TF expression was detected on the epithelial cells of malignant glands (73%; $n = 67$) of prostate cancer specimens, and TF expression was significantly correlated with tumor angiogenesis and preoperative level of prostate specific antigen. Similarly, Kaushal et al. (Kaushal et al., 2008) reported that TF expression was positively stained in malignant gland in 78% specimens of early-stage and 60% of specimens of advanced stage prostate cancer, but not in the benign gland. TF was also detected in 75.3% of metastatic prostate cancer from patients (Akashi et al., 2003).

5.2.8 TF in gynecological cancers

TF is also over-expressed by ovarian cancer cells (Chinen et al., 2009; Uno et al., 2007; Yokota et al., 2009). Particularly, when Uno et al. studied the TF expression as a possible determinant of thromboembolism in ovarian cancer, they found that TF was over-expressed on the ovarian cancer cells in 27 out of 32 ovarian cancer patients (84%) by immunohistochemical staining (Uno et al., 2007). However, tumor endothelial TF in ovarian cancer and other gynecological cancers remains to be investigated. In addition, our unpublished studies showed that TF was expressed by human ovarian cancer lines OVCAR-5 and Hey and cervical cancer line HeLa.

5.2.9 TF in brain cancer

Hamada and co-workers studied the TF expression in surgical specimens of glioma in 1996 (Hamada et al., 1996). They showed that 75% gliomas were positive for TF, with a correlation of malignancy grade (1 of 10 benign gliomas (10%) in malignancy Grade I-II, 13 of 14 anaplastic astrocytomas (86%) in malignancy grade III and 19 of 20 glioblastomas (95%) in malignancy grade IV. Takano and co-workers (Takano et al., 2000) reported that TF antigen was detected in the brain tumor cells of 21 out of 23 glioblastomas, 6 of 13 anaplastic astrocytomas, and 5 of 32 low-grade astrocytomas, indicating that TF was expressed by 47% (32/68) of glioma patients. Importantly, TF antigen was also detected in the tumor-associated vessels of 17 out of 23 glioblastomas, 7 of 13 anaplastic astrocytomas, and none of 32 low grade astrocytomas. Later, Guau et al. (Guan et al., 2002) also investigated the expression of TF in 5 normal brain tissues and 32 glioma tissues from Chinese patients, and the glioma cell line U251. They similarly showed that the higher grade, the more TF expression (90% of the grade IV cases, 58% of grade III, 43% of grade II, and 20% of grade I). Importantly they showed that TF expression was not detected in any of 5 normal brain tissues, indicating that TF expression could be a specific biomarker in brain tumor.

5.2.10 TF in leukemia

Leukemia is a malignant neoplasm of hematopoietic tissue originating in the bone marrow and infiltrating the peripheral blood and often also the spleen, liver, and lymph nodes. Acute leukemia, including AML and ALL are characterized by proliferation of immature cells or blasts. If untreated, death usually occurs within 6 months in most cases. It was reported that TF is expressed on the human leukemic HL-60 (Freeburn et al., 1995; Hair et al., 1996; Kubota et al., 1991; Rickles et al., 1995; Tanaka et al., 1989), Molt-4 (Tanaka, 1989), THP-1 (Tanaka, 1989) cell lines , and on leukemic cells from patients with AML (Andoh et al., 1987; Bauer et al., 1989; Nakasaki et al., 2000b; Tanaka et al., 1989; Tanaka and Kishi, 1990; Tanaka and Yamanishi, 1993) and ALL (Kubota et al., 1991; Nakasaki et al., 2000a). TF is not expressed on the normal peripheral mononuclear cells unless stimulated by endotoxin or other cytokines (Rickles et al., 1995), nor on myeloid precursor cells (Bauer et al., 1989). TF was also detected in the plasma of patients with leukemia (Kubota et al., 1991; Nakasaki et al., 2000a) and in HL-60 culture medium (Kubota et al., 1991). The evidence for TF expression on proliferating leukemic cells identifies leukemia as a potential candidate for TF-targeting therapies.

Another target in leukemia patients for TF-targeting therapy is the bone marrow vascularity (Yang and Han, 2002). Angiogenesis does occur in leukemia (Aguayo et al., 2000; Dickson

and Shami, 2001; Hussong et al., 2000; Korkolopoulou et al., 2003; Litwin et al., 2002; Padro et al., 2000; Perez-Atayde et al., 1997; Pule et al., 2002). Microvessel density is increased in bone marrow biopsies obtained from patients with childhood ALL (Aguayo et al., 2000; Dickson and Shami, 2001), and in bone marrow of newly diagnosed AML patients (Padro et al., 2000). Further indication of leukemia-associated angiogenesis is an increased level of vascular endothelial growth factor (VEGF) in bone marrow blast cells of AML patients (Litwin et al., 2002). These findings have led to tests of anti-angiogenic therapy for leukemia (Thomas et al., 2001). VEGF induces TF expression on vascular endothelial cells (Hu et al., 2010, 2011; Zucker et al., 1998) and therefore also could induce TF expression in the tumor neovasculature in bone marrow of leukemia patients (to be investigated), providing an additional target for TF-targeting therapies including Icon immunotherapy (Hu and Garen, 2000, 2001; Hu and Li, 2010; Hu et al., 1999) and fVII-targeted PDT (Duanmu et al., 2011; Hu et al., 2010, 2011).

5.2.11 TF on normal cells is sequestered by semi-permeable vessel walls

Although TF is a normal cell surface receptor (Morrissey et al., 1987; Spicer et al., 1987) and is expressed defensively on extravascular cells of several normal tissues and in the adventitial layer of the blood vessel wall (readily to initiate coagulation cascade upon the damage to vessel wall integrity), it is sequestered from direct contact with circulating fVII at a low concentration (10 nM) at these sites by the tight endothelial cell layer of the normal vasculature (Osterud, 1997; Rao and Pendurthi, 1998; Semeraro and Colucci, 1997). In contrast, newly formed tumor capillary blood vessels are leaky because they lack tight junctions between endothelial cells, and their walls may even lack endothelial cells entirely (Carmeliet and Jain, 2000; McDonald and Choyke, 2003). Thus, only the TF on angiogenic VECs and tumor cells is accessible to TF-targeting therapeutic agents via leaky tumor blood vessels.

Based on its selective expression and the leakiness of tumor neovasculature, TF is regarded as a common but specific target molecule in tumor blood vessels and cancer cells for the development of novel neovasculature- and cancer cell-targeting therapies, such as fVII-targeted PDT (fVII-tPDT) (Duanmu et al., 2011; Hu et al., 2010, 2011).

6. Successful development of fVII-tPDT by targeting TF for simultaneous, selective and effective killing of angiogenic VECs and breast cancer cells

As reported in our three recent published studies, we have chosen the receptor TF as a target molecule and its ligand, fVII, as a targeting vehicle for the development of ligand-targeted PDT and have successfully developed fVII-targeted PDT using fVII-SnCe6 or fVII-VP conjugates for treatment of breast cancer (Duanmu et al., 2011; Hu et al., 2010, 2011) and wet macular degeneration (Lu et al., 2009). We showed that TF as the target molecule and fVII as the targeting vehicle met all of the requirements for successful development of a dual-action tPDT, as discussed below.

6.1 Identification of a targeting vehicle that can specifically bind the target molecule and be internalized by the diseased cells

We identified fVII as the targeting vehicle to bind the target molecule TF. The reason for choosing the natural ligand fVII for TF, instead of antibodies to TF, is that the dissociation

constant (Kd) for fVII binding to TF (pM level) is about 100-1000 fold lower than those of antibodies to TF (nM levels), i.e., the affinity for fVII to TF is 100-1000 fold higher that antibodies to TF, and that fVII can be made in human sequence without need for humanization process (Hu and Li, 2010; Hu et al., 2010, 2011).

Regarding the ability of fVII to internalize into cells, Rao and colleagues had shown that wild-type human fVII protein could be endocytosed by TF-transfected baby hamster kidney cells (Hansen et al., 2001). But it was unknown if fVII with a coagulation active site mutation (K341A) can be internalized into cells, which was a key process for successful development of tPDT in order for the targeting vehicle fVII to internalize the covalently-conjugated photosensitizer into the target cells. Using co-localization of an intracellular F-actin cytoskeleton with a fVII(K341A)/human IgG1 Fc Icon immunoconjugate, which contained an fVII peptide containing a K341A mutation fused to the Fc portion of an IgG1 antibody for cancer immunotherapy (Hu and Garen, 2000, 2001; Hu and Li, 2010; Hu et al., 1999), we showed that fVII with K341A mutation could be endocytosed into breast cancer cells and VEGF-stimulated angiogenic HUVEC with a maximal detection of intracellular fVII at 30 min after binding (Hu et al., 2011) (confocal imagings at other time points were not shown). Thus we conclude that fVII(K341A) as the targeting vehicle can specifically bind its cognate receptor TF, the target molecule, and be internalized by the diseased cells.

6.2 The ability to covalently conjugate fVII as the targeting vehicle to a photosensitizer without affecting its binding activity or the photoactivity of the photosensitizer

To covalently conjugate fVII protein with photosensitizers verteporfin and Sn IV chlorin e6 (SnCe6), we used the cross linker EDC with the ability to bind to –COOH groups on photosensitizers and then to –NH2 groups on protein molecules (Hu et al., 2010, 2011). In principle, this procedure using EDC can be used to conjugate fVII proteins to any other photosensitizers that have –COOH groups. We tested two photosensizers that have –COOH groups, namely verteporfin (689nm) and SnCe6 (635 nm). At the time when we carried out the study reported in (Hu et al., 2010), the chemically pure form of verteporfin was not commercially available to us. Now both SnCe6 and VP can be synthesized and commercially available in chemically pure form. It is worth noting that SnCe6 is stable at 4°C for at least one year without any loss of its photoactivity to cancer cells (when it was prevented from light. See supplementary information in ref. (Hu et al., 2011)). Since we used excess photosensitizer in the chemical reaction, we used size exclusion spin columns (Sephadex G50) to separate the unconjugated photosensitizers (being hold in the column resin) from the fVII-conjugated photosensitizer (being collected in the collection tubes after spin).

Using breast cancer cell-ELISA and flow cytometry assays, we showed that the binding activity of fVII in the photosensitizer conjugate either with verteporfin or with SnCe6 was retained (Hu et al., 2010, 2011). To address the photoactivity of fVII-PS conjugate, we used a crystal violet staining for the loss of monolayer membrane to determine the effect of PDT on killing cancer cells and compared to ntPDT *in vitro*. We showed that TF-targeting PDT by fVII enhanced 3-4 fold the effect of verteporfin PDT (Hu et al., 2010) and 12-13 fold of SnCe6 PDT (Hu et al., 2011) on killing breast cancer cells *in vitro*. The reason for the less effect of fVII-verteporfin PDT was probably because chemically pure verteporfin (Benzoporphyrin

derivative-monacid ring A, BPD-MA) was not available to us so we had to extract it from its commercial formula Visudyne (QLT Inc) and the extracts still might contain trace of other components that might interfere with the killing effect, whereas SnCe6 was available in chemically pure form (Frontier Scientific, Logan, UT). We conclude that covalent conjugation reaction did not affect the binding activity of fVII and the photoactivity of the photosensitizer in the fVII-photosensitizer conjugates.

Interestingly, we observed that fVII-tPDT, either with fVII-verteporfin or with fVII-SnCe6, reached the maximal killing effect on a variety of cancer cells *in vitro* for drug-laser light interval at 90 min (Hu et al., 2010, 2011), whereas the maximal intracellular localization of fVII/TF reached at 30 min after incubation of fVII/Fc with target cells (Hu et al., 2011).

6.3 The selectivity of fVII-tPDT for cancer cells and angiogenic VECs

In order to test whether fVII-tPDT is selective on killing cancer cells and has no adverse effects on normal cells that do not express TF, we use a high (MDA-MB-231) and a low (MCF7) TF breast cancer cell lines and a normal human embryonic kidney 293 cells as a non-TF expressing line. We showed that fVII-tPDT was selectively killing both TF-expressing breast cancer cells with a TF-level dependent response, but it had no killing at all on 293 cells, indicating that this fVII-tPDT was selective and effective in killing TF-breast cancer cells.

Moreover, we used VEGF-stimulated HUVEC and unstimulated HUVEC as models of angiogenic VECs and normal, resting VECs. We showed that fVII-tPDT was able to selectively kill VEGF-stimulated angiogenic HUVEC but had no killing effect on normal, resting HUVEC (Hu et al., 2011), indicating that fVII-tPDT was also selective and effective in killing angiogenic VECs.

6.4 Mechanism of action of fVII-tPDT

Using the assays for Caspase-3/7 and lactate dehydrogenase (LDH) activities, we showed that fVII-tPDT, either with verteporfin or SnCe6, could induce apoptosis and necrosis right after PDT treatment (Duanmu et al., 2011; Hu et al., 2010, 2011), as the underlying mechanism.

6.5 The effect and safety of fVII-tPDT *in vivo* in mouse models of breast cancer

We tested the effect of fVII-tPDT using verteporfin or SnCe6 in three mouse models of breast cancer, the first with murine breast cancer EMT6 in immunocompetent Balb/c mice, the second with human breast tumor MDA-MD-231 in immunodeficient nude mice and the third with chemoresistant breast cancer MCF-7/MDR in nude mice. The results showed that fVII-tPDT (2 μM SnCe6 in fVII-SnCe6 PDT for EMT6 and MDA-MB-231 (72 J/cm^2) and MCF-7/MDR (65 J/cm^2); or 2 μM, 105 J/cm^2 in verteporfin PDT for EMT6) was effective in inhibiting tumor growth in mice, whereas ntPDT at the same conditions had no effect. In all three studies, there were no differences in mouse body weight, complete blood counts and differential analyses of leukocytes between the control mice and fVII-tPDT treated mice, and none of those mice had any other signs of toxicity during or at the end of the experiments (Duanmu et al., 2011; Hu et al., 2010, 2011). We thus conclude that fVII-tPDT was effective and safe in the treatment of human and murine breast tumors in mice, including chemoresistant breast tumor.

7. Previous studies on the development of other TF-targeting therapeutics

In the course of the development of TF-targeting therapeutics, Hu and Garen reported for the first time in 1999 an antibody-like fVII-human IgG1 Fc fusion protein (Hu et al., 1999), which later was called an Icon (Hu and Garen, 2001). Icon has been tested for immunotherapy by eradicating pathological neovasculature for treatment of tumors (Cocco et al., 2010; Hu and Garen, 2000, 2001; Hu and Li, 2010; Hu et al., 1999; Tang et al., 2007) and non-cancerous diseases such as wet form macular degeneration (Bora et al., 2003; Tezel et al., 2007) and endometriosis (Krikun et al., 2010). Similar to the studies in which Hu and Garen introduced a mutation (K341A) in the fVII protein in order to reduce its coagulation activity, Shoji and colleagues in 2008 (Shoji et al., 2008) reported the use of an active site-chemically inactivated fVIIa (FFRck-fVIIa) as a carrier for the targeted delivery of a potent synthetic curcumin analog (EF24) to TF-expressing tumor-associated VECs and tumor cells. The study by Shoji et al. provides independent evidence in support of our previous concept and findings regarding the efficacy of TF-targeting therapeutics.

As a different TF-targeting therapeutic approach, fVII-tPDT can be used as a stand-alone modality, and potentially in combination with Icon immunotherapy, surgery, and other modality for treatment of breast cancer and potentially of other laser light-accessible tumors on skin surfaces or in the internal cavity. To further enhance the effect of fVII-tPDT, we can increase the laser irradiation time and use better photosensitizers with longer wavelength (for deeper penetration) and better laser light sources with larger irradiation area in future preclinical and clinical studies.

8. Conclusion

In conclusion, fVII-targeted verteporfin or SnCe6 PDT is selective and effective in killing angiogenic VECs and breast cancer cells *in vitro* and can significantly inhibit the tumor growth of murine and human breast cancer *in vivo*. As TF is over-expressed by many types of cancer cells, including solid cancer and leukemia, and selectively expressed by angiogenic VECs of pathological neovasculature in tumor, macular generation and endometriosis, we anticipate that fVII-tPDT will have broad therapeutic potential for breast tumor and other tumors as well as for other pathological neovasculature-associated non-cancerous diseases (including but not limited to wet macular degeneration and endometriosis), in which the diseased lesions abnormally express TF and can be accessed by laser light, for instance, delivered by optical fibers through endoscopic, interstitial, or intracavitary techniques.

9. Acknowledgement

The studies on fVII-tPDT were supported by CT DPH Biomedical Research Grant (RFP#2009-0096), the Breast Cancer Alliance Exceptional Project Grant, the Susan Komen Award (BCTR0601204) and the Swebilius Translational Cancer Research Award from the Yale Cancer Center.

10. Competing interests

The author is a co-inventor of the US patents on Neovascular-Targeted Immunoconjugates (US patents no. 6,924,359, 7,858,092 and 7,887,809).

11. References

Abdulkadir, S.A., Carvalhal, G.F., Kaleem, Z., Kisiel, W., Humphrey, P.A., Catalona, W.J., and Milbrandt, J. (2000). Tissue factor expression and angiogenesis in human prostate carcinoma. Hum Pathol *31*, 443-447.

Abe, K., Shoji, M., Chen, J., Bierhaus, A., Danave, I., Micko, C., Casper, K., Dillehay, D.L., Nawroth, P.P., and Rickles, F.R. (1999). Regulation of vascular endothelial growth factor production and angiogenesis by the cytoplasmic tail of tissue factor. Proceedings of the National Academy of Sciences of the United States of America *96*, 8663-8668.

Abe, R. (2008). Angiogenesis in tumor growth and metastasis. Curr Pharm Des *14*, 3779.

Aden, D.P., Fogel, A., Plotkin, S., Damjanov, I., and Knowles, B.B. (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature *282*, 615-616.

Aguayo, A., Kantarjian, H., Manshouri, T., Gidel, C., Estey, E., Thomas, D., Koller, C., Estrov, Z., O'Brien, S., Keating, M., *et al.* (2000). Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. Blood *96*, 2240-2245.

Akashi, T., Furuya, Y., Ohta, S., and Fuse, H. (2003). Tissue factor expression and prognosis in patients with metastatic prostate cancer. Urology *62*, 1078-1082.

Alessi, P., Ebbinghaus, C., and Neri, D. (2004). Molecular targeting of angiogenesis. Biochimica et biophysica acta *1654*, 39-49.

Altomare, D.F., Rotelli, M.T., Pentimone, A., Rossiello, M.R., Martinelli, E., Guglielmi, A., De Fazio, M., Marino, F., Memeo, V., Colucci, M., *et al.* (2007). Tissue factor and vascular endothelial growth factor expression in colorectal cancer: relation with cancer recurrence. Colorectal Dis *9*, 133-138.

Andoh, K., Kubota, T., Takada, M., Tanaka, H., Kobayashi, N., and Maekawa, T. (1987). Tissue factor activity in leukemia cells. Special reference to disseminated intravascular coagulation. Cancer *59*, 748-754.

Armesilla, A.L., Lorenzo, E., Gomez del Arco, P., Martinez-Martinez, S., Alfranca, A., and Redondo, J.M. (1999). Vascular endothelial growth factor activates nuclear factor of activated T cells in human endothelial cells: a role for tissue factor gene expression. Molecular and cellular biology *19*, 2032-2043.

Bauer, K.A., Conway, E.M., Bach, R., Konigsberg, W.H., Griffin, J.D., and Demetri, G. (1989). Tissue factor gene expression in acute myeloblastic leukemia. Thrombosis research *56*, 425-430.

Bechet, D., Tirand, L., Faivre, B., Plenat, F., Bonnet, C., Bastogne, T., Frochot, C., Guillemin, F., and Barberi-Heyob, M. (2010). Neuropilin-1 targeting photosensitization-induced early stages of thrombosis via tissue factor release. Pharm Res *27*, 468-479.

Blood, C.H., and Zetter, B.R. (1990). Tumor interactions with the vasculature: angiogenesis and tumor metastasis. Biochimica et biophysica acta *1032*, 89-118.

Bohle, A.S., and Kalthoff, H. (1999). Molecular mechanisms of tumor metastasis and angiogenesis. Langenbecks Arch Surg *384*, 133-140.

Bora, P.S., Hu, Z., Tezel, T.H., Sohn, J.H., Kang, S.G., Cruz, J.M., Bora, N.S., Garen, A., and Kaplan, H.J. (2003). Immunotherapy for choroidal neovascularization in a laser-

induced mouse model simulating exudative (wet) macular degeneration. Proc Natl Acad Sci U S A *100*, 2679-2684.

Bromberg, M.E., Konigsberg, W.H., Madison, J.F., Pawashe, A., and Garen, A. (1995). Tissue factor promotes melanoma metastasis by a pathway independent of blood coagulation. Proc Natl Acad Sci U S A *92*, 8205-8209.

Callander, N.S., Varki, N., and Rao, L.V. (1992). Immunohistochemical identification of tissue factor in solid tumors. Cancer *70*, 1194-1201.

Camera, M., Giesen, P.L., Fallon, J., Aufiero, B.M., Taubman, M., Tremoli, E., and Nemerson, Y. (1999). Cooperation between VEGF and TNF-alpha is necessary for exposure of active tissue factor on the surface of human endothelial cells. Arterioscler Thromb Vasc Biol *19*, 531-537.

Capella, M.A., and Capella, L.S. (2003). A light in multidrug resistance: photodynamic treatment of multidrug-resistant tumors. J Biomed Sci *10*, 361-366.

Carmeliet, P., and Jain, R.K. (2000). Angiogenesis in cancer and other diseases. Nature *407*, 249-257.

Castano, A.P., Mroz, P., and Hamblin, M.R. (2006). Photodynamic therapy and anti-tumour immunity. Nat Rev Cancer *6*, 535-545.

Chinen, K., Fujino, T., Horita, A., Sakamoto, A., and Fujioka, Y. (2009). Pulmonary tumor thrombotic microangiopathy caused by an ovarian cancer expressing tissue factor and vascular endothelial growth factor. Pathol Res Pract *205*, 63-68.

Cocco, E., Hu, Z., Richter, C.E., Bellone, S., Casagrande, F., Bellone, M., Todeschini, P., Krikun, G., Silasi, D.A., Azodi, M., *et al.* (2010). hI-con1, a factor VII-IgGFc chimeric protein targeting tissue factor for immunotherapy of uterine serous papillary carcinoma. Br J Cancer *103*, 812-819.

Cohen, S.J., and Burtness, B.A. (2006). Novel targets in pancreatic cancer: focus on future paths to therapy. Expert opinion on therapeutic targets *10*, 771-775.

Contrino, J., Hair, G., Kreutzer, D.L., and Rickles, F.R. (1996). In situ detection of tissue factor in vascular endothelial cells: correlation with the malignant phenotype of human breast disease. Nature medicine *2*, 209-215.

Dickson, D.J., and Shami, P.J. (2001). Angiogenesis in acute and chronic leukemias. Leuk Lymphoma *42*, 847-853.

Duanmu, J., Cheng, J., Xu, J., Booth, C.J., and Hu, Z. (2011). Effective treatment of chemoresistant breast cancer in vitro and in vivo by a factor VII-targeted photodynamic therapy. British journal of cancer *104*, 1401-1409.

Ellis, L.M. (2004). Angiogenesis and its role in colorectal tumor and metastasis formation. Semin Oncol *31*, 3-9.

Fernandez, P.M., and Rickles, F.R. (2002). Tissue factor and angiogenesis in cancer. Curr Opin Hematol *9*, 401-406.

Fisher, K.L., Gorman, C.M., Vehar, G.A., O'Brien, D.P., and Lawn, R.M. (1987). Cloning and expression of human tissue factor cDNA. Thrombosis research *48*, 89-99.

Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. N Engl J Med *285*, 1182-1186.

Folkman, J. (2002). Role of angiogenesis in tumor growth and metastasis. Semin Oncol *29*, 15-18.

Forster, Y., Meye, A., Albrecht, S., Kotzsch, M., Fussel, S., Wirth, M.P., and Schwenzer, B. (2003). Tissue specific expression and serum levels of human tissue factor in patients with urological cancer. Cancer Lett *193*, 65-73.

Freeburn, J.C., Gilmore, W.S., and Strain, J.J. (1995). The effect of cytokines on tissue factor expression in HL-60 and U937 cell lines. Biochem Soc Trans *23*, 286S.

Gomer, C.J. (1989). Photodynamic therapy in the treatment of malignancies. Semin Hematol *26*, 27-34.

Guan, M., Jin, J., Su, B., Liu, W.W., and Lu, Y. (2002). Tissue factor expression and angiogenesis in human glioma. Clin Biochem *35*, 321-325.

Haas, S.L., Jesnowski, R., Steiner, M., Hummel, F., Ringel, J., Burstein, C., Nizze, H., Liebe, S., and Lohr, J.M. (2006). Expression of tissue factor in pancreatic adenocarcinoma is associated with activation of coagulation. World J Gastroenterol *12*, 4843-4849.

Hair, G.A., Padula, S., Zeff, R., Schmeizl, M., Contrino, J., Kreutzer, D.L., de Moerloose, P., Boyd, A.W., Stanley, I., Burgess, A.W., *et al.* (1996). Tissue factor expression in human leukemic cells. Leuk Res *20*, 1-11.

Hamada, K., Kuratsu, J., Saitoh, Y., Takeshima, H., Nishi, T., and Ushio, Y. (1996). Expression of tissue factor correlates with grade of malignancy in human glioma. Cancer *77*, 1877-1883.

Hansen, C.B., Pyke, C., Petersen, L.C., and Rao, L.V. (2001). Tissue factor-mediated endocytosis, recycling, and degradation of factor VIIa by a clathrin-independent mechanism not requiring the cytoplasmic domain of tissue factor. Blood *97*, 1712-1720.

Hobbs, J.E., Zakarija, A., Cundiff, D.L., Doll, J.A., Hymen, E., Cornwell, M., Crawford, S.E., Liu, N., Signaevsky, M., and Soff, G.A. (2007). Alternatively spliced human tissue factor promotes tumor growth and angiogenesis in a pancreatic cancer tumor model. Thrombosis research *120 Suppl 2*, S13-21.

Hu, Z., and Garen, A. (2000). Intratumoral injection of adenoviral vectors encoding tumor-targeted immunoconjugates for cancer immunotherapy. Proc Natl Acad Sci U S A *97*, 9221-9225.

Hu, Z., and Garen, A. (2001). Targeting tissue factor on tumor vascular endothelial cells and tumor cells for immunotherapy in mouse models of prostatic cancer. Proc Natl Acad Sci U S A *98*, 12180-12185.

Hu, Z., and Li, J. (2010). Natural killer cells are crucial for the efficacy of Icon (factor VII/human IgG1 Fc) immunotherapy in human tongue cancer. BMC Immunol *11*, 49.

Hu, Z., Rao, B., Chen, S., and Duanmu, J. (2010). Targeting tissue factor on tumour cells and angiogenic vascular endothelial cells by factor VII-targeted verteporfin photodynamic therapy for breast cancer in vitro and in vivo in mice. BMC Cancer *10*, 235.

Hu, Z., Rao, B., Chen, S., and Duanmu, J. (2011). Selective and effective killing of angiogenic vascular endothelial cells and cancer cells by targeting tissue factor using a factor VII-targeted photodynamic therapy for breast cancer. Breast Cancer Res Treat *126*, 589-600.

Hu, Z., Sun, Y., and Garen, A. (1999). Targeting tumor vasculature endothelial cells and tumor cells for immunotherapy of human melanoma in a mouse xenograft model. Proc Natl Acad Sci U S A *96*, 8161-8166.

Hussong, J.W., Rodgers, G.M., and Shami, P.J. (2000). Evidence of increased angiogenesis in patients with acute myeloid leukemia. Blood *95*, 309-313.

Idusogie, E., Rosen, E., Geng, J.P., Carmeliet, P., Collen, D., and Castellino, F.J. (1996a). Characterization of a cDNA encoding murine coagulation factor VII. Thrombosis and haemostasis *75*, 481-487.

Idusogie, E., Rosen, E.D., Carmeliet, P., Collen, D., and Castellino, F.J. (1996b). Nucleotide structure and characterization of the murine blood coagulation factor VII gene. Thrombosis and haemostasis *76*, 957-964.

Iijima, K., Fukuda, C., and Nakamura, K. (1991). Measurements of tissue factor-like activity in plasma of patients with DIC. Thrombosis research *61*, 29-38.

Kageshita, T., Funasaka, Y., Ichihashi, M., Wakamatsu, K., Ito, S., and Ono, T. (2001). Tissue factor expression and serum level in patients with melanoma does not correlate with disease progression. Pigment Cell Res *14*, 195-200.

Kaido, T., Oe, H., Yoshikawa, A., Mori, A., Arii, S., and Imamura, M. (2005). Tissue factor is a useful prognostic factor of recurrence in hepatocellular carcinoma in 5-year survivors. Hepato-gastroenterology *52*, 1383-1387.

Kakkar, A.K., Lemoine, N.R., Scully, M.F., Tebbutt, S., and Williamson, R.C. (1995a). Tissue factor expression correlates with histological grade in human pancreatic cancer. The British journal of surgery *82*, 1101-1104.

Kakkar, A.K., Lemoine, N.R., Stone, S.R., Altieri, D., and Williamson, R.C. (1995b). Identification of a thrombin receptor with factor Xa receptor and tissue factor in human pancreatic carcinoma cells. Clinical molecular pathology *48*, M288-M290.

Kaushal, V., Mukunyadzi, P., Siegel, E.R., Dennis, R.A., Johnson, D.E., and Kohli, M. (2008). Expression of tissue factor in prostate cancer correlates with malignant phenotype. Appl Immunohistochem Mol Morphol *16*, 1-6.

Keller, T., Salge, U., Konig, H., Dodt, J., Heiden, M., and Seitz, R. (2001). Tissue factor is the only activator of coagulation in cultured human lung cancer cells. Lung cancer (Amsterdam, Netherlands) *31*, 171-179.

Khorana, A.A., Ahrendt, S.A., Ryan, C.K., Francis, C.W., Hruban, R.H., Hu, Y.C., Hostetter, G., Harvey, J., and Taubman, M.B. (2007). Tissue factor expression, angiogenesis, and thrombosis in pancreatic cancer. Clin Cancer Res *13*, 2870-2875.

Khorana, A.A., and Fine, R.L. (2004). Pancreatic cancer and thromboembolic disease. The lancet oncology *5*, 655-663.

Knowles, B.B., Howe, C.C., and Aden, D.P. (1980). Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science (New York, NY *209*, 497-499.

Konigsberg, W.H., and Nemerson, Y. (1988). Molecular cloning of the cDNA for human tissue factor. Cell *52*, 639-640.

Koomagi, R., and Volm, M. (1998). Tissue-factor expression in human non-small-cell lung carcinoma measured by immunohistochemistry: correlation between tissue factor and angiogenesis. International journal of cancer *79*, 19-22.

Korkolopoulou, P., Viniou, N., Kavantzas, N., Patsouris, E., Thymara, I., Pavlopoulos, P.M., Terpos, E., Stamatopoulos, K., Plata, E., Anargyrou, K., *et al.* (2003). Clinicopathologic correlations of bone marrow angiogenesis in chronic myeloid leukemia: a morphometric study. Leukemia *17*, 89-97.

Krikun, G., Hu, Z., Osteen, K., Bruner-Tran, K.L., Schatz, F., Taylor, H.S., Toti, P., Arcuri, F., Konigsberg, W., Garen, A., *et al.* (2010). The immunoconjugate "icon" targets aberrantly expressed endothelial tissue factor causing regression of endometriosis. Am J Pathol *176*, 1050-1056.

Kubota, T., Andoh, K., Sadakata, H., Tanaka, H., and Kobayashi, N. (1991). Tissue factor released from leukemic cells. Thrombosis and haemostasis *65*, 59-63.

Lacal, P.M., Failla, C.M., Pagani, E., Odorisio, T., Schietroma, C., Falcinelli, S., Zambruno, G., and D'Atri, S. (2000). Human melanoma cells secrete and respond to placenta growth factor and vascular endothelial growth factor. J Invest Dermatol *115*, 1000-1007.

Langer, F., Chun, F.K., Amirkhosravi, A., Friedrich, M., Leuenroth, S., Eifrig, B., Bokemeyer, C., and Francis, J.L. (2007). Plasma tissue factor antigen in localized prostate cancer: distribution, clinical significance and correlation with haemostatic activation markers. Thrombosis and haemostasis *97*, 464-470.

Lindahl, A.K., Boffa, M.C., and Abildgaard, U. (1993). Increased plasma thrombomodulin in cancer patients. Thrombosis and haemostasis *69*, 112-114.

Lindahl, A.K., Odegaard, O.R., Sandset, P.M., and Harbitz, T.B. (1992). Coagulation inhibition and activation in pancreatic cancer. Changes during progress of disease. Cancer *70*, 2067-2072.

Litwin, C., Leong, K.G., Zapf, R., Sutherland, H., Naiman, S.C., and Karsan, A. (2002). Role of the microenvironment in promoting angiogenesis in acute myeloid leukemia. Am J Hematol *70*, 22-30.

Lopez-Terrada, D., Cheung, S.W., Finegold, M.J., and Knowles, B.B. (2009). Hep G2 is a hepatoblastoma-derived cell line. Hum Pathol *40*, 1512-1515.

Lu, F., Hu, Z., Sinard, J., Garen, A., and Adelman, R.A. (2009). Factor VII-verteporfin for targeted photodynamic therapy in a rat model of choroidal neovascularization. Invest Ophthalmol Vis Sci *50*, 3890-3896.

Lwaleed, B.A., Francis, J.L., and Chisholm, M. (2000). Urinary tissue factor levels in patients with bladder and prostate cancer. Eur J Surg Oncol *26*, 44-49.

Mayo, G.L., Melendez, R.F., Kumar, N., McKinnon, S.J., and Glickman, R.D. (2003). Antibody-targeted photodynamic therapy. American journal of ophthalmology *136*, 1151-1152.

McDonald, D.M., and Choyke, P.L. (2003). Imaging of angiogenesis: from microscope to clinic. Nature medicine *9*, 713-725.

Merlin, J.L., Gautier, H., Barberi-Heyob, M., Teiten, M.H., and Guillemin, F. (2003). The multidrug resistance modulator SDZ-PSC 833 potentiates the photodynamic activity of chlorin e6 independently of P-glycoprotein in multidrug resistant human breast adenocarcinoma cells. International journal of oncology *22*, 733-739.

Milsom, C., and Rak, J. (2008). Tissue factor and cancer. Pathophysiol Haemost Thromb *36*, 160-176.

Minamiya, Y., Matsuzaki, I., Sageshima, M., Saito, H., Taguchi, K., Nakagawa, T., and Ogawa, J. (2004). Expression of tissue factor mRNA and invasion of blood vessels by tumor cells in non-small cell lung cancer. Surgery today *34*, 1-5.

Morrissey, J.H., Fakhrai, H., and Edgington, T.S. (1987). Molecular cloning of the cDNA for tissue factor, the cellular receptor for the initiation of the coagulation protease cascade. Cell *50*, 129-135.

Nakasaki, T., Wada, H., Mori, Y., Okugawa, Y., Watanabe, R., Nishikawa, M., Gabazza, E.C., Masuya, M., Kageyama, S., Kumeda, K., *et al.* (2000a). Decreased tissue factor and tissue-plasminogen activator antigen in relapsed acute promyelocytic leukemia. Am J Hematol *64*, 145-150.

Nakasaki, T., Wada, H., Shigemori, C., Miki, C., Gabazza, E.C., Nobori, T., Nakamura, S., and Shiku, H. (2002). Expression of tissue factor and vascular endothelial growth factor is associated with angiogenesis in colorectal cancer. Am J Hematol *69*, 247-254.

Nakasaki, T., Wada, H., Watanabe, R., Mori, Y., Gabazza, E.C., Kageyama, S., Nishikawa, M., and Shiku, H. (2000b). Elevated tissue factor levels in leukemic cell homogenate. Clin Appl Thromb Hemost *6*, 14-17.

Nieva, J. (2007). The clinical significance of circulating tissue factor in prostate cancer. Thrombosis and haemostasis *97*, 329-330.

Nitori, N., Ino, Y., Nakanishi, Y., Yamada, T., Honda, K., Yanagihara, K., Kosuge, T., Kanai, Y., Kitajima, M., and Hirohashi, S. (2005). Prognostic significance of tissue factor in pancreatic ductal adenocarcinoma. Clin Cancer Res *11*, 2531-2539.

O'Hara, P.J., Grant, F.J., Haldeman, B.A., Gray, C.L., Insley, M.Y., Hagen, F.S., and Murray, M.J. (1987). Nucleotide sequence of the gene coding for human factor VII, a vitamin K-dependent protein participating in blood coagulation. Proceedings of the National Academy of Sciences of the United States of America *84*, 5158-5162.

Ohta, S., Wada, H., Nakazaki, T., Maeda, Y., Nobori, T., Shiku, H., Nakamura, S., Nagakawa, O., Furuya, Y., and Fuse, H. (2002). Expression of tissue factor is associated with clinical features and angiogenesis in prostate cancer. Anticancer Res *22*, 2991-2996.

Oleinick, N.L., and Evans, H.H. (1998). The photobiology of photodynamic therapy: cellular targets and mechanisms. Radiat Res *150*, S146-156.

Osterud, B. (1997). Tissue factor: a complex biological role. Thrombosis and haemostasis *78*, 755-758.

Padro, T., Ruiz, S., Bieker, R., Burger, H., Steins, M., Kienast, J., Buchner, T., Berdel, W.E., and Mesters, R.M. (2000). Increased angiogenesis in the bone marrow of patients with acute myeloid leukemia. Blood *95*, 2637-2644.

Park, J.G., Lee, J.H., Kang, M.S., Park, K.J., Jeon, Y.M., Lee, H.J., Kwon, H.S., Park, H.S., Yeo, K.S., Lee, K.U., *et al.* (1995). Characterization of cell lines established from human hepatocellular carcinoma. International journal of cancer *62*, 276-282.

Perez-Atayde, A.R., Sallan, S.E., Tedrow, U., Connors, S., Allred, E., and Folkman, J. (1997). Spectrum of tumor angiogenesis in the bone marrow of children with acute lymphoblastic leukemia. Am J Pathol *150*, 815-821.

Poon, R.T., Lau, C.P., Ho, J.W., Yu, W.C., Fan, S.T., and Wong, J. (2003). Tissue factor expression correlates with tumor angiogenesis and invasiveness in human hepatocellular carcinoma. Clin Cancer Res *9*, 5339-5345.

Pule, M.A., Gullmann, C., Dennis, D., McMahon, C., Jeffers, M., and Smith, O.P. (2002). Increased angiogenesis in bone marrow of children with acute lymphoblastic leukaemia has no prognostic significance. Br J Haematol *118*, 991-998.

Rak, J., Milsom, C., May, L., Klement, P., and Yu, J. (2006). Tissue factor in cancer and angiogenesis: the molecular link between genetic tumor progression, tumor neovascularization, and cancer coagulopathy. Semin Thromb Hemost *32*, 54-70.

Rak, J., Milsom, C., and Yu, J. (2008). Tissue factor in cancer. Curr Opin Hematol *15*, 522-528.

Rao, L.V., and Pendurthi, U.R. (1998). Tissue factor on cells. Blood Coagul Fibrinolysis *9 Suppl 1*, S27-35.

Rauch, U., Antoniak, S., Boots, M., Schulze, K., Goldin-Lang, P., Stein, H., Schultheiss, H.P., and Coupland, S.E. (2005). Association of tissue-factor upregulation in squamous-cell carcinoma of the lung with increased tissue factor in circulating blood. The lancet oncology *6*, 254.

Rickles, F.R., and Brenner, B. (2008). Tissue factor and cancer. Semin Thromb Hemost *34*, 143-145.

Rickles, F.R., Hair, G.A., Zeff, R.A., Lee, E., and Bona, R.D. (1995). Tissue factor expression in human leukocytes and tumor cells. Thrombosis and haemostasis *74*, 391-395.

Rickles, F.R., Patierno, S., and Fernandez, P.M. (2003). Tissue factor, thrombin, and cancer. Chest *124*, 58S-68S.

Romanque, P., Piguet, A.C., and Dufour, J.F. (2008). Targeting vessels to treat hepatocellular carcinoma. Clin Sci (Lond) *114*, 467-477.

Rouhi, P., Lee, S.L., Cao, Z., Hedlund, E.M., Jensen, L.D., and Cao, Y. (2010). Pathological angiogenesis facilitates tumor cell dissemination and metastasis. Cell Cycle *9*, 913-917.

Ruf, W., and Mueller, B.M. (1996). Tissue factor in cancer angiogenesis and metastasis. Curr Opin Hematol *3*, 379-384.

Ruf, W., Yokota, N., and Schaffner, F. (2010). Tissue factor in cancer progression and angiogenesis. Thrombosis research *125 Suppl 2*, S36-38.

Salge, U., Seitz, R., Wimmel, A., Schuermann, M., Daubner, E., and Heiden, M. (2001). Transition from suspension to adherent growth is accompanied by tissue factor expression and matrix metalloproteinase secretion in a small cell lung cancer cell line. Journal of cancer research and clinical oncology *127*, 139-141.

Sawada, M., Miyake, S., Ohdama, S., Matsubara, O., Masuda, S., Yakumaru, K., and Yoshizawa, Y. (1999). Expression of tissue factor in non-small-cell lung cancers and its relationship to metastasis. British journal of cancer *79*, 472-477.

Schabbauer, G., Schweighofer, B., Mechtcheriakova, D., Lucerna, M., Binder, B.R., and Hofer, E. (2007). Nuclear factor of activated T cells and early growth response-1 cooperate to mediate tissue factor gene induction by vascular endothelial growth factor in endothelial cells. Thrombosis and haemostasis *97*, 988-997.

Semeraro, N., and Colucci, M. (1997). Tissue factor in health and disease. Thrombosis and haemostasis *78*, 759-764.

Sharman, W.M., van Lier, J.E., and Allen, C.M. (2004). Targeted photodynamic therapy via receptor mediated delivery systems. Advanced drug delivery reviews *56*, 53-76.

Shen, B.Q., Lee, D.Y., Cortopassi, K.M., Damico, L.A., and Zioncheck, T.F. (2001). Vascular endothelial growth factor KDR receptor signaling potentiates tumor necrosis factor-induced tissue factor expression in endothelial cells. The Journal of biological chemistry *276*, 5281-5286.

Shigemori, C., Wada, H., Matsumoto, K., Shiku, H., Nakamura, S., and Suzuki, H. (1998). Tissue factor expression and metastatic potential of colorectal cancer. Thrombosis and haemostasis *80*, 894-898.

Shoji, M., Hancock, W.W., Abe, K., Micko, C., Casper, K.A., Baine, R.M., Wilcox, J.N., Danave, I., Dillehay, D.L., Matthews, E., *et al.* (1998). Activation of coagulation and angiogenesis in cancer: immunohistochemical localization in situ of clotting proteins and vascular endothelial growth factor in human cancer. Am J Pathol *152*, 399-411.

Shoji, M., Sun, A., Kisiel, W., Lu, Y.J., Shim, H., McCarey, B.E., Nichols, C., Parker, E.T., Pohl, J., Mosley, C.A., *et al.* (2008). Targeting tissue factor-expressing tumor angiogenesis and tumors with EF24 conjugated to factor VIIa. J Drug Target *16*, 185-197.

Silberberg, J.M., Gordon, S., and Zucker, S. (1989). Identification of tissue factor in two human pancreatic cancer cell lines. Cancer research *49*, 5443-5447.

Spicer, E.K., Horton, R., Bloem, L., Bach, R., Williams, K.R., Guha, A., Kraus, J., Lin, T.C., Nemerson, Y., and Konigsberg, W.H. (1987). Isolation of cDNA clones coding for human tissue factor: primary structure of the protein and cDNA. Proceedings of the National Academy of Sciences of the United States of America *84*, 5148-5152.

Sturm, U., Luther, T., Albrecht, S., Flossel, C., Grossmann, H., and Muller, M. (1992). Immunohistological detection of tissue factor in normal and abnormal human mammary glands using monoclonal antibodies. Virchows Arch A Pathol Anat Histopathol *421*, 79-86.

Taber, S.W., Fingar, V.H., and Wieman, T.J. (1998). Photodynamic therapy for palliation of chest wall recurrence in patients with breast cancer. J Surg Oncol *68*, 209-214.

Takano, S., Tsuboi, K., Tomono, Y., Mitsui, Y., and Nose, T. (2000). Tissue factor, osteopontin, alphavbeta3 integrin expression in microvasculature of gliomas associated with vascular endothelial growth factor expression. British journal of cancer *82*, 1967-1973.

Tanaka, H., Narahara, N., Kurabayashi, H., Sadakata, H., Andoh, K., Uchiyama, T., Kobayashi, N., and Maekawa, T. (1989). Studies on leukemic cell tissue factor. Thrombosis research *53*, 535-549.

Tanaka, M. (1989). Induction of tissue factor-like activity of human monoblastic leukemia cell line by tumor necrosis factor-alpha. Thrombosis research *56*, 201-211.

Tanaka, M., and Kishi, T. (1990). Induction of tissue factor by interleukin-2 in acute myelogenous leukemia (AML) cells. Growth Factors *4*, 1-8.

Tanaka, M., and Yamanishi, H. (1993). The expression of tissue factor antigen and activity on the surface of leukemic cells. Leuk Res *17*, 103-111.

Tang, Y., Borgstrom, P., Maynard, J., Koziol, J., Hu, Z., Garen, A., and Deisseroth, A. (2007). Mapping of angiogenic markers for targeting of vectors to tumor vascular endothelial cells. Cancer Gene Ther *14*, 346-353.

Taniguchi, T., Kakkar, A.K., Tuddenham, E.G., Williamson, R.C., and Lemoine, N.R. (1998). Enhanced expression of urokinase receptor induced through the tissue factor-factor VIIa pathway in human pancreatic cancer. Cancer research *58*, 4461-4467.

Tesselaar, M.E., Romijn, F.P., Van Der Linden, I.K., Prins, F.A., Bertina, R.M., and Osanto, S. (2007). Microparticle-associated tissue factor activity: a link between cancer and thrombosis? J Thromb Haemost *5*, 520-527.

Tezel, T.H., Bodek, E., Sonmez, K., Kaliappan, S., Kaplan, H.J., Hu, Z., and Garen, A. (2007). Targeting tissue factor for immunotherapy of choroidal neovascularization by intravitreal delivery of factor VII-Fc chimeric antibody. Ocul Immunol Inflamm *15*, 3-10.

Thomas, D.A., Giles, F.J., Cortes, J., Albitar, M., and Kantarjian, H.M. (2001). Antiangiogenic therapy in leukemia. Acta Haematol *106*, 190-207.

Ueda, C., Hirohata, Y., Kihara, Y., Nakamura, H., Abe, S., Akahane, K., Okamoto, K., Itoh, H., and Otsuki, M. (2001). Pancreatic cancer complicated by disseminated intravascular coagulation associated with production of tissue factor. Journal of gastroenterology *36*, 848-850.

Ueno, T., Toi, M., Koike, M., Nakamura, S., and Tominaga, T. (2000). Tissue factor expression in breast cancer tissues: its correlation with prognosis and plasma concentration. British journal of cancer *83*, 164-170.

Uno, K., Homma, S., Satoh, T., Nakanishi, K., Abe, D., Matsumoto, K., Oki, A., Tsunoda, H., Yamaguchi, I., Nagasawa, T., *et al.* (2007). Tissue factor expression as a possible determinant of thromboembolism in ovarian cancer. British journal of cancer *96*, 290-295.

Wang, B., Berger, M., Masters, G., Albone, E., Yang, Q., Sheedy, J., Kirksey, Y., Grimm, L., Wang, B., Singleton, J., *et al.* (2005). Radiotherapy of human xenograft NSCLC tumors in nude mice with a 90Y-labeled anti-tissue factor antibody. Cancer biotherapy & radiopharmaceuticals *20*, 300-309.

Watanabe, Y., Lee, S.W., Detmar, M., Ajioka, I., and Dvorak, H.F. (1997). Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) delays and induces escape from senescence in human dermal microvascular endothelial cells. Oncogene *14*, 2025-2032.

Weidner, N., Semple, J.P., Welch, W.R., and Folkman, J. (1991). Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med *324*, 1-8.

Yang, R., and Han, Z.C. (2002). Angiogenesis in hematologic malignancies and its clinical implications. Int J Hematol *75*, 246-256.

Yokota, N., Koizume, S., Miyagi, E., Hirahara, F., Nakamura, Y., Kikuchi, K., Ruf, W., Sakuma, Y., Tsuchiya, E., and Miyagi, Y. (2009). Self-production of tissue factor-coagulation factor VII complex by ovarian cancer cells. Br J Cancer *101*, 2023-2029.

Zacharski, L.R., Schned, A.R., and Sorenson, G.D. (1983). Occurrence of fibrin and tissue factor antigen in human small cell carcinoma of the lung. Cancer research *43*, 3963-3968.

Zetter, B.R. (1998). Angiogenesis and tumor metastasis. Annu Rev Med *49*, 407-424.

Zucker, S., Mirza, H., Conner, C.E., Lorenz, A.F., Drews, M.H., Bahou, W.F., and Jesty, J. (1998). Vascular endothelial growth factor induces tissue factor and matrix metalloproteinase production in endothelial cells: conversion of prothrombin to thrombin results in progelatinase A activation and cell proliferation. International journal of cancer *75*, 780-786.

10

Ectopic Synthesis of Coagulation Factor VII in Breast Cancer Cells: Mechanisms, Functional Correlates, and Potential for a New Therapeutic Target

Shiro Koizume and Yohei Miyagi
Molecular Pathology & Genetics Division,
Kanagawa Cancer Center Research Institute,
Japan

1. Introduction

Recent advances in the development of therapeutic strategies have enabled the cure of a considerable amount of cases of breast cancer patients. However, breast cancer is a worldwide problem since this disease remains a common cause of cancer death in women throughout the world (Álvarez 2010). Although breast cancer can be regulated by chemotherapy, there are still difficulties with treating recurrence and triple-negative breast cancer without therapeutic molecular targets (HER2 and hormone receptors) (Foulkes et al. 2010). Therefore, it is necessary to increase the knowledge of breast cancer biology and investigate target molecules to facilitate therapeutic strategy toward aggressive breast cancers.

Hypercoagulation is a common complication of cancer patients and also correlates with mortality (Ten Cate & Falanga 2007). In fact, it has been reported that the risk of venous thromboembolism (VTE) is highest for cancers of the ovary, pancreas, liver (Iodice et al., 2008), and breast during chemotherapy (Kirwan et al. 2008).

Blood coagulation factor VII (fVII) is a key enzyme of the extrinsic coagulation cascade that is produced predominantly by hepatocytes (Furie & Furie 1988). Tissue factor (TF) is a 47-kD transmembrane glycoprotein and a cellular receptor of fVII. Blood coagulation factor VII from blood plasma bound to TF is converted to its active form (fVIIa) and activates a downstream extrinsic coagulation cascade, leading to fibrin deposition (Fig. 1).

It has been reported that plasma TF levels are higher in cancer patients including advanced breast cancer (Ueno et al. 2000). Furthermore, breast cancer cells secrete cell membrane-derived microvesicles containing TF antigen under pathological conditions, resulting in coagulation activation (Davila et al. 2008). Therefore, TF-fVIIa formation can be a major cause of thromboembolic disease.

A number of studies have demonstrated that tissue factor-fVIIa complex formation on the cell surface also initiates key pathogenic events including activation of cell motility, invasiveness, cell survival, and angiogenesis (Milsom & Rak 2009). Recently, growing experimental evidence has also suggested that TF contributes to tumor initiation (Milsom & Rak 2009). Given that fVII presents on the invasive edge of various cancer tissues (Fischer et al. 1999;

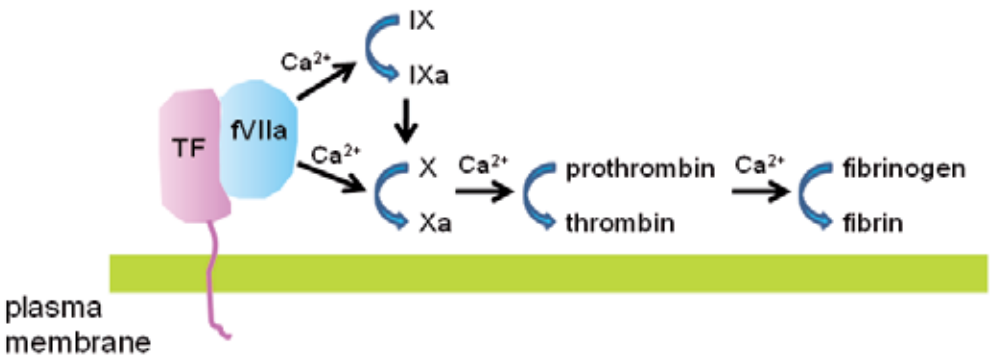


Fig. 1. Extrinsic coagulation cascade initiated by TF-fVIIa complex formation.
TF-fVIIa complex formation on the cell surface triggers an extrinsic coagulation cascade. TF-fVIIa initiates coagulation by activating factor X, resulting in fibrin deposition *via* formation of thrombin. The mechanism of mediation *via* factor IX activation is also possible.

Zacharski et al. 1993), this suggests that TF-fVIIa complex formation may play a critical role in malignant phenotype expression of clinical tumors. Therefore, anti-TF strategy may be applicable to breast cancer although a probable side effect of bleeding should be considered. Blood plasma has been considered to be a predominant source of fVII present on cancer cell surfaces since most plasma proteins including fVII are produced in the liver. Blood coagulation factor VII is believed to penetrate through hyperpermeabilized blood vessels around tumor tissues (McDonald & Baluk 2002), and then associate with the integral membrane protein TF of cancer cells. However, various cancer cells can ectopically synthesize fVII (Koizume et al. 2006). Notably, breast cancer cells were found to constitutively and highly express fVII mRNA by RT-PCR analysis. Further, functional fVII exists as a complex with TF on the cell surface (Koizume et al. 2006). Unsurprisingly, fVII mRNA expression is frequently found in surgical specimens of breast cancer (Koizume et al. 2009), suggesting that fVII is synthesized in cancer tissues of patients and that ectopic fVII synthesis may play an important role in the biology of breast cancer. Therefore, inhibition of ectopic fVII synthesis without affecting fVII secreted from the liver may be a useful therapeutic approach for breast cancer patients. In this chapter, recent progress in breast cancer biology associated with TF-fVIIa signaling and in the biology of ectopic synthesis of fVII in breast cancer cells will be summarized. In addition to descriptions based on previously published data, new data mainly concerning cell growth accelerating characteristics of ectopic fVII in breast cancer cells will be presented. Finally, potential future therapeutic strategies of breast cancer targeting ectopic fVII will be discussed. Recently a target molecule specifically associated with the ectopically active *FVII* gene promoter but not with the *FVII* promoter in hepatocytes (Koizume et al. 2009) was found. Therefore, ectopic fVII synthesis can be selectively blocked without disturbing the normal haemostatic process.

2. Biology of TF-fVIIa signaling in breast cancer cells

Considerable progress has been made towards understanding of how cancer cells depend on TF-fVIIa complex formation to express their malignant phenotypes (Milsom & Rak 2009;

Mackman & Taubman 2009; Schaffner & Ruf 2009). To date, a number of studies concerning TF-fVIIa signaling have been performed using breast cancer cell lines such as MDA-MB-231, MCF-7, and BT549, possibly because they express relatively high levels of TF, and their phenotypes such as motility and invasiveness are TF-dependent (Jiang et al. 2008; Morris et al. 2006). Among these cell lines, MDA-MB-231 synthesizes a considerable amount of TF and is a frequently used cell line as a good TF-dependent breast cancer model. Its characteristics such as motility, invasiveness, and growth are highly TF-dependent in response to fVII *in vitro* (Hjortoe et al. 2004; Jiang et al. 2004; Morris et al. 2006) and *in vivo* (Versteeg et al. 2008a & b).

TF-fVIIa signaling mechanisms potentially resulting in breast cancer phenotypes can be classified into three categories. The first is signaling mediated by TF-fVIIa binary complex formation. Various experimental evidence has suggested that activation of a G protein-coupled receptor, protease activated receptor 2 (PAR2) rather than PAR1 by the TF-fVIIa complex plays a crucial role in phenotypic expression of breast cancer cells through activation of the mitogen-activated protein kinase cascade (Hjortoe et al. 2004; Morris et al. 2006; Versteeg et al. 2008a). Second, TF-fVIIa signaling could involve coagulation factor X (fX) to transmit signals. The TF-fVIIa complex may further interact with fX to produce the TF-fVIIa-fXa ternary complex (Jiang et al. 2004). Breast cancer phenotypes mediated *via* TF-fVIIa-fXa complex formation may not only cause PAR2 signaling but also activate PAR1 to initiate thrombin signaling. This ternary complex may also activate the mTOR pathway in breast cancer cells (Jiang et al. 2008). Third, the G protein–independent pathway of PAR2 signaling is also possible in breast cancer cells (Schaffner et al. 2010). PAR2 recruits a scaffold protein, β-arrestin, to support extracellular signal-regulated kinase signaling to enhance cell motility. In any case, TF-fVIIa signaling may eventually enhance expression of downstream effectors, such as IL-8 (Hjortoe et al. 2004), Cyr61 (Pendurthi et al. 2000), CTGF (Pendurthi et al. 2000), VEGF (Liu & Mueller 2006), CXCL1 (Albrektsen et al. 2007; Versteeg et al. 2008b), Birc3 (Albrektsen et al. 2007), CUX1 (Wilson et al. 2009), and CSF (Albrektsen et al. 2007), which then contribute to malignant phenotype expression of breast cancers.

While phenotypes regulated by TF-fVIIa formation seem dependent on PAR2 signaling, breast cancer cells could augment invasiveness and tumorigenesis by PAR1 signaling (Booden et al. 2004). PAR1 is a thrombin receptor that is highly expressed in invasive breast cancer cells. Treatment of breast cancer cells with thrombin increases cellular invasion in a PAR1-dependent manner. On the other hand, PAR1 can be a receptor of matrix metalloprotease-1 secreted from stromal cells and it enhances invasiveness and tumorigenesis of MDA-MB-231 cells (Boire et al. 2005). Therefore, it is likely that the relative importance of PAR1 and PAR2 signaling in breast cancer progression is dependent on the tumor microenvironment.

Characteristics of breast cancer cells might be influenced by exogenous TF in patients. Recent *in vitro* experiments revealed that growth and metastatic properties of breast cancer cells can also be affected by TF in a paracrine manner (Collier et al. 2008). Treatment with recombinant TF mimicking stroma-derived TF enables breast cancer cells to be more invasive and proliferative *via* activation of β1-integrins and/or PAR2 signaling cascades, followed by down-regulation of estrogen receptor gene expression.

3. Expression of TF in breast cancer tissues

3.1 Expression of TF activity in breast cancer tissues

Immunohistochemical analyses have revealed that breast cancer tissues as well as ovarian and pancreatic cancer tissues highly express TF (Contrino et al. 1996; Ueno et al. 2000).

Although the mechanisms of this overexpression have not been clearly defined, transcriptional up-regulation appears to be a major cause of this aberrant expression. Mechanisms of transcriptional regulation of the TF gene (*F3*) have been well characterized (Mackman 1995). Basal *F3* gene expression is known to be regulated by various transcription factors such as Sp1, AP-1, and NFκB. Given that AP-1 and NFκB are major factors activated in breast cancer (Benz & Yau 2008), it is likely that aberrant activation of these factors is associated with higher TF levels. Indeed, it has been reported that the *F3* gene promoter is highly occupied by these transcription factors in MDA-MB-231 cells, whereas the promoter is weakly bound in TF low-expressing MCF-7 cells (Zhou et al. 1998).

Inducible expression of the *F3* gene is another plausible cause of high TF expression in cancer tissues. Expression of TF is known to be affected by various environmental stimuli such as exposure to cytokines, growth factors, and hypoxia (Milsom & Rak 2009), leading to activation of AP-1 and NFκB. Therefore, expression levels of TF can be varied depending on the tumor microenvironment. Besides environmental factors, expression of TF is also affected by oncogenic events such as activation of ras, p53, PTEN, and EGFR (Milsom & Rak 2009). Expression of the *F3* gene can be up-regulated through signaling cascade downstream of these effectors.

The transcription factor Egr-1 is also known to play a major role in inducible TF expression (Mackman 1995). Briefly, Sp1 mainly contributes to basal up-regulation of *F3* transcription. However, once cells are stimulated with various factors associated with the cellular microenvironment, expression of Egr-1 is induced in cancer cells. Since Egr-1 shares DNA binding sites with Sp1, it could thus substitute Sp1 binding within the promoter region to enhance TF transcription under pathological conditions.

Post transcriptional regulation may also determine TF expression or activity level in breast cancer cells. A major mechanism is inhibition by tissue factor pathway inhibitor (TFPI), directly inhibiting serine protease activity of TF-fVIIa (Milsom & Rak 2009). Indeed, it was recently reported that breast cancer tissues highly express TFPI (Sierko et al. 2010). In addition, TFPI may augment cell adhesion (Fischer et al. 1999) and invasiveness (Sierko et al. 2010) through interaction with the TF-fVIIa complex. Another post-transcriptional mechanism is functional modification by conformational change. Procoagulant activity of TF can be regulated by conformational change called encryption/decryption, possibly mediated *via* multiple mechanisms (Bach 2006). However, recent studies using MDA-MB-231 cell suggested that de-encryption of TF is caused *via* interaction with anionic phospholipids rather than intra-molecular disulfide exchange (Pendurthi et al. 2007). Only decrypted TF can exert its procoagulant activity, while encrypted TF still has the ability to transmit signals. It was recently found that a microRNA (miR-19) could bind to 3′-UTR of the TF transcript to repress translation in breast cancer cells (Zhang et al. 2010). This study showed that miR-19 is abundant in a TF low-expressing breast cancer cell line, MCF-7, suggesting that regulation by this microRNA may be a determinant of TF levels in breast cancer tissues.

In addition to cell surface expression, breast cancer cells can secrete TF as microparticles derived from cell membrane fragments in response to various stimuli *in vitro* and *in vivo* (Tilley et al. 2008; Davila et al. 2008); therefore, this circulating TF may increase the risk of thrombosis in patients. Further, besides expression in tumor parenchyma cells, tumor stroma produces TF to promote breast cancer metastasis (Vrana et al. 1996). Growth factors secreted from breast cancer cells may stimulate stromal cells to produce TF, resulting in breast cancer progression.

3.2 Relationship between TF expression and clinical outcome of breast cancer patients

Activation of platelets (Kirwan et al. 2008) and elevation of plasma TF levels (Davila et al. 2008) are candidate determinants for the development of thrombosis in cancer patients. Indeed, VTE post chemotherapy is relatively frequent for breast cancer patients, and therefore, the relationship between haemostatic markers and thrombosis was investigated (Kirwan et al. 2008). This study showed that plasma TF levels of patients were significantly higher than those of non-cancer controls, but they were not elevated in response to chemotherapy, suggesting that chemotherapy-induced VTE is due to TF-independent mechanisms.

On the other hand, analysis with clinical samples revealed that TF is highly expressed in breast cancer patients. It has been reported that TF is expressed in vascular endothelial cells in invasive breast cancer tissues, suggesting that TF may be a marker for angiogenic phenotypes of breast cancer (Contrino et al. 1996). As well as tissue expression including stromal, vascular endothelial, and monocytic expression, TF levels are elevated in plasma of breast cancer patients. Although it was found that there was no difference in plasma TF levels between normal and benign tumors, primary and recurrent cancer patients showed significantly higher TF levels (Ueno et al. 2000). These expression patterns of TF are correlated with those in tissue samples. These TF levels are associated with tumor grade and can be an independent prognostic indicator. Further, TF levels in urine of breast cancer patients also correlate with disease malignancy (Lwaleed et al. 1999), suggesting that measurement of TF expression has some clinical advantages.

Although molecular mechanisms linking TF levels and cancer phenotypes can be explained by multiple cellular events downstream of TF-fVIIa signaling pathways, recent immunohistochemical analyses have revealed that phosphorylation of the cytoplasmic domain of TF correlates with expression levels of TF and other proteins known to be associated with recurrence and aggressive phenotypes of breast cancers (Rydén et al. 2010). This study showed that TF phosphorylation associated with PAR2 expression correlates with recurrence in breast cancer patients. A recent *in vivo* study by the same group showed that the cytoplasmic domain of TF cooperates with PAR2 to promote breast cancer development through modulating the angiogenic response (Schaffner et al. 2010), providing a rationale for the observed clinical association.

4. Ectopic expression of coagulation factor VII in breast cancer cells

Although the major source of fVII is the liver under normal conditions, various cancer cells could ectopically synthesize fVII (Koizume et al. 2006). Notably, breast cancer cell lines, such as YMB-1, MDA-MB-453, and MCF-7, constitutively and highly express fVII mRNA as shown by RT-PCR analysis (Koizume et al. 2006). Recently, we additionally found by real-time RT-PCR analysis that a breast cancer cell line, T47D, highly expresses fVII, and its mRNA level is comparable with that of YMB-1 (data not shown). Cancer cells that express fVII exhibit fXa-generating activity depending on TF-fVIIa complex formation, suggesting that ectopically synthesized fVII is functional and may play an important role in the progression of breast cancer. As mentioned above, fVII expression is frequent in surgical specimens of breast cancer by RT-PCR and immunohistochemical analyses (Koizume et al. 2009).

4.1 Mechanism of constitutive fVII synthesis in breast cancer cells

Transcript levels of fVII are higher in breast cancer cells, and therefore, it was examined whether the *FVII* gene is activated in breast cancer cells. It is known that binding of a transcription factor, HNF-4 is crucial for *FVII* gene activation in hepatocytes. However, HNF-4 is not responsible for *FVII* expression in breast cancer cells as shown by immunoblotting and chromatin immunoprecipitation (ChIP) analyses (Koizume et al. 2009). Reporter gene analysis showed that the authentic *FVII* promoter region could fully activate reporter activity in breast cancer cells. As expected, deletion of the HNF-4 binding site from the *FVII* promoter construct showed that reporter activity is not significantly impaired, further supporting the idea that HNF-4 is dispensable for ectopic *FVII* expression. Further, analysis with a reporter construct without a Sp1 transcription factor binding site showed a marked decrease in promoter activity in breast cancer cells (Koizume et al. 2009). These results indicate that Sp1 binding to the promoter region is essential for constitutive *FVII* activation in breast cancer cells.

Histone acetylation within the gene promoter region is a crucial step for transcriptional activation, and therefore, epigenetic factors responsible for ectopic *FVII* expression were further explored (Koizume et al. 2009). Histone acetyltransferases (HATs) associated with the *FVII* promoter in breast cancer cells were examined. ChIP analysis revealed that major HATs, p300 and CBP are associated with the *FVII* promoter region in breast cancer cells. On the other hand, other HATs, PCAF and SRC-1, can also interact with the hepatocytic *FVII* promoter. Notably, the *FVII* promoter in HepG2 cells is devoid of p300 and CBP. ChIP analysis showed that histone H4 is hyperacetylated within the active *FVII* promoter in various cells, suggesting that p300 and CBP may be responsible for histone acetylation within the *FVII* promoter region in breast cancer cells, although various HATs can contribute to the acetylation in hepatocytes.

fVII production in hepatocellular carcinoma and breast cancer cells was detected by western blotting and colorimetric analysis (Koizume et al. 2009). Unlike hepatocytes, fVII synthesized in YMB-1 cells is not secreted into culture media, suggesting that ectopically synthesized fVII is involved in TF-fVIIa complex formation on the cell surface in an autocrine manner.

4.2 Induction of fVII expression in breast cancer cells under hypoxic conditions

FVII transcription is inducible under hypoxia and hypoxia mimetic ($CoCl_2$ treatment) conditions depending on the cell type (Koizume et al. 2006). To date, several cell lines have been found to express the fVII transcript in response to hypoxia. As ovarian cancer cells tend to express fVII in response to hypoxia (Koizume et al. 2006; Yokota et al. 2009), it was next tested whether breast cancer cell lines inducibly express fVII mRNA under hypoxic conditions. It was revealed that cell lines with high fVII expression, such as YMB-1 and MDA-MB-453, do not enhance fVII transcript levels in response to hypoxic stimuli (Koizume et al. 2009). However, we recently found that fVII mRNA is inducible in MDA-MB-468 cells under $CoCl_2$ treatment conditions (Fig. 2A), suggesting that induction of fVII in breast cancer cells is cell-type dependent.

Detailed mechanisms of this hypoxic induction have not been defined yet. However, it is likely that a hypoxia inducible factor, HIF-2α, may contribute to the activation. Chromatin immunoprecipitation analysis revealed that although both HIF-1α and HIF-2α are inducible under hypoxia-mimetic conditions (Fig. 2B), HIF-2α predominantly binds to the *FVII* promoter region in MDA-MB-468 cells (Fig. 2C), as in the case of OVSAYO cells (Koizume et al. 2006).

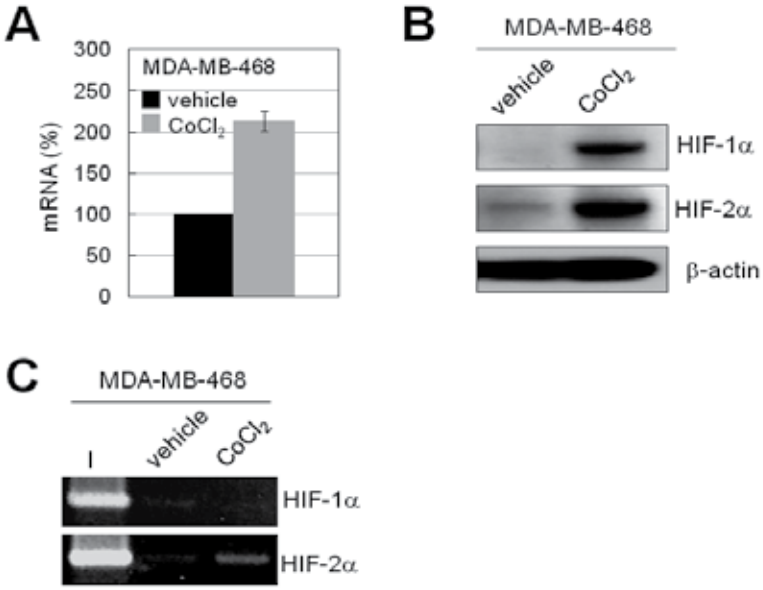


Fig. 2. Induction of the fVII transcript in MDA-MB-468 cells.
(A) *FVII* activation in MDA-MB-468 cells treated with $CoCl_2$. Cells were cultured for 4 hours in the presence or absence of 500 μM $CoCl_2$, and then fVII mRNA levels were analyzed by real-time RT-PCR analysis. *Columns*, mean (n = 2); *bars*, SD. (B) Western blotting analysis of HIF expression in MDA-MB-468 cells cultured with or without 500 μM $CoCl_2$ for 4 hours. (C) Chromatin immunoprecipitation (ChIP) analysis of HIFs binding to the *FVII* promoter in MDA-MB-468 cells. Cells were cultured in the presence or absence of 500 μM $CoCl_2$ for 4 hours, and then subjected to ChIP analysis. I indicates input sonicated DNA fragments without immunoprecipitation.

Notably, unlike typical transcriptional activation mechanisms by HIFs (Majmundar et al. 2010), the promoter region bound by HIF-2α is devoid of the hypoxia responsible element (Koizume et al. 2006), and therefore, novel mechanisms of transcriptional regulation may contribute to *FVII* activation under hypoxia.

5. Ectopically expressed fVII can enhance proliferation of breast cancer cells

As in the case of exogenous fVII treatment of cancer cells, ectopically expressed fVII augments motility and invasiveness of breast cancer cells, MDA-MB-453, as well as ovarian cancer cells, OVSAYO (Koizume et al. 2006). Growth and development of some breast tumors are dependent on TF-fVIIa formation, followed by activation of PAR2 signaling (Versteeg et al. 2008a & b). This is characteristic of *in vivo* conditions since TF expression does not contribute to cell growth in monolayer culture (Yu et al. 2005; Versteeg et al. 2008a). These results may simply be due to the absence of fVII in culture media and/or reflect the importance of host components of the tumor microenvironment (Jessani et al. 2004). Therefore, we tested whether proliferation of breast cancer cells *in vitro* is affected by ectopic fVII synthesis. The cell lines YMB-1 and MDA-MB-453 were selected for this purpose since these cells highly express fVII mRNA (Koizume et al. 2006).

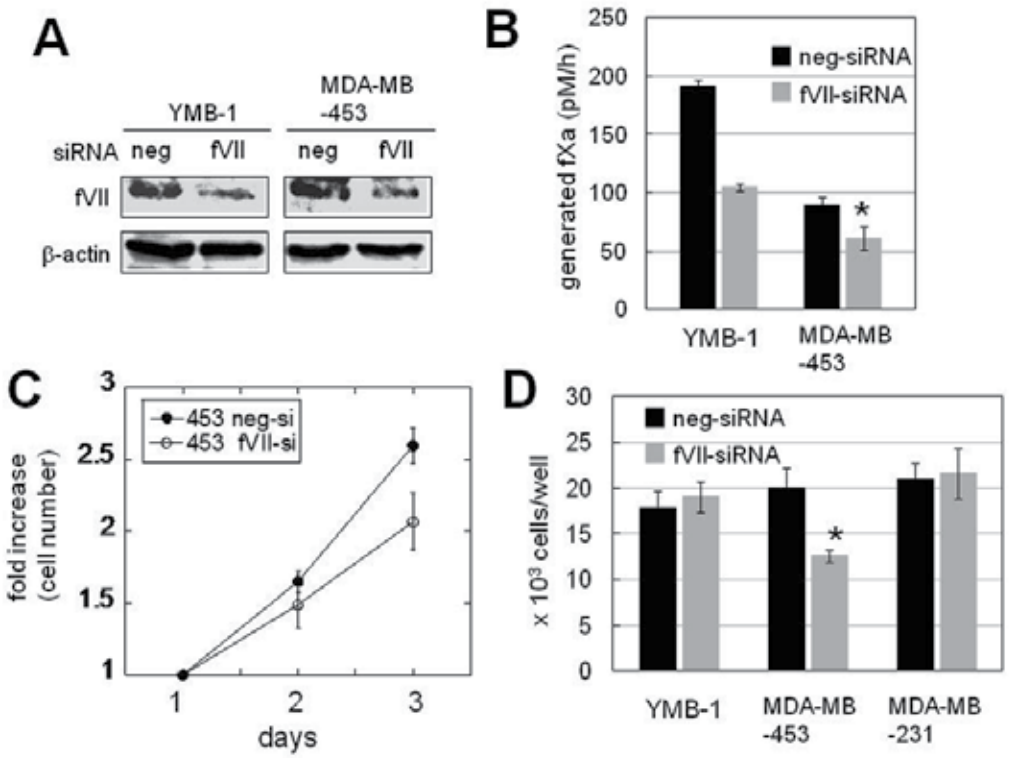


Fig. 3. Blood coagulation factor VII contributes to cell proliferation of MDA-MB-453 cells. (A) Knockdown of fVII expressed in YMB-1 and MDA-MB-453 cells. Cells were transfected with negative control- or fVII-siRNA. After 43 hours of transfection, whole cell lysates were analyzed by immunoblotting. (B) fXa generation analysis of breast cancer cells transfected with siRNA of fVII. Forty-three hours post transfection, cells were examined for fXa generation activity. *Columns*, mean (n = 3); *bars*, SD. **P* < 0.05 (two-sided Student's *t* test). (C, D) Cell proliferation analysis of fVII-expressing (YMB-1 and MDA-MB-453) and non-expressing (MDA-MB-231) breast cancer cells after transfection of negative control- or fVII-siRNA. *Columns*, mean (n = 3); *bars*, SD. **P* < 0.05 (two-sided Student's *t* test).

Expression of fVII in YMB-1 and MDA-MB-453 cells is suppressed by small interference RNA (siRNA) transfection, resulting in reduction of protein levels (Fig. 3A) and cell surface procoagulant activity (Fig. 3B). After 24 hours post transfection, monitoring of cell growth was obtained by colorimetric assay. Growth of MDA-MB-453 cells was considerably diminished by fVII suppression (Fig. 3C and D). On the other hand, proliferation of YMB-1 cells was not affected after 3 days post transfection (Fig. 3D). As expected, proliferation of MDA-MB-231 cells without fVII expression was not affected by siRNA transfection (Fig. 3D), suggesting that fVII can enhance breast cancer cell proliferation, but the effect is cell-type dependent. Western blotting of hormone receptors (Koizume et al. 2009) and fluorescent *in situ* hybridization analysis of *ERBB2* amplification showed that YMB-1 cells correspond to the "triple negative" molecular subtype, although MDA-MB-453 cells are HER-2 positive (data not shown).

To test whether triple-negative breast cancer cells are unresponsive to ectopically synthesized fVII to activate cell proliferation, we next tested the effect of forced fVII expression on proliferation of other triple-negative breast cancer cells.

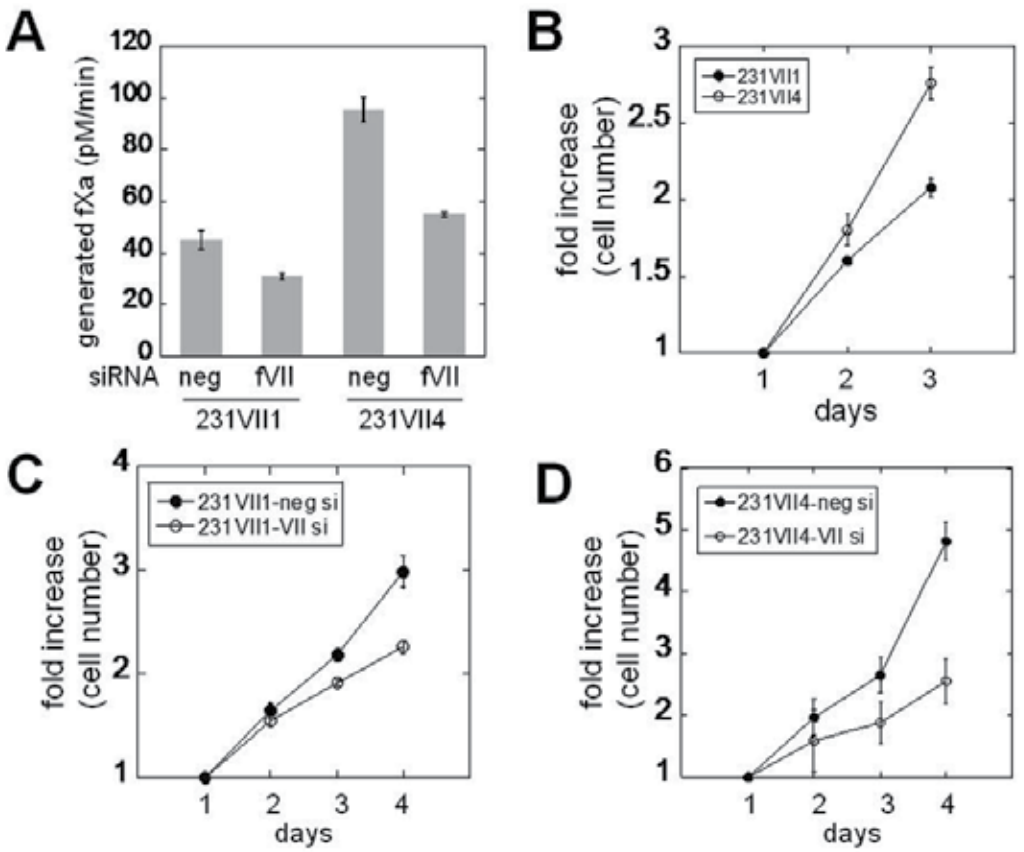


Fig. 4. Effect of fVII overexpression on proliferation of MDA-MB-231 cells.
(A) Procoagulant activity of fVII in MDA-MB-231 cells transfected with fVII. Cloned cells (231VII1 and 231 VII4) constitutively overexpressing fVII were transfected with negative control- or fVII-siRNA and then, examined for fXa generation activity. *Columns*, mean (n = 2); *bars*, SD. (B) Cell proliferation analysis of fVII-expressing cells with different procoagulant activity shown in (A). *Columns*, mean (n = 3); *bars*, SD. (C) Effect of fVII suppression on cell proliferation of 231VII1 cells. After 24 hours of fVII- or negative control-siRNA transfection, cells were examined for proliferation activity. *Columns*, mean (n = 3); *bars*, SD. (D) Effect of fVII suppression on cell proliferation of 231VII4 cells. After 24 hours of fVII- or negative control- siRNA transfection, cells were examined for proliferation activity. *Columns*, mean (n = 3); *bars*, SD.

MDA-MB-231 cell was used for experiments since this cell line without fVII expression highly expresses TF and its growth *in vivo* is dependent on TF-fVIIa signaling (Versteeg et al. 2008a & b). Cell clones stably expressing fVII were prepared and fVII expression levels

were analyzed by western blotting (data not shown) and fXa generation assay (Fig. 4A). Two cell clones with variation in fVII expression levels (Fig. 4A) were selected. Cell proliferation assay with these cells showed that the higher fVII is expressed, the faster breast cancer cells proliferate (Fig. 4B). Suppression of fVII synthesis by RNAi restored cell proliferation efficiency (Fig. 4C & D), while it did not affect proliferation of parental MDA-MB-231 cells (Fig. 3D). These results show that ectopically expressed fVII can accelerate proliferation of triple-negative breast cancer cells.

6. Strategy to inhibit ectopic fVII synthesis in breast cancer cells

6.1 Inhibition of ectopic fVII synthesis in breast cancer cells by curcumin

The expression of fVII may contribute to breast cancer metastasis and growth. Therefore, targeting fVII expression may be therapeutically beneficial if ectopic fVII could be specifically inhibited without precluding function of fVII secreted from the liver. We showed that the ectopically activated *FVII* promoter is associated with p300 and CBP, while the *FVII* promoter in hepatocytes can be bound by various HATs, suggesting that targeting p300 and CBP activities may fulfil selective inhibition (Koizume et al. 2009). To date, curcumin, a component of spice turmeric, is the only known compound to selectively block p300/CBP HAT activity. Therefore, the effect of this small compound on ectopic *FVII* expression in breast cancer cells was tested (Koizume et al. 2009). The breast cancer cells YMB-1 and MDA-MB-453 were cultured in the presence of curcumin, and then fVII mRNA levels were analyzed by real-time RT-PCR analysis. It was found that curcumin markedly reduced fVII transcript levels in these cells in a dose-dependent manner, while normal expression of *FVII* in hepatic cells was only weakly impaired (Koizume et al. 2009). Additionally, TF mRNA was not significantly diminished by the same curcumin concentrations in these cells. Furthermore, anacardic acid, another natural small compound inhibitor for p300 and PCAF, did not selectively inhibit ectopic *FVII* expression, suggesting that curcumin targets p300/CBP activity (Koizume et al. 2009). The effect of curcumin on *FVII* expression was further examined at the protein level and it was confirmed that curcumin selectively inhibits ectopic fVII synthesis, consistent with the results of mRNA analysis. However, HAT activity associated with the *FVII* promoter in hepatocytes including p300 and CBP is heterogeneous. Furthermore, we showed selective blockade of ectopic fVII synthesis using a limited number of cell lines; therefore it is possible that hepatocytic fVII synthesis can be considerably impaired by curcumin if p300/CBP is a major coactivator of the *FVII* gene.

We tested whether curcumin treatment inhibits proliferation of fVII-expressing breast cancer cells. YMB-1 and MDA-MB-453 cells were cultured in the presence of curcumin as in the case of transcript analysis, and then cell proliferation was evaluated. As in the case of siRNA experiments, it was found that growth of YMB-1 was not reduced (data not shown) but proliferation of MDA-MB-453 cells was significantly impaired (~50% inhibition after 3 days post curcumin addition). We further tested whether the pro-proliferation effect of ectopic fVII is dependent on TF-fVIIa formation on the cell surface. MDA-MB-453 cells were then cultured with anti-TF (5G9 or 10H10) antibody or negative control IgG. Cell proliferation after antibody addition was analyzed by colorimetric assay. We found that treatment of cells with these antibodies did not affect cell proliferation (Fig. 5A).

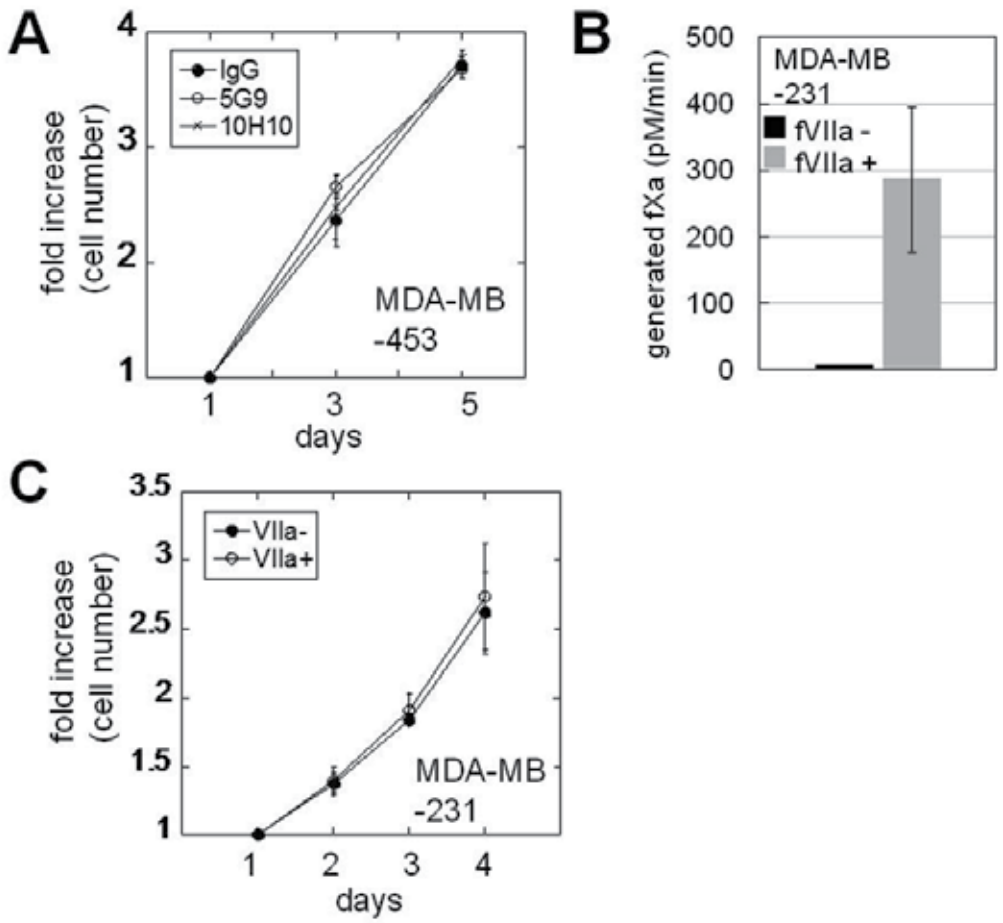


Fig. 5. Effect of TF-fVIIa formation on proliferation of breast cancer cells.
(A) Cell proliferation analysis of MDA-MB-453 cells treated with anti-TF antibodies. Cells had 50 μg/ml of IgG, Mab-5G9, or Mab-10H10 added, and then viable cells were monitored by colorimetric assay. *Columns*, mean (n = 3); *bars*, SD. (B) fXa generation analysis of MDA-MB-231 cells treated with fVIIa. After 1 hour post addition of 10 nM fVIIa, cells were washed twice and then fXa-generating activity due to TF-fVIIa formation on the cell surface was examined by colorimetric assay. *Columns*, mean (n = 2); *bars*, SD. (C) Cells were cultured with or without 20 nM fVIIa. After 2 hours post fVIIa addition, cell proliferation was estimated by colorimetric assay. *Columns*, mean (n = 3); *bars*, SD.

The same results were obtained by cell proliferation experiments (data not shown) with anti-PAR1 and –PAR2 antibodies (Koizume et al. 2006). We further tested the effect of TF-fVIIa protease activity on cell proliferation by adding fVIIa to cell culture media. We confirmed that MDA-MB-231 cells pre-treated with fVIIa strongly generate pro-coagulant activity (Fig. 5B), but cell proliferation was not affected in the presence of fVIIa (Fig. 5C). These data indicate that mechanisms independent of TF-fVIIa formation on the cell surface may be involved in acceleration of cell proliferation driven by ectopic fVII synthesis.

6.2 Other effects of curcumin may augment anti-breast cancer efficacy of ectopic fVII repression

Curcumin has been widely studied because this compound exhibits various anti-cancer effects such as pro-apoptotic, anti-angiogenic, and anti-metastatic activities (Sa & Das 2008). Therefore, anti-ectopic fVII activity in combination with other effects of curcumin may cooperate to suppress malignant phenotype expression of breast cancers. Many investigators have reported that curcumin has pro-apoptotic activity in cancer cells. The molecular mechanism of this effect can be attributed to repression of transcription factors crucial for cell survival. Curcumin can inhibit binding of Egr-1, NFκB, and AP-1 to target genes *in vitro* (Bierhaus et al. 1997; Pendurthi et al. 1997), resulting in down-regulation of proteins essential for tolerance to cell death. Indeed, pro-apoptotic effects of curcumin have also been observed *in vivo* using mouse xenograft breast tumor models (Adams et al. 2004; Shoji et al. 2008). A chemosensitizing effect of curcumin *via* inhibition of the above transcription factors (Goel & Aggarwal 2010) or by targeting proteasomes (Landis-Piwowar et al. 2006; Zhang et al. 2007) has also been reported. Therefore, anti-cancer activity of curcumin can be a result of complex cellular events induced by inhibition of multiple target molecules. In addition, induction of TF expression under cellular stress can be suppressed by curcumin since the *F3* gene is a target of Egr-1, NFκB, and AP-1 for regulation (Bierhaus et al. 1997; Pendruthi et al. 1997). This indicates that curcumin can block a pathologically increased fraction of TF without impairing normal TF expression responsible for extrinsic hemostasis. Since anti-TF strategy may be therapeutically beneficial (Schaffner & Ruf 2009), use of curcumin may be advantageous since this compound could synergistically block malignant phenotypes caused by TF-ectopic fVIIa signaling without causing bleeding.

7. Future directions

Many breast cancer cell lines constitutively and highly express fVII (Fig. 6). Given that TF-fVIIa signaling can be a major mechanism of malignant phenotype expression of breast cancers through enhancement of metastasis, angiogenesis, and anti-apoptotic effects, breast cancer cells possibly synthesize fVII as an optional means to survive in a severe tumor microenvironment unique to mammary tissue. In addition, ectopic fVII can promote cell proliferation. Since transcript and immunohistochemical analyses of clinical samples showed that breast cancer tissues frequently express fVII, anti-breast cancer strategies targeting ectopic fVII should be considered.

It was found that curcumin selectively inhibits ectopic fVII synthesis by precluding p300/CBP binding to the *FVII* promoter region. Since animal studies have shown that curcumin can cure cardiovascular diseases by targeting p300 activity (Morimoto et al. 2008; Li et al. 2008), anti-p300 strategy by curcumin treatment is clinically applicable without significant toxicity. Therefore, for the next step, it will be necessary to examine whether an anti-p300/CBP strategy against fVII-expressing breast cancer is also effective *in vivo*. In addition, an anti-p300/CBP strategy may be compromised since HATs can be targeted to the hepatocytic *FVII* promoter. It is therefore necessary to discover novel molecular targets more specifically associated with ectopic fVII synthesis. Furthermore, although we showed that blockade of ectopic fVII synthesis by RNAi or curcumin treatment could repress cell proliferation, this inhibitory effect is cell-type dependent. Therefore, mechanistic understanding of this difference is also required to predict breast cancer cells responsive to an anti-ectopic fVII strategy.

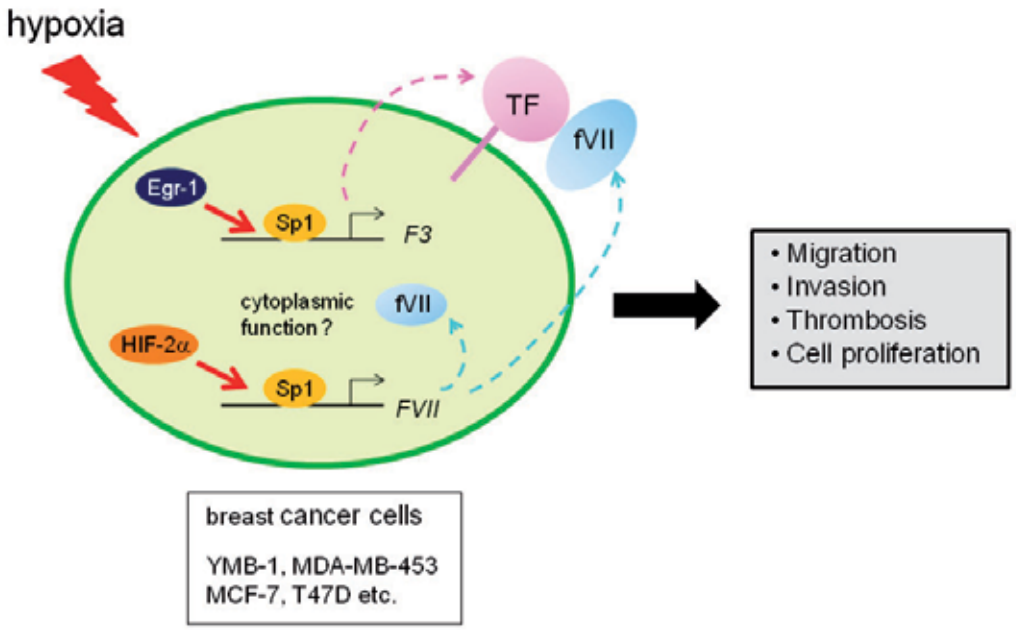


Fig. 6. Breast cancer phenotypes potentially augmented by ectopic fVII expression.
Blood coagulation factor VII can be synthesized *via* transcription from the *FVII* gene constitutively activated by activators such as Sp1 in breast cancer cells. Hypoxic stress may enhance this transcription *via* activation of the *FVII* promoter by binding of the transcription factor HIF-2α. Ectopic fVII activity may be strengthened since expression of its cellular receptor, TF, can also be increased *via F3* gene activation *via* Sp1 substitution by Egr-1 under pathological conditions such as hypoxia. Red symbols are indicative of events expected to occur under hypoxia. Bent arrows are indicative of transcriptional activation. Dotted arrows designate protein expression.

From the clinical point of view, it is essential to reveal relationships between fVII expression and various clinical parameters, such as chemoresistance, relapse, and overall survival to predict patients who may benefit from anti-TF-fVIIa treatment. Notably, breast cancer prognosis is known to closely correlate with pathological complete response after adjuvant chemotherapy (Ross et al. 2008). Therefore, it is worth investigating whether chemoresistant patients express high levels of fVII as a survival factor so that fVII can be targeted to improve prognosis.

Finally, many questions concerning the biology of ectopic fVII synthesis remain unanswered. Why do breast cancer cells tend to synthesize more fVII compared with other cancer cells? How does ectopically expressed fVII associate with TF to express TF-fVIIa complex on the cell surface? It would also be intriguing to investigate whether ectopically expressed fVII has novel functions other than authentic TF-fVIIa signaling. Notably, ectopic fVII might have a cytoplasmic function since we found that ectopic fVII accelerates breast cancer cell proliferation, possibly *via* a cell surface TF-fVIIa-independent mechanism. More specifically, does cytoplasmic fVII exist without complexing with TF to play a biological role? A more detailed understanding of how ectopic fVII expression is regulated and how it

can contribute to breast cancer biology will be essential for translating current knowledge to therapeutic strategies.

8. Materials and methods

8.1 Cell lines and reagents

Human cancer cell lines, antibodies, and curcumin were as previously described (Koizume et al. 2009). fVIIa was kindly provided by Wolfram Ruf (Scripps Research Institute, La Jolla, CA). MDA-MB-231 cell lines constitutively expressing fVII were primarily prepared as previously described (Koizume et al. 2006). Briefly, the parent cell line was transfected with pIRES/fVII expression vector, and then clones resistant to puromycin exposure were isolated by passaging. The expression activity of fVII of cloned cells was primarily confirmed by fXa generation assay (Koizume et al. 2006).

8.2 Chromatin immunoprecipitation, real-time PCR, and fXa generation analysis

These experiments were performed as previously described (Koizume et al., 2006).

8.3 RNAi experiments

Small interference RNA (siRNA) for fVII was ON-TARGET plus SMART pool (Dharmacon, Lafayette, CA). Silencer Negative Control 1 RNAi (Ambion, Austin, TX) was used for non-specific siRNA transfection. Transfection of siRNA was performed using the Neon™ transfection system (Invitrogen, Carlsbad, CA).

8.4 Cell proliferation assay

Cell proliferation was analyzed using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI).

9. Acknowledgments

This work was partly supported by a Grant-in-Aid for the Encouragement of Basic Science and Technology from the Science and Technology Office of the Kanagawa Prefectural Government (S. Koizume), and by a grant from the Japanese Ministry of Education, Culture, Sports, Science and Technology (Y. Miyagi).

10. References

Adams, B. K., Ferstl, E. M., Davis, M. C., Herold, M., Kurtkaya, S., Camalier R. F., Hollingshead, M. G., Kaur, G., Rickles, F. R., Snyder, J. P., Liotta, D. C., Shoji, M. (2004) *Bioorg. Med. Chem.*, 12, 3871-3883.

Albrektsen, T., Sorensen, B. B., Hjortoe, G. M. Fleckner, J., Rao, L. V. M., Petersen, L. C. (2007) *J. Thromb. Haemost.*, 5, 1588-1597.

Álvarez, R. H., (2010) *Breast Cancer Res.*, 12, S1-18.

Bach, R. R. (2006) *Arterioscler. Thromb. Vasc. Biol.*, 26, 456-461.

Benz, C. C. & Yau, C. (2008) *Nat. Rev. Cancer*, 8, 875-879.

Bierhaus, A., Zhang, Y., Quehenberger, P., Luther, T., Haase, M., Muller, M., Mackman, N., Ziegler, R., Nawroth P. P. (1997) *Thromb. Haemost.*, 77, 772-782.

Boire, A., Covic, L., Agarwal, A., Jacques, S., Sherifi, S., Kuliopulos, A. (2005) *Cell*, 120, 303-313.

Booden, M. A., Eckert, L. B., Der, C. J., Trejo, J. (2004) *Mol. Cell. Biol.*, 24, 1990-1999.

Collier, M. E. W., Li Cao, Ettelaie, C. (2008) *Mol. Cancer. Res.*, 6, 1807-1818.

Contrino, J., Hair, G., Kreutzer, D., Rickles, F. R. (1996) *Nature Med.* 2, 209-215.

Davila, M., Amirkhosravi, A., Coll, E., Desai, H., Robles, L., Colon, J., Baker, C. H., Francis, J. L. (2008) *J. Thromb. Haemost.*, 6, 1517-1524.

Fischer, E. G., Riewald, M., Huang, H. Y., Miyagi, Y., Kubota, Y., Mueller, B. M., Ruf, W. (1999) *J. Clin. Invest.*, 104, 1213-1221.

Foulkes, W. D., Smith, I. E., Reis-Filho, J. S., (2010) *N. Engl. J. Med.*, 363, 1938-1948.

Furie, B. & Furie, B. C. (1988) *Cell*, 53, 505-518.

Goel, A. & Aggarwal, B. B., (2010) *Nutr. Cancer*, 62, 919-930.

Hjortoe, G. M. Petersen, L. C., Albrektsen, T., Sorensen, B. B., Norby, P. L., Mandal, S. K., Pendurthi, U. R., Rao, M. V. (2004) *Blood*, 103, 3029-3037.

Iodice, S., Gandini, S., Lohr, M., Lowenfels, A. B., Maisonneuve, P. (2008) *J. Thromb. Haemost.*, 6, 781-788.

Jiang, X.; Bailly, M. A.; Panneti, T. S.; Cappello, M.; Konigsberg, W. H.; Bromberg, M. E. (2004) *J. Thromb. Haemost.*, 2, 93-101.

Jessani, N., Humphrey, M., McDonald, W. H., Niessen, S., Masuda, K., Gangadharan, B., Yates, J. R. III., Mueller, B. M., Cravatt, B. F. (2004) *Proc. Natl. Acad. Sci. USA.* 101, 13756-13761.

Jiang, X.; Zhu, S., Panetti, T. S.; Bromberg, M. E. (2008) *Thromb. Haemost.*, 100, 127-133.

Kirwan, C. C., McDowell, G., McCollum, C. N., Kumar, S., Byrne, G. J., (2008) *Br. J. Cancer.* 99, 1000-1006.

Koizume, S., Jin, M-S., Miyagi, E., Hirahara, F., Nakamura, Y., Piao, J-H., Asai, A., Yoshida, A., Tsuchiya, E., Ruf, W., Miyagi, Y., (2006) *Cancer Res.*, 66, 9453-9460.

Koizume, S., Yokota, Y., Miyagi, E., Hirahara, F., Nakamura, Y., Sakuma, Y., Yoshida, A., Kameda, Y., Tsuchiya, E., Ruf, W., Miyagi, Y. (2009) *Mol. Cancer Res.*, 7, 1928-1936.

Li, H-L., Liu, C., de Couto, G., Ouzounian, M., Sun, M., Wang, A-B., Huang, Y., He, C-W., Shi, Y., Chen, X., Nghiem, M. P., Liu, Y., Chen, M., Dawood, F., Fukuoka, M., Maekawa, Y., Zhang, L., Leask, A., Ghosh, A. K., Kirshenbaum, L. A., Liu, P. P. (2008) *J. Clin. Invest.*, 118, 879-893.

Liu, Y. & Mueller, B. M. (2006) *Biochem. Biophys. Res. Commun.*, 344, 1263-1270.

Landis-Piwowar, K. R., Milacic, V., Chen, D., Yang, H., Zhao, Y., Chan, T. H., Yan, B., Dou, Q. P. (2006) *Drug. Resist. Updat.*, 9, 263-273.

Lwaleed, B. A., Chisholm, M., Francis, J. L., (1999) *J. Pathol*. 187, 291-294.

Mackman, N. (1995) *FASEB J.*, 9, 883-889.

Mackman, N. & Taubman, M. (2009) *Arterioscler. Thromb. Vasc. Biol.*, 29, 1986-1988.

Majmundar, A. J., Wong, W. J., Simon, M. C. (2010) *Mol. Cell,* 40, 294-309.

McDonald, D. M. & Baluk, P., (2002) *Cancer Res.*, 62, 5381-5385.

Milsom, C. & Rak, J. (2009) *Pathol. Haemost. Thromb.*, 36, 160-176.

Morimoto, T., Sunagawa, Y., Kawamura, T., Takaya, T., Wada, H., Nagasawa, A., Komeda, M., Fujita, M., Shimatsu, A., Kita, T., Hasegawa, K. (2008) *J. Clin. Invest.*, 118, 868-878.

Morris, D. R., Ding, Y., Ricks, T. K., Gullapalli, A., Wolfe, B. L., Trejo, J. (2006) *Cancer Res.*, 66, 307-314.

Pendurthi, U. R., Williams, J. T., Rao, L. V. (1997), *Arterioscler Thromb. Vasc. Biol.*, 17, 3406-3413.

Pendurthi, U. R., Allen, K. E., Ezban, M., Rao, V. M. (2000) *J. Biol. Chem.*, 275, 14632-14641.

Pendurthi, U. R., Ghosh, S., Mandal, S. K., Rao, V. M. (2007) *Blood*, 110, 3900-3908.

Ross, J. S., Hatzis, C., Symmans, W. F., Pusztai, L., Hortobagyi, G. N. (2008) *The Oncologist*, 13, 477-493.

Rydén, L., Grabau, D., Schaffner, F., Jönson, P-E., Ruf, W., Belting, M., (2010) *Int. J. Cancer*, 126, 2330-2340.

Sa, G. & Das, T. (2008) *Cell Division*, 3, 1-14.

Schaffner, F. & Ruf. W. (2009) *Arterioscler. Thromb. Vasc. Biol.*, 29, 1999-2004.

Schaffner, F., Versteeg, H. H., Schillert, A., Yokota, N., Petersen. L. C., Mueller, B. M., Ruf, W. (2010) *Blood*, 116, 6106-6113.

Shoji, M., Sun, A., Kisiel, W., Lu, Y. J., Shim, H., McCarey, B. E., Nichols, C., Parker, E. T., Pohl, J., Mosley, C. A., Alizadeh, A. R., Liotta, D. C., Snyder, J. P., (2008) *J. Drug Target*, 16, 185-197.

Sierko, E., Wojtukiewicz, M. Z., Zimnoch, L., Kisiel, W. (2010) *Thromb. Haemost.*, 103, 198-204.

Ten Cate, H. & Falanga, A. (2007) *Pathophysiol Haemost. Thromb.*, 36, 122-130.

Tilley, R. E., Holsher, T., Belani, R., Nieva, J., Mackman, N., (2008) *Thromb. Res.*, 122, 604-609.

Ueno, T., Toi, M., Koike, M., Nakamura, S., Tominaga, T., (2000) *Br. J. Cancer.* 83, 164-170.

Versteeg, H. H., Schaffner, F., Kerver, M., Petersen, H. H., Ahamed, J., Felding-Habermann, Takada, Y., Mueller, B. M., Ruf, W. (2008a) *Blood*, 111, 190-199.

Versteeg, H. H., Schaffner, F., Kerver, M., Ellies, L. G., Andre-Gordon, P., Mueller, B. M., Ruf, W. (2008b) *Cancer Res*., 68, 7219-7227.

Vrana, J. A., Stang, M. T., Grande, J. P., Getz, M. J., (1996) *Cancer Res.*, 56, 5063-5070.

Wilson, B. J., Harada, R., leDuy, L., Hollenberg, M. D., Nepveu, A. (2009) *J. Biol. Chem.* 284, 36-45.

Yokota, N., Koizume, S., Miyagi, E., Hirahara, F., Nakamura, Y., Kikuchi, K., Ruf, W., Sakuma, Y., Tsuchiya, E., Miyagi, Y. (2009) *Br. J. Cancer*, 101, 2023-2029.

Yu, J. L., May, L., Lhotak, V., Shahrzad, S., Shirasawa, S., Weitz, J. I., Coomber, B. L. Mackman, N., Rak, J. W. (2005) *Blood*, 105, 1734-1741.

Zacharski, L. R., Memoli, V. A., Ornstein, D. L., Rousseau, S. M., Kisiel, W., Kudryk, B. J. (1993) *J. Natl. Cancer Inst.*, 85, 1225-1230.

Zhang, H. G., Kim, H., Liu, C., Wang, J., Grizzle, W. E., Kimberly, R. P., Barnes, S. (2007) *Biochim. Biophys. Acta.*, 1173, 1116-1123.

Zhang, X., Yu, H., Lou, J. R., Zheng, J., Zhu, H., Popescu, N-I., Lupu, F., Lind, S. E., Ding, W-Q. (2010) *J. Biol. Chem., 2*86, 1429-1435.

Zhou, J. N., Ljungdahl, S., Shoshan, M. C., Swedenborg, J., Linder, S. (1998) *Mol. Carcinog.,* 21, 234-243.

Part 4

Use of Herbal Medicine and Derivatives

11

Lunasin, a New Breast Cancer Chemopreventive Seed Peptide

Chia-Chien Hsieh[1], Blanca Hernández-Ledesma[2] and Ben O. de Lumen[1]
[1]*Department of Nutritional Sciences and Toxicology, University of California Berkeley, CA*
[2]*Institute of Food Science Research (CIAL, CSIC-UAM), Nicolás Cabrera, 9. Campus de la Universidad Autónoma de Madrid, Madrid*
[1]*USA*
[2]*Spain*

1. Introduction

With a prevalence of about 4.4 million women and a lethality rate of more than 410,000 cases per year, breast cancer is the most common cancer disease and the leading cause of death in women worldwide (Mangiapane et al., 2008). Advances in early detection and improved treatment for breast cancer have led to a steady decrease in the overall breast cancer mortality rate. However, breast cancer remains a significant cause of morbidity and mortality. In general, breast cancer is categorized into the estrogen receptor (ER)–positive type and the ER-negative type, based on the prevalence of ERs within the cell. About 70-80% of all breast cancers are estrogen sensitive and they are treated by conventional procedures including surgery, radiation chemotherapy, and estrogen analogues. Results from clinical trials have demonstrated that it is possible to prevent estrogen-responsive breast cancers by targeting the ER with selective modulators (i.e. tamoxifen, raloxifene, or lasofoxifene) or with aromatase inhibitors (i.e. anastrozole, letrozole, or exemestene). While the ER-positive tumors respond to anti-estrogen therapy and have better prognosis, the ER-negative tumors are more aggressive and resistant to treatments (Uray & Brown, 2006; Li & Brown, 2007; Cuzick, 2008). The high aggressiveness and therapeutic resistance of ER-negative breast tumors have made necessary the development of new and efficient strategies for their prevention and/or treatment.

Recently, the World Cancer Research Fund/American Institute for Cancer Research has reported that changes in diet and lifestyle are good strategies for cancer prevention. Evidence based on a systematic review of the published literature has demonstrated benefits of diet modification approach to reduce cancer risk (Greenwald & Dunn, 2009). The rising prevalence of cancer worldwide and the corresponding rise in health care costs is propelling interest among researchers and consumers for multiple health benefits of food compounds, including reduction in cancer risk and modification of tumor behavior (Béliveau, 2007; Kaefer and Milner, 2008). A large number of natural compounds present in the diet have been demonstrated to lower breast cancer risk and sensitize tumor cells to anti-cancer therapies (Kaefer & Milner, 2008; Ramos, 2008; Hauner & Hauner, 2010). Among these dietary compounds, those present in plant foods and collectively termed phytochemicals,

have been identified as the most promising chemopreventive agents. They may affect different targets of the signal transduction pathways that modulate gene expression, cell cycle progression, proliferation, metabolism and/or apoptosis (Ramos, 2008; van Breda et al., 2008). High priority should be given to research aimed at the study of natural compounds that could potentially prevent the development of breast cancer in susceptible patients.

1.1 Role of dietary compounds against estrogen-receptor negative breast cancer

It is well-known that endocrine interventions are not an effective strategy to reduce the risk of ER-negative breast cancers (Uray & Brown, 2006; Li & Brown, 2007; Cuzick, 2008). Thus, in the last few years, the searching and development of chemopreventive agents against ER-negative breast cancer are attracting more attention. Recently, a number of novel chemopreventive agents targeting non-endocrine pathways have been developed and their capacity to prevent ER-negative mammary tumorigenesis has been demonstrated (Li & Brown, 2007). These agents include retinoids, cyclooxygenase-2 inhibitors, tyrosine kinase inhibitors, and growth factors. Further studies have shown the other compounds possess critical role on cell growth of ER-negative breast cancer, such as retinoid X receptors, vitamin D receptors, peroxisome proliferator-activated receptors, n-3 polyunsaturated fatty acids (PUFA) and several phytochemicals (Simeone & Tari, 2004; Uray & Brown, 2006; Spencer et al., 2009). This chapter is focused on those dietary compounds that have demonstrated a promising potential as chemopreventive agents against ER-negative breast cancer. These compounds, including vitamins, lipids, phytochemicals, proteins and bioactive peptides, have been reported to provide important protection against initiation, promotion or progression of breast cancer. Effects of these compounds as well as their mechanism of action are summarized in this chapter, as shown in Table 1. Moreover, special attention is given to peptide lunasin, identified in soybean and other plants, and which chemopreventive properties against breast cancer have been recently demonstrated.

1.1.1 Vitamins

Retinoids is a family of compounds that include both natural and synthetic derivatives of vitamin A or retinol. They have been found to play important regulatory roles in cellular proliferation, development, metabolism and differentiation (Gudas et al., 1994). It has been reported that these compounds prevent carcinogenesis in a variety of tissues including the breast (Yang et al., 1999). *All-trans*-retinoic acids (ATRA), the oxidized form of retinol, inhibit the proliferation of breast cancer cells predominantly by blocking the transition from G1 to S phase, and activating proteasome function, caspase cleavage and apoptosis (Toma et al., 1997; Son et al., 2007). The main mechanisms of 4-haptoglobin-related protein's apoptotic action have been associated with nitric oxide and reactive oxygen species production, ceramide function, and mitochondrial permeability transition (Simeone & Tari, 2004). Recently, it has shown a rapid decrease of histone H3 acetylation at position Lys 9 at the human telomerase reverse transcriptase promoter. It could be an important mechanism by which ATRA shuts down telomerase activity, thus mediating its antitumor effects in ER-negative breast cancer cells (Phipps et al., 2009). Unfortunately, their side effects, including hyperlipidaemia, muco-cutaneous and liver toxicity, have limited their extensive use in humans (Lee et al., 1993). Therefore, there is a growing body of studies developing novel synthetic retinoids or combination therapies to decrease their toxicity and increase their

Dietary compound	Cell line/Animal model	Chemopreventive activity	Reference
Vitamin A	- MDA-MB-231 and MCF-7 cells	- ↓ Cell proliferation, blocking of G1 to S phase transition	Toma et al.,1997
	- SKBR-3 cells	- ↓ Cell proliferation, G1 phase arrest; ↓ PKCα expression; ↓ ERK/MAPK phosphorylation, and RB dephosphorylation	Nakagawa et al., 2003
	- SKBR-3 cells	- Activates proteasome function, caspase cleavage, and apoptosis	Son et al., 2007
	- SKBR-3 and MCF-7 cells	- ↓ Telomerase activity, histone H3-lysine 9 acetylation inhibition, and ↑ apoptosis	Phipps et al., 2009
Vitamin D	- SUM-159PT cells	- ↑ Apoptosis associated with an enrichment of membrane bound bax, a redistribution of cytochome c from the mitochondria to the cytosol and PARP cleavage; ↓ cell invasion	Flanagan et al., 2003
	- MDA-MB-231 and MCF-7 cells	- ↓ RelB/RELB gene expression and pro-survival targets Survivin, MnSOD and Bcl-2 levels; ↑ sensitivity to gamma-irradiation, and ↑ MAP-kinases ERK1 and ERK2 activity	Mineva et al., 2009; Cordes et al., 2006
	- Xenograft MDA-MB-231-TxSA cells in a murine model	- Vitamin D deficiency promotes the tumor growth	Ooi et al., 2010
n-3 PUFAs	- MDA-MB-231 cells	- ↓ Surface expression of CXCR4 and attenuate the migration/invasion	Altenburg and Siddiqui, 2009
	- MDA-MB-231 cells	- ↓ Cell proliferation, ↑ apoptosis via a transient ↑ caspase-3 activity, the promotion of nuclear condensation, and ↓ invasive potential	Blanckaert et al., 2010
	- Xenograft MDA-MB-435 cells in nude mice	- ↓ Cancer growth and metastasis, ↓ insulin-like growth factor I and epidermal growth factor receptor expression	Chen et al., 2002
	- Xengraft MDA-MB-231 cells metastasis to bone in nude mice	- Prevents the formation of osteolytic lesions in bone, indicating suppression of cancer cell metastasis to bone through ↓ CD44 expression in mRNA and protein levels	Mandal et al., 2010
MUFAs	-Carcinogen-induced mammary tumor in rats	- Lower degree of morphological malignancy in mammary tumor	Costa et al., 2002
	- *In vitro* and *in vivo* laboratory studies	- Influences on stages of carcinogenesis, modification on cell membranes, actions on signal transduction pathways, effects on gene expression and protein activity	Escrich et al., 2006, 2007
Olive oil phenols	- *In vitro* and *in vivo* laboratory studies	- Protect DNA from oxidative damage, inhibit carcinogen activation, and activate carcinogen-detoxifying system	Escrich et al., 2006, 2007
Flavonoids	- MDA-MB 231 and MCF-7 cells	- ↑ Cell apoptosis and maintenance of genome stability through ↑ BRCA1 and BRCA2 gene expression and modulate the different genes, such as BAX, RB1, BRIP, p53	Caëtano et al., 2006; Bernard-Gallon et al., 2010
	- *In vitro* laboratory studies	- Modulation of epigenetic events, such as DNA methylation and/or histone acetylation	Banerjee et al., 2008; Gaur & Bhatia, 2009
	- HS578T, MDA-MB-231, and MCF-7 cells	- ↑ Cell apoptosis through a caspase-3-dependent pathway; arrest cell cycle in the G2/M phase	Lin et al., 2009
	- MDA-MB-231 cells	- ↑ Anti-invasion potency and MMP-3 inhibitory activity	Phromnoi et al., 2009

Table 1. Summary of the potential mechanisms of dietary compounds against ER-negative breast cancer.

Dietary compound	Cell line/Animal model	Chemopreventive activity	Reference
Green tea polyphenols	- MDA-MB-231 cells	- ↑ Cell apoptosis and ↓ invasion through beta-catenin and AKT signaling pathway modulation	Thangapazham et al., 2007a
	- Xenograft MDA-MB-231 cells in nude mice	- Arrest cell cycle at G1 phase by down regulation the expression of Cyclin D, Cyclin E, CDK 4, CDK1 and PCNA; delaying the tumor incidence as well as ↓ tumor burden	Thangapazham et al., 2007b
Indole-3-carbinol	- MDA-MB-468 cells	- ↑ Cell apoptosis mediated ↓ phosphorylation and activation of protein kinase B/Akt, and ↓ NF-κB DNA binding activity	Howells et al., 2002
BBI	-Carcinogen-induced mammary mouse tumor in culture system	- Prevents carcinogen-induced transformation	Du et al., 2001
Lactoferrin	- MDA-MB-231 cells	- ↓ Cell proliferation	Hurley et al, 1994
	- MDA-MB-231, HBL-100, and MCF-7 cells	- Arrest cell growth at G1 to S associated with ↓ CDk2 and cyclin E protein levels, ↓ CDk2 and CDk4 activity, and ↑ p21 expression to maintain pRb in a hypophosphorylated form	Damiens et al., 1999
	- HS578T and T47D cells	- ↓ Cell viability and cell migration, and ↑ cell apoptosis	Duarte et al., 2011
Lactoferricin	- MDA-MB-435 cells	- DNA fragmentation and morphological changes consistent with apoptosis	Furlong et al., 2006
Lectins	- MCF-7, T47D, HBL100 and BT 20 cells	- ↓ Cell proliferation	Valentiner et al., 2003
	- MDA-MB-231 cells	- ↑ Cell cytotoxity	Park et al., 2004
	- Xenograft MDA-MB-231 cells in SCID mice	- ↓ Cell proliferation, ↓ HER2 gene expression; ↑ mice survival and delayed tumor development	Lee-Huang et al., 2000
Lunasin	- MDA-MB-231 cells	- ↓ Cell proliferation; arrest cell cycle in S-phase, ↓ expression of CDC25A, Caspase 8, and Ets2, Myc, Erbb2, PIK3R1 and JUN signaling genes; ↓ expression of cyclins D1 and D3, CDK4 and CDK6 protein levels	Hsieh et al., 2010b; Hernández-Ledesma et al., 2011
	- DMBA-induced transformed NIH/3T3 cell	- ↓ Foci formation and cell proliferation	Hsieh et al., 2011b
	- DMBA-induced mammary cancer in SENCAR mice	- ↓ Tumor incidence	Hsieh et al., 2010c
	- Xenograft MDA-MB-231 cells in nude mice	- ↓ Tumor incidence, ↓ cell proliferation, induction of apoptosis	Hsieh et al., 2010a

Table 1. Summary of the potential mechanisms of dietary compounds against ER-negative breast cancer.

efficacy against breast cancer (Gediya et al., 2008). Several clinical studies using retinoids as breast cancer chemopreventive agents are currently undergoing. Phase III clinical trials are showing that retinoids potently reduced incidence of second breast malignancies in premenopausal women (Bonanni et al., 2007).

1,25-Dihydroxyvitamin D3 (1,25(OH)2D3), the biologically active form of vitamin D, is not only a secosteroid hormone essential for bone and mineral homeostasis but also it is a compound exerting a number of biological functions. Epidemiological investigations indicate that higher level of vitamin D intake is inversely associated with breast cancer risk (Shin et al., 2002). In contrast, an epidemiological study carried out in 107 countries has demonstrated that deficiency in vitamin D increases the risk of breast cancer (Mohr et al., 2008). Recently, it has also been demonstrated that vitamin D deficiency promotes the growth of human breast cancer MDA-MB-231-TxSA cells in a murine model of malignant bone lesions (Ooi et al., 2010). Treatment of 1,25(OH)2D3 has been found to decrease RelB/RELB gene expression and pro-survival targets Survivin, MnSOD and Bcl-2 levels, and to stimulate the MAP-kinases ERK1 and ERK2 activity in both ER-positive MCF-7 cells and ER-negative MDA-MB-231 breast cancer cells (Mineva et al., 2009; Cordes et al., 2006). Vitamin D affects different pathways, such as cell cycle, apoptosis, hormone receptors, angiogenesis, and hypoxia, all of which are related to the breast cancer growth, progression and metastasis by mechanisms independent of estrogen signaling. Moreover, vitamin D may have synergistic, additive, or antagonistic effects when combined with other therapeutic agents against breast cancer (Flanagan et al., 2003; Bertone-Johnson, 2009). Well-designed clinical trials should be needed to further address whether vitamin D is likely to play an important role in reducing risk, mortality and recurrence of breast cancer.

1.1.2 Dietary lipids

Relationship between breast cancer and dietary lipids has been extensively studied. Studies in animal models and observations in humans have provided evidence that a high intake of n-6 PUFAs stimulate several stages in the development of mammary cancer, from an increase in oxidative DNA damage to effects on cell proliferation, free estrogen levels to hormonal catabolism (Bartsch et al., 1999). In contrast, fish oil-derived n-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), seem to prevent cancer by influencing the activity of enzymes and proteins related to intracellular signaling, and ultimately cell proliferation. Recently, it has been demonstrated that these n-3 PUFAs exert potent anti-inflammatory, anti-apoptotic, anti-proliferative and anti-angiogenic effects (Spencer et al., 2009). These authors have reported that these compounds act through regulation of several growth factors, cyclooxygenase 2 (COX-2), prostaglandin-E2 (PGE2), nitric oxide, nuclear factor kappa beta (NF-κB), matrix metalloproteinases (MMP) and beta-catenin. Mandal and co-workers (2010) used a xenograft metastasis MDA-MB-231 mouse model, demonstrating that fish oil diet prevents the formation of osteolytic lesions in bone by inhibiting pro-metastatic molecule CD44 expression both in mRNA and protein levels. It has been also shown that both DHA and EPA significantly reduce surface expression of CXCR4 and attenuate the migration/invasion of MDA-MB-231 cells *in vitro* (Altenburg and Siddiqui, 2009; Mandal et al., 2010). Recently, Blanckaert et al. (2010) reported that n-3 PUFAs have anti-proliferative effect, induce apoptosis via a transient increase in caspase-3 activity, promote nuclear condensation, and reduce the invasive potential of MDA-MB-231

cells. Flaxseed is the richest source of n-3 PUFA alpha-linolenic acid among the vegetable oils. Chen et al. (2002) have shown that nude mice fed flaxseed enriched diet inhibited the MDA-MB-435 human breast cancer growth and metastasis in a xenograft model, and this effect was partly due to its down-regulation of insulin-like growth factor I and epidermal growth factor receptor expression. These studies indicate that naturally occurring n-3 PUFAs are emerging because of their potential to increase efficacy to breast cancer treatment without having any additional side effects.

Abundant epidemiological studies have attributed a potential chemopreventive effect to extra-virgin olive oil, rich in antioxidants and monounsaturated fatty acids (MUFA) such as oleic acid, which is associated with low incidence and mortality rates from cardiovascular disease and some cancers, including breast cancer (Escrich et al., 2007). Interestingly, a negative modulatory role of a high-virgin olive oil diet in the appearance and progression of experimental breast cancer has been described (Escrich et al., 2007). Moreover, mammary tumors from animals fed this kind of diet not only showed a benign clinical behavior but also a lower degree of morphological malignancy compared with control and high n-6 fat diet (Costa et al., 2002; Escrich et al., 2006). Oleic acid as well as other minor components could contribute for the biological effects of olive oil on the distinct carcinogenesis stages through different molecular mechanisms of action.

1.1.3 Phytochemicls

The low incidence of breast cancer among Asians may be explained in part by dietary habits. Epidemiological and experimental studies have shown convincing evidence that people consuming phytoestrogens-rich diets have lower incidence and mortality of breast cancer (Messina & Flickinger, 2002). Asian diets are rich in soybean products containing different compounds found to provide important protection against initiation, promotion and/or progression of breast cancer (Messina et al., 2006). These include isoflavones, saponins, phenolic acids, phytosterols, protease inhibitors, and bioactive proteins and peptides, such as lectins and lunasin.

Genistein, daidzein and glycitein are the three major isoflavonoids, with a chemical structure similar to estrogens, found in soybean and soy products which properties have been extensively studied (Park and Surh, 2004). Genistein has been identified as the predominant isoflavone contained in soybean. Accumulating experiments have concluded that genistein functions as a promising carcinogenesis inhibitor through different molecular mechanisms of action (Banerjee et al., 2008). In addition to its estrogenic effects, genistein has been reported to possess anti-carcinogenic effect through an ER independent pathway, thus being beneficial for ER-negative breast cancer. Genistein possesses free radicals scavenging activity, inhibits the expression of stress-response related genes, and inhibits the growth of several cancer cell lines through the modulation of genes intimately related to the regulation of cell cycle and apoptosis (Vissac-Sabatier et al., 2003; Liao et al., 2004). Mutations of the onco-suppressor genes BRCA1 and BRCA2 are associated with a hereditary risk of breast cancer. It has been demonstrated that genistein or daidzein treatments up-regulate BRCA1 and BRCA2 gene expression and modulate the different genes involved these pathways, such as BAX, RB1, BRIP and p53 in both MCF-7 and MDA-MB-231 cells, suggested a potential chemopreventive effect in promoting apoptosis and maintenance of genome stability (Caëtano et al., 2006; Bernard-Gallon et al., 2010). Genistein also intervenes in several cellular transduction signaling pathways and involves in the

regulation of gene activity by modulating epigenetic events such as DNA methylation and/or histone acetylation (Banerjee et al., 2008; Gaur & Bhatia, 2009). *Puerariae radix* is a popular natural herb and a traditional food in Asia. Isoflavones contained in this plant induce cell apoptosis through a caspase-3-dependent pathway and mediate cell cycle arrest in the G2/M phase in HS578T, MDA-MB-231 and MCF-7 cell lines (Lin et al., 2009). Recently, it has demonstrated that the flavonols quercetin and kaempferol have higher anti-invasion potency and higher MMP-3 inhibitory activity than genistein, genistin and daidzein in the MDA-MB-231 cells (Phromnoi et al., 2009). Phytochemicals, ingested through soybean or other legumes, exerts anti-carcinogenic effects through pleiotropic molecular mechanisms of action on cell cycle, apoptosis, angiogenesis, invasion and metastasis. As an adjuvant therapy in many chronic diseases like cancer, its use is almost established due to less or no side effects.

Epidemiological studies have shown that consumption of green tea polyphenols (GTP) reduces the incidence and progression of breast cancer. An inverse association between the risk of breast cancer and the intake of green tea has also been reported in Asian Americans. The breast cancer progression is delayed in the Asian population that consumes green tea on regular basis (Wu et al., 2003). Green tea is an important source of antioxidants that may be useful for chemoprevention of cancer. It was demonstrated that treatments of GTP and its principal constituent epigallocatechin gallate (EGCG) significantly induce apoptosis and decrease invasion of MDA-MB-231 cells, through beta-catenin and AKT signaling pathway modulation (Thangapazham et al., 2007a). Moreover, both GTP and EGCG treatments had the ability to arrest the cell cycle at G1 phase by down-regulation the protein expression of cyclin D, cyclin E, cyclin-dependent kinases (CDK) 4, CDK 1 and PCNA. In an *in vivo* study, nude mice were inoculated with MDA-MB-231 cells and treated with GTP and EGCG, observing that these compounds were effective in delaying the tumor incidence as well as reducing the tumor burden compared to the control group (Thangapazham et al., 2007b). The tumor sections were also observed that GTP and EGCG induce apoptosis and inhibit cell proliferation by immunohistochemistry analysis. Similarly, indole-3-carbinol, abundant in cruciferous vegetables, induces apoptosis inhibiting phosphorylation and activation of protein kinase B/Akt, and decreasing NF-κB DNA binding activity in the tumor-derived ER-alpha-negative breast cell line MDA-MB-468 (Howells et al., 2002).

1.1.4 Food peptides

Natural peptides have attracted attention as drug candidates owing to their possession of certain key advantages over alternative chemotherapy molecules. In contrast to most small-molecule drugs, peptides have high affinity, strong specificity for targets and low toxicity. Moreover, peptides have good penetration of tissue due to the small size, thus attractive as alternative cell surface targeting agents for cancer therapy (Bhutia and Maiti, 2008). Proteins and peptides have become one group of nutraceuticals exerted biological functions that shows potential results in preventing the different stages of cancer including initiation, promotion and progression (de Mejia and Dia, 2010). Recently, there has been increased interest in the potential health benefits of different food proteins and peptides, including plant protease inhibitors, lactoferrin and lactoferricin, lectins and lunasin.

Protease inhibitors, found in plant tissues particularly legumes, act as possible protective agents against several types of cancer, such as breast cancer. The Bowman-Birk inhibitor (BBI) is a soybean-derived protease inhibitor with 71 amino acids that has been shown to be

an effective suppressor of carcinogenesis in both *in vitro* and *in vivo* assays (Losso, 2008). The role of BBI in carcinogenesis was evaluated either as a purified BBI or as BBI concentrate (BBIC). BBI is involved in inflammation processes, decreases the amount of oxidative damage, and suppresses carcinogenesis by affecting the amount of certain types of proteolytic activities or the expression of certain proto-oncogenes. BBIC achieved Investigational New Drug status from the FDA in 1992, and human trials are currently undergoing to evaluate its use as an anticarcinogenic agent for prostate and oral carcinomas (Armstrong et al., 2000, 2003; Malkowicz et al., 2001). Although BBI has a broad spectrum of cancer-protective activities, its effects on breast cancer remains limited. Treatment of BBI was effective in preventing 7,12-dimethylbenz[a]anthracene (DMBA)-induced transformation using mouse mammary glands in culture system (Du et al., 2001). However, no *in vivo* study reporting the effect of BBI as breast cancer preventive peptide has been published. Nevertheless, there are some *in vitro* studies showing that BBI decreased estrogen-dependent human breast cancer cell growth. These studies demonstrated that suppressed proliferation of MCF-7 cells through abating proteasome function that resulted in up-regulation of MAP kinase phosphatase-1, which turns to suppress ERK1/2 activity and induce apoptosis and lysosome membrane permeabilization (Zhang et al., 1999; Chen et al., 2005; Joanitti et al., 2010). Kunitz trypsin inhibitor (KTI) is another protease inhibitor originally isolated from soybean. The biological significance of this protein in carcinogenesis is mainly attributed to suppress invasion and metastasis of cancer cells (de Mejia and Dia, 2010). KTI isolated from seeds of Chinese black soybean, suppressed proliferation of MCF-7 cells and HepG2 hepatoma cells (Ye and Ng, 2009). However, there are few data about the role of protease inhibitors against ER-negative breast cancer.

Lactoferrin, a globular glycoprotein with a molecular mass of 80 kDa, is a multifunctional protein of the transferrin family that is widely represented in various secretory fluids, such as milk, saliva, tears, and nasal secretions (Lönnerdal, 2009). Early study showed that bovine and human lactoferrin had no effect on growth of MCF-7 cells and only a minimal inhibitory activity toward the MDA-MB-231 line was observed (Hurley et al., 1994). Lactoferrin induces growth arrest at the G1 to S transition, through decreasing protein levels of CDK2 and cyclin E. CDK2 and CDK4 kinase activities are also decreased and p21 expression is increased, maintaining the retinoblastoma protein (Rb) in a hypophosphorylated form in MDA-MB-231 cells and other epithelial cell lines such as HBL-100, MCF-7 and HT-29 (Damiens et al., 1999). Recently, Duarte et al. (2011) has also provided evidences that bovine lactoferrin decreases the cell viability and cell migration, and increases apoptosis in HS578T and T47D cells. Bovine lactoferricin is a cationic peptide produced by gastric-pepsin hydrolysis of bovine lactoferrin, with potent cytotoxic activity against cancer cells (Bellamy et al., 1992). Lactoferricin caused DNA fragmentation and morphological changes consistent with apoptosis in MDA-MB-435 cell cultures, but did not affect the viability of untransformed mammary epithelial cells (Furlong et al., 2006). Although the mechanisms of action are not fully known, the results gathered in this work suggest that lactoferricin interferes with some of the most important steps involved in cancer development.

Extensive studies have revealed that a number of lectins from plants can be used for prevention and/or treatment of cancer. Soy lectins are a significant group of biologically active glycoproteins that have been shown to possess cancer chemopreventive activity by *in vitro*, *in vivo* and human case studies (de Mejia et al., 2003). A sialic acid-specific lectin has been purified from the mushroom *Paecilomyces Japonica*, which exerts cell cytotoxic effects on

the human breast cancer MDA-MB-231, human stomach cancer SNU-1 and pancreas cancer AsPc-1 cells (Park et al., 2004). The suggested mechanisms of action for lectins include effects on membranes of tumor cells, reducing cell proliferation and inducing apoptosis, as well as effects on macrophages increasing their tumor-specific cytotoxicity. Another potential mechanism of action includes lectins's effects on the immune system by altering the production of various interleukins (de Mejia and Prisecaru, 2005). GAP31 (Gelonium protein of 31 kDa) and MAP30 (Momordica protein of 30 kDa) are glycoproteins isolated from the medicinal plants *Gelonium multiflorum* and *Momordica charantia*, respectively. Lee-Huang et al. (2000) conducted a study demonstrated the efficacy of GAP31 and MAP30 inhibiting MDA-MB-231 cells proliferation and expression of HER2 gene, and also increasing survival delayed tumor development in human breast cancer bearing SCID mice. Moreover, some dietary lectins can inhibit cell growth of human breast cancer MCF-7, T47D, HBL100 and BT 20 cells *in vitro*, suggesting a protective effect of these plant lectins for breast cancer (Valentiner et al., 2003). There is still much to learn about the effects of plant lectins on cancer risk. However, they are currently being used as therapeutics agents in cancer treatment studies and this area of research holds considerable potential.

2. Lunasin's discovery and beyond

Lunasin is a peptide composed of 43 amino acid residues with a MW of 5.5 kDa, which sequence is SKWQHQQDSCRKQKQGVNLTPCEKHIMEKIQGRGDDDDDDDDDD. It was initially identified in the soybean cotyledon when a cDNA encoding for a post-translationally processed 2S-albumin (Gm2S-1) was cloned from mid-maturation soybean seed (Galvez et al., 1997). Gm2S-1 coded not only for the methionine-rich protein that was sought to promote the nutritional quality of soy protein but also for other three proteins, a signal peptide, a linker peptide, and a small subunit. This subunit was termed lunasin from the Tagalog word "lunas" for cure. Galvez and co-workers observed that transfection and constitutive expression of the lunasin gene into mammalian cells disrupted mitosis and induced chromosomal fragmentation and apoptosis (Galvez and de Lumen, 1999). The authors attributed lunasin's effects to its negatively charged poly-D carboxyl end that could bind to the highly basic histones found within the nucleosomes of condensed chromosomes, probably to regions that contain more positively charged, such as the hypoacetylated chromatin found in telomeres and centromeres. The displacement by lunasin of the kinetochore proteins normally bound to the centromeres could lead to the failure of spindle fiber attachment and eventually to mitotic arrest and cell death. In addition, lunasin contains the sequence RGD, a cell adhesion motif, that is responsible for the attachment of lunasin to extracellular matrix (Galvez and de Lumen, 1999) thereby allowing its internalization in mammalian cells within a few minutes and its localization in the nucleus in approximately 18 h (Lam et al., 2003). The tri-peptide RGD is the cell attachment site recognized by integrins present in extracellular matrix and cell surface proteins (Ruoslahti and Pierschbacher, 1986). Previous studies have shown the role of RGD peptides inducing apoptosis in different cell lines via a caspase-dependent mechanism (Matsuki et al., 2008; Anuhadra et al., 2000). This motif has been also found to cause cytotoxicity in established human cancer cell lines, including HL 60 (Anuhadra et al., 2000).

Lunasin has been identified in soybean and other beans, grains and herbal plants, such as wheat, barley, rye, amaranth, sunberry, wonderberry, bladder-cherry and jimson weed etc., at concentrations ranged from 0.013 to 8.1 mg lunasin/g of protein (Jeong et al., 2002; de

Mejia et al., 2004; Jeong et al., 2007a, 2007b, 2007c; Silva-Sanchez et al., 2008; Jeong et al., 2009; Jeong et al., 2010a). Lunasin's concentration in seeds and its products has been reported to depend on the genotype varieties, some environmental factors, such as temperature and soil moisture, and the processing conditions (de Mejia et al., 2004; Wang et al, 2008). The stages of seed development have also been found to affect lunasin's concentration, and thus, a notable increase of this concentration has been found to happen during seed maturation. However, the content of lunasin is continually decrease accompanied with the soaking time of sprouting, but is not affected by light and dark conditions (Park et al., 2005). Recently, it has been found that environmental factors, such as germination time and temperature has a significant influence on the composition and concentration of bioactive compounds in germinated soybean flour from the Brazilian soybean cultivars BRS 133 and BRS 258 (Paucar-Menacho et al., 2010b, c). These authors reported that protein concentration also affect the final distribution of nutrients and bioactive components in soybean, including lunasin (Paucar-Menacho et al., 2010a).

A first study has demonstrated lunasin's presence in US commercially available soy foods, including soy milk, infant formulas, tofu, bean curd, soybean cake, tempeh, and su-jae (Hernández-Ledesma et al., 2009a). Concentrations of this peptide in soy milk and other soybean products seem to be determined by the soybean variety and the process used during manufacturing, indicating that these two parameters can be used to control contents of these two peptides. Previously it had been demonstrated that large-scale processing of soy to produce different protein fractions influences lunasin concentration. This content varied from 12 to 44 mg lunasin/g of flour when different commercially available soy proteins were analyzed (de Mejia et al., 2004; Jeong et al., 2003).

2.1 Lunasin's bioavailability

Oral administration of an anti-carcinogenic agent has been recognized as a plausible and cost-effective approach to reduce cancer morbidity and mortality by inhibiting precancerous events before the occurrence of clinical disease (Prasain & Barnes, 2007). Since lunasin is a peptide, it is crucial to establish whether it, once orally ingested, survives digestion and gets absorbed, reaching the target tissues and organs in an intact and bioactive state. Lunasin's bioavailability has been demonstrated by both *in vitro* and *in vivo* studies. Preliminary bioavailability studies carried out in mice and rats fed lunasin-enriched soy protein have found that 35% of ingested lunasin reaches the target tissues and organs in an intact and active form (Jeong et al., 2007a, 2007b). Park and coworkers carried out *in vitro* studies demonstrating the role of proteases inhibitors, such as BBI and KTI, in protecting lunasin from digestion by gastrointestinal enzymes when soy protein was orally consumed (Park et al., 2007). This protection plays a major role in making lunasin bioavailable in soy protein. Recently, it has been demonstrated that lunasin extracted from the blood and liver of lunasin-enriched soy diet fed rats is bioactive and able to suppress foci formation as synthetic lunasin does (Hsieh et al., 2010a). Lunasin's bioavailability has been also reported in a human study. Dia and co-workers demonstrated 4.5% of lunasin ingested in the form of soy protein reached plasma of healthy volunteer men (Dia et al., 2009a). The capacity of lunasin to survive degradation by gastrointestinal and serum proteinases and peptidases reaching blood and other organs in a bioactive form, make lunasin as a perfect candidate to exert a potent *in vivo* cancer preventive activity. This fact supports future clinical trials to investigate the chemopreventive activities of lunasin.

2.2 Lunasin's chemopreventive properties against breast cancer

Lunasin's chemopreventive activities have been demonstrated by both *in vitro* and *in vivo* studies. *In vitro* studies have demonstrated that cancer preventive properties of this peptide in mammalian cells induced by chemical carcinogens and viral oncogenes. *In vivo* studies, lunasin's preventive properties have also been confirmed in both skin and a breast cancer mouse model induced by a chemical carcinogen. Recently, it has been demonstrated that lunasin exerts a promising role against breast cancer both by using MDA-MB-231 cell culture and a breast cancer xenograft mouse model. Lunasin's chemopreventive properties against ER-negative breast cancer and its possible mechanisms are described in this section.

2.2.1 *In vitro* lunasin's chemopreventive properties – Mechanism of action

Lunasin was considered as a "watchdog" agent that sits in the nucleus of the cells and effectively does nothing when there is no transformation event. When a transformation event occurs, lunasin is triggered into action (de Lumen, 2005). Interestingly, recent studies have revealed that lunasin also acts on well-established cancer cell lines. Up to one third on breast cancers that are initially ER-independent making tumors resistant to endocrine therapy during tumor progression (Im et al, 2008). Due to this emergence of hormone-resistance, it is necessary to search for alternative therapies. Lunasin has been demonstrated to inhibit cell proliferation in ER-negative breast cancer MDA-MB-231 cells in a dose-dependent manner, showing an IC_{50} value of 181 μM (Hsieh et al., 2010b). Studies carried out to establish a structure/activity relationship showed an IC_{50} value of 138 μM for the 21 amino acid sequence localized at the C-terminus of lunasin, thus being the main responsible for lunasin's inhibitory effect on breast cancer cells proliferation (Hernandez-Ledesma et al., 2011). Possible mechanisms of action have been recognized as responsible for lunasin's chemopreventive action against breast cancer (Figure 1). First studies demonstrated that lunasin inhibits histone acetylation that is considered as one of the most important epigenetic modifications acting on signal transduction pathways involved in cancer development (Dwarakanath et al., 2008; Dalvai and Bystricky, 2010). Early mistargeted and deregulated histone acetyltransferase (HATs) activities occurring in the common tumor types, such as breast cancer might determine the subsequent genetic changes leading to tumor development and progression (Gayther et al., 2000; Stearns et al., 2007). Because epigenetic changes may be reversible, they represent an active area for new drug investigation and are promising targets for cancer therapy. Therefore, accumulating studies focus on investigating and developing the HATs modulators either for mechanistic studies or for anticancer values.

When the cells are in the steady-state conditions, the core H3 and H4 histones are mostly deacetylated, as a repressed state. After cells treatment with lunasin and sodium butyrate, a known deacetylase inhibitor, the process of histone acetylation was found to be inhibited in C3H10T1/2 fibroblasts and MCF-7 breast cancer cells (Galvez et al., 2001; Jeong et al., 2003). Furthermore, lunasin has demonstrated to compete with different HATs, such as yGCN5 and PCAF, inhibiting the acetylation and repressing the cell cycle progression (Jeong et al., 2002, 2007a, 2007b). According to these findings, an epigenetic mechanism of action for lunasin has been proposed. This model reveals that lunasin can selectively kill cells that are being transformed or are newly transformed when tumor suppressor proteins, like Rb, p53 and pp32, are inactivated by chemical carcinogens and/or viral oncogenes. When lunasin is present in the nucleus, it is acting as a surrogate tumor suppressor and tightly binding to

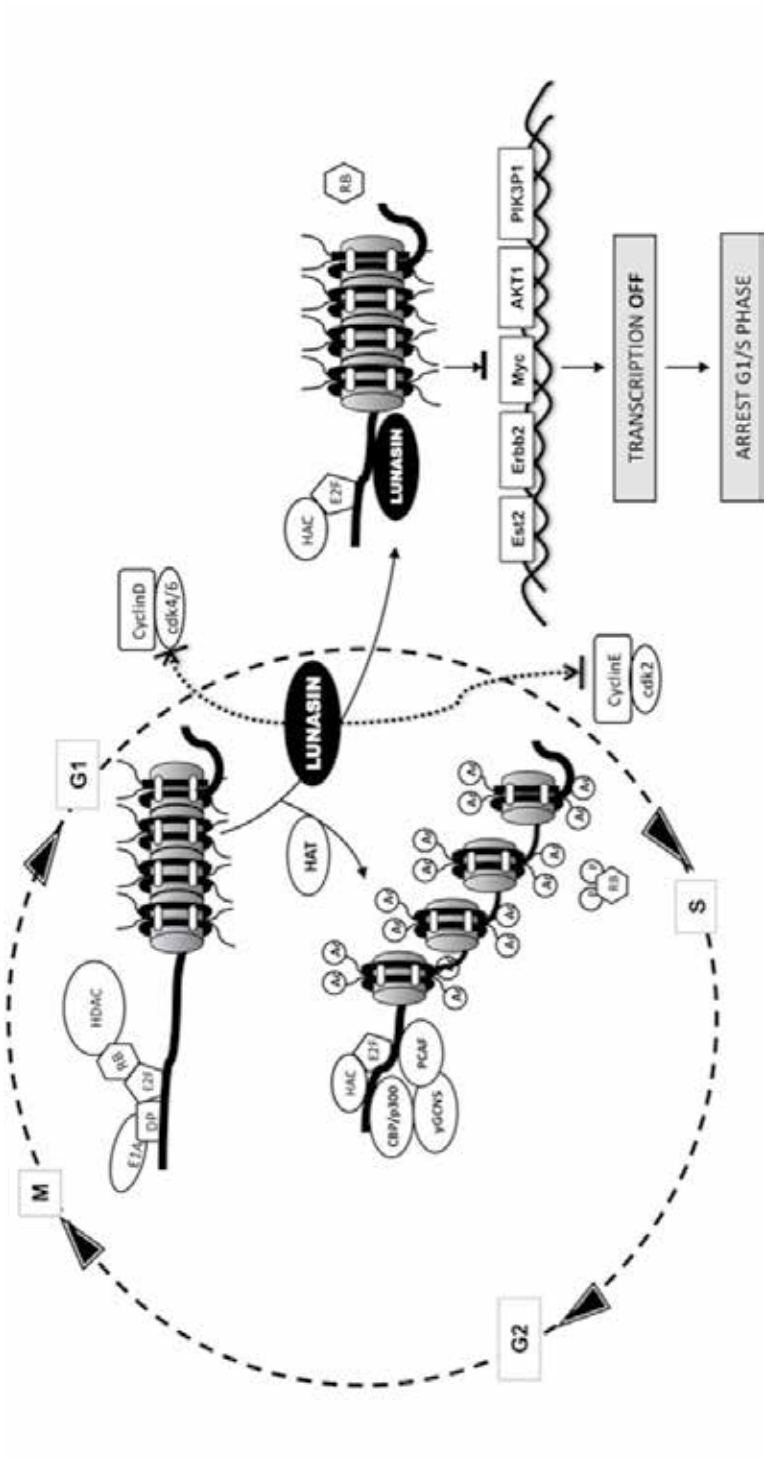

RB
LUNASIN
HAC
E2F
PIK3P1
AKT1
Myc
Erbb2
Est2
TRANSCRIPTION OFF
ARREST G1/S PHASE
CyclinD
cdk4/6
CyclinE
cdk2
G1
S
G2
M
HAT
HDAC
pRB
E2F
DP
E2A
PCAF
CBP/p300
pCAF/SRC1

Fig. 1.

deacetylated core histones and disrupting the balance between acetylation-deacetylation, which is perceived by the cell as abnormal and leads to cell death (de Lumen, 2005). Recently, we have demonstrated that lunasin is a potent inhibitor of H3 and H4 histone acetylation (Hernández-Ledesma et al., 2011). This activity was higher than that demonstrated by other compounds, such as anacardic acid and curcumin, which chemopreventive properties have been demonstrated (Balasubramanyam et al., 2003, 2004a, 2004b). Studies focused on elucidating lunasin's structure-activity relationship establish that lunasin's sequence is essential for inhibiting H4 acetylation whereas poly-D sequence is the main active sequence responsible for H3 acetylation inhibition (Hernández-Ledesma et al., 2011).

Acetylation of specific lysine residues in histones is generally linked to chromatin disruption and the transcriptional activation of genes (Strahl & Allis, 2000). A plethora of chromatin alterations appears to be responsible for the development and progression of various types of cancers, including breast cancer. A global histone modification analysis revealed that in the majority of breast cancers, histone H4 acetylation at position Lys16 was reduced or absent, suggesting that this alteration may represent an early sign of breast cancer (Fraga et al., 2005). In addition, moderate to low levels of histone H3 acetylation at positions Lys9 and Lys18, and histone H4 acetylation at Lys12 were observed in breast carcinomas, and they were associated with poor prognosis and clinical outcome (Elsheikh et al., 2009). Therefore, evaluation of inhibitory effect on specific lysine residues of histones seems to be very promising for searching new therapies against breast cancer. A dose-dependent inhibitory effect on H4 acetylation at positions H4-Lys 8 and H4-Lys 12 was observed after addition of lunasin to breast cancer MDA-MB-231 cells, reaching 17% and 19% for both positions, respectively, when lunasin was treated at 75 μM (Hernández-Ledesma et al., 2011). It should be needed to extensively study the relevance of these results on lunasin's chemopreventive activity to provide data about lunasin's molecular mechanism of action on epigenetic alterations that would be very useful to define new prognostic markers and therapeutic targets.

Studies conducted in our laboratory have revealed different mechanisms of action than histone acetylation inhibition. We have demonstrated that lunasin modulates expression of different genes and proteins involved in cell cycle, apoptosis and signaling transduction (Hsieh et al., 2010b). Inhibition of deregulated cell cycle progress in cancer cells is being considered an effective strategy to delay or halt tumor growth. The cell cycle is regulated through the sequential activation and inactivation of CDKs that drive cell cycle progression (Kato et al., 1993). A pivotal regulatory pathway determining rates of cell cycle transition from G1 to S phase is the CCN/CDK/p16/RB pathway. Over-expression of cyclins D1 and D3 is one of the most frequent alterations present in breast tumors. Cyclins D interacts with CDK4 or CDK6 to form a catalytically active complex, which phosphorylates RB to free active E2F (Sutherland and Musgrove, 2004). Up-regulatory lunasin's effect of RB gene expression (Hsieh et al., 2010b), as well as its inhibitory effect of RB phosphorylation (Jeong et al., 2007b), suggest that both transcriptional and post-translational modifications may be responsible for lunasin's inhibitory effect on cancer cell cycle progression. Moreover, lunasin has been found to inhibit cell proliferation, arrest the cell cycle in the S phase in 45% and down-regulated the mRNA levels of CDk2, CDk4, CDC25A, Caspase 8, and Ets2, Myc, Erbb2, AKT1, PIK3R1 and Jun signaling genes in MDA-MB-231 cells (Hsieh et al., 2010b, 2011a). Lunasin was also demonstrated to reduce protein levels of cyclin D1, cyclin D3,

CDK4 and CDK6 in a dose-dependent form in these breast cancer cells (Hernández-Ledesma et al., 2011), that might also contribute on this lunasin's suppressive effect. This action also affects cell cycle control pathway, especially in the G1/S phase arrest. However, further research should be needed to elucidate the complete molecular and epigenetic lunasin's mechanism of action in breast cancer and other type of cancer cell lines.

Inflammation and oxidative stress are two of the most critical factors implicated in carcinogenesis and other degenerative disorders. Accumulating evidences have revealed that chronic inflammation is involved in the development of approximately 15–20% of malignancies worldwide (Kuper et al., 2000), being clearly associated with increased cancer risk and progression (Allavena et al, 2008). In the last years, there was an increasing body of evidence supporting the role of COX-2 in breast cancer development and progression. COX-2 has been found to be inappropriately induced and up-regulated in human breast cancer. Molecular studies have linked over-expression of COX-2 to a number of critical components of breast carcinogenesis including mutagenesis, angiogenesis, inhibition of apoptosis and aromatase-catalysed oestrogen biosynthesis. Moreover, high levels of COX-2 have been also associated with poor prognosis (Ristimäki et al., 2002; Singh-Ranger et al., 2008). Lunasin has been found to exert anti-inflammatory activity that might contribute to its chemopreventive properties against breast cancer. First studies demonstrated that lunasin potently inhibits lipopolysaccharide-induced production of pro-inflammatory mediators interleuquine-6, tumor necrosis factor-α, and PGE2 in macrophage cells (Hernández-Ledesma et al., 2009b), through modulation of COX-2/PGE2 and inducible nitric oxide synthase/nitric oxide pathways, and suppressing of NF-κB pathways (Dia et al., 2009b; de Mejia & Dia, 2009). Larkins and co-workers (2006) have demonstrated that COX-2 inhibition can decrease breast cancer cells motility, invasion and matrix metalloproteinase expression. Abnormally up-regulated COX and PGs expression are features in human breast tumors, so lunasin might have a role in treatment and prevention of this kind of cancer.

Large amounts of reactive oxygen species (ROS) have been shown to participate in the etiology of several human degenerative diseases, including inflammation, cardiovascular and neurodegenerative disorders, and cancer (Ames et al., 1993). Oxidative stress and ER-associated proliferative changes are suggested to play important roles in estrogen-induced breast carcinogenesis. Several transcription factors and tumor suppressors are involved during stress response such as Nrf2, NFκB and BRCA1 (Acharya et al., 2010). Physiologically achievable concentrations of estrogen or estrogen metabolites have been shown to induce ROS production. Estrogen-induced ROS by increasing genomic instability and by transducing signal through influencing redox sensitive transcription factors play important role in cell transformation, cell cycle, migration and invasion of the breast cancer (Okoh et al., 2011). Lunasin has also been found to exert potent antioxidant properties, reducing lipopolysaccharide-induced production of ROS by macrophage cells, and acting as a potent free radical scavenger (Hernández-Ledesma et al., 2009b). Recently, lunasin purified from *Solanum nigrum* L. has been found to protect DNA from oxidative damage by suppressing the generation of hydroxyl radical via blocking fenton reaction (Jeong et al., 2010b).

2.2.2 *In vivo* lunasin's chemopreventive properties

Lunasin's role as chemopreventive agent against breast cancer has also been demonstrated in *in vivo* mouse models. Our first findings show a relevant inhibitory effect on mammary

tumors development when a lunasin-enriched diet was administered to DMBA-induced SENCAR mice (Hsieh et al., 2010c). Six-week-old SENCAR mice were fed experimental diets before, during and after DMBA treatment by gavage once per week for 6 weeks, until they were sacrificed at 24-week-old. Tumor generation and tumor incidence were reduced by 38% and 25%, respectively, in the mice fed with lunasin-enriched diet (containing 0.23% lunasin) compared with control group. Moreover, the tumor sections obtained from mice fed the lunasin-enriched diet showed slight stromal invasion and degree of morphological aggressiveness due to the effect of this peptide contained in the soy protein preparation (Hsieh et al., 2010c). Park and co-works have reported that an isoflavone-deprived soy peptides prevent DMBA-induced rat mammary tumorigenesis, as well as inhibits the growth of human breast cancer MCF-7 cells in a dose-dependent manner, and induce cell death (Park et al., 2009). Lunasin might be responsible for the effects reported by these authors.

A recent study has demonstrated that lunasin reduces tumor incidence and generation in a xenograft mouse model using human breast cancer MDA-MB-231 cells (Hsieh et al., 2010a). The nude mice were intraperitoneal injected with lunasin, at 20 mg/kg and 4 mg/kg body weight three times per week for two months, and then transplanted with MDA-MB-231 cells and followed up for the other seven weeks. The tumor incidence was 49% and 33%, respectively, in the two doses of lunasin groups compared to the control group. The tumor generation was significantly reduced in the lower dose of lunasin group by 70% lower relative to control group. Lunasin's inhibitory effect was also found on the tumor weight and volume. In contrast, injection with BBI at 20 mg/kg body weight showed no effect on tumor development. The breast tumor histological sections obtained from the lunasin group showed cell proliferative inhibition and cell apoptosis induction. These mice studies show lunasin as promising alternative to prevent and/or treat skin and breast cancer. Further research should be needed to demonstrate chemopreventive role of this peptide against other types of cancer, as well as to elucidate its *in vivo* mechanism of action.

2.2.3 Lunasin's combinations as a novel strategy against breast cancer

Cancer chemotherapeutic strategies commonly require multiple agents. Combination of two or more chemopreventive agents is becoming the best strategy to prevent and/or treat cancer because of its ability to achieve greater inhibitory effects on cancer cells with lower toxicity potential on normal cells (Li et al., 2005; de Kok et al., 2008). Studies based on molecular mechanisms are needed to optimize this combination, increasing tumor response and reducing toxicity levels in non-cancerous cells (de Kok et al., 2008).

Aspirin (acetylsalicylic acid) has been demonstrated as one of the most promising agents with chemopreventive efficacy against several types of cancer. However, aspirin use has been associated with undesirable side effects, peptic ulcer complications, particularly bleeding and mucosal injury in the stomach, small intestine, and colon (Lanas et al., 2000; Laine, 2006). In an attempt to increase aspirin's efficacy and to avoid its side effects, some researchers have explored the potentially beneficial effects of its combination with several agents that may produce synergisms, resulting in considerably stronger protective effects against carcinogenesis than individually agent use. Since lunasin is present in various seeds and food products, and no safety concerns have been noted. Hsieh and co-workers (2010b) have demonstrated that lunasin promotes the cell proliferation inhibitory and apoptosis inducing activities of aspirin in human breast cancer MDA-MB-231 cells (Hsieh et al.,

2010b). Significant synergistic effects were observed when 10 μM lunasin was combined with 0.5 mM aspirin, resulting in a 73% reduction of cell number. This synergistic effect, at least partially, was mediated through modulating the expression of genes encoding G1 and S-phase regulatory proteins and the extrinsic-apoptosis dependent pathway. Synergistic down-regulatory effects were observed for ERBB2, AKT1, PIK3R1, FOS and JUN signaling genes, whose amplification has been reported as being responsible for breast cancer cell growth and resistance to apoptosis. Moreover, additional studies have demonstrated that lunasin/aspirin combination inhibits foci formation and cell proliferation in chemical carcinogens DMBA and MCA induced-NIH/3T3 cells (Hsieh et al., 2011b). The effect was notably higher than that observed when compounds of the combination acted as a single agent.

Anacardic acid (6-pentadecylsalicylic acid), found in the shell of the cashew nut, has been linked to anti-oxidative, anti-microbial, anti-inflammatory and anti-carcinogenic activities (Kubo et al., 1993; Sung et al., 2008). Synergistic effects have also been observed when lunasin (1–25 μM) was combined with anacardic acid (25 to 100 μM) to treat human breast cancer MDA-MB-231 cells resulting in a concentration-dependent inhibition (Hsieh et al., 2011a). Our findings revealed that lunasin/anacardic acid combination arrests cell cycle in S-phase and induces apoptosis at higher levels than that observed when each compound is used individually. This combination also promotes the inhibition of ERBB2, AKT1/PI3K, JUN and RAF1 signaling gene expression. Importantly, lunasin is demonstrated to promote the anti-carcinogenic properties of anacardic acid, suggesting the role of lunasin is not only in cancer prevention, but also in cancer auxiliary therapy.

Lunasin enhances anti-cancer ability of other chemopreventive compounds from natural or synthetic sources, making them perfect candidate strategies to prevent and/or treat breast cancer. The safety and efficacy of chronic use of these combinations should be further tested in animal models and human studies to establish the optimal dose and duration of treatment. Moreover, studies derived from these findings about mechanisms of action of these lunasin's combinations would open a new vision in the development of novel therapies against breast cancer.

3. Conclusion / Future perspectives

Breast cancer is the most common cancer disease and the leading cause of death in women worldwide. Different food compounds have been demonstrated to be effective against this type of cancer. Among them, peptide lunasin is becoming as one of the most promising agents. This peptide, found in soybean and other plants, has been demonstrated to be bioavailable after resisting gastrointestinal and serum degradation, and to reaches blood and target organs in an intact and active form. Lunasin has been shown to act as a promising agent against breast cancer in both *in vitro* and *in vivo* assays. This peptide inhibits ER-independent breast cancer MDA-MB-231 cells proliferation and promotes other chemopreventive agents' activities, inhibiting proliferation and inducing apoptosis. Moreover, lunasin reduces tumor incidence in a chemical carcinogen-induced mammary tumor and in a xenograft breast cancer mouse model. Moreover, genomics, proteomics and biochemical tools are being applied to complete elucidate its molecular mechanism of action. An array of mechanisms have been revealed for this peptide, including antioxidant and anti-inflammatory properties, histone acetylation inhibitory activity, and modulatory activity of expression of genes and proteins involved in different breast carcinogenesis pathways.

Obtained results from all these studies make lunasin a good candidate for new generation of cancer preventive agents derived from foods. However, there is still much to be learned about lunasin's effects on cancer prevention. The major challenge on the use of lunasin in treating cancer would be the conversion of *in vitro* and *in vivo* results into clinical outcomes. Therefore, it should be needed to design clinical trials that confirm lunasin's chemopreventive properties against breast cancer. Other aspects, such as searching for lunasin in other seeds, optimization of techniques to enrich products with this peptide and studying lunasin's interactions with other food constituents affecting its activity should also be conducted.

4. Acknowledgments

The authors want to acknowledge the support by the American Institute for Cancer Research (AICR). B.H.-L. thanks Spanish National Research Council (CSIC) for her post-doctoral JAE-Doc contract.

5. References

Acharya, A.; Das, I.; Chandhok, D. & Saha, T. (2010). Redox regulation in cancer: a double-edged sword with therapeutic potential. *Oxidative Medicine and Cellular Longevity*, Vol. 3, No. 1, (January-February 2010), pp. 23-34, ISSN 1942-0900.

Allavena, P.; Garlanda, C.; Borrello, M.G.; Sica, A. & Mantovani, A. (2008). Pathways connecting inflammation and cancer. *Current Opinion in Genetics Development*, Vol. 18, No. 1, (Rebruary 2008), pp. 3-10, ISSN 0959-437X.

Altenburg, J.D. & Siddiqui, R.A. (2009). Omega-3 polyunsaturated fatty acids down-modulate CXCR4 expression and function in MDA-MB-231 breast cancer cells. *Molecular Cancer Research*, Vol. 7, No. 7, (July 2009), pp. 1013-1020, ISSN 1541-7786.

Ames, B.N.; Shigenaga, M.K. & Hagen, T.M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of USA*, Vol. 90, No. 17, (September 1993), pp. 7915-7922, ISSN 0027-8424.

Anuhadra, C.D.; Kanno, S. & Hirano, S. (2000). RGD peptide-induced apoptosis in human leukemia HL-60 cells required caspase-3 activation. *Cell Biology and Toxicology,* Vol. 16, No. 5, (August 2000), pp.275–83, ISSN 0742-2091.

Armstrong, W.B.; Kennedy, A.R.; Wan, X.S.; Atiba, J.; McLaren, E. & Meyskens, F.L. (2000). Single-dose administration of Bowman-Birk inhibitor concentrate in patients with oral leukoplakia. *Cancer Epidemiology Biomarkers & Prevention*, Vol. 9, No. 1, (January 2000), pp. 43-47, ISSN 1055-9965.

Armstrong, W.B.; Wan, X.S.; Kennedy, A.R.; Taylor, T.H. & Meyskens, F.L. (2003). Development of the Bowman-Birk inhibitor for oral cancer chemoprevention and analysis of neu immunohistochemical staining intensity with Bowman-Birk inhibitor concentrate treatment. *Laryngoscope*, Vol. 113, No. 10, (October 2003), pp. 1687-1702, ISSN 0023-852X.

Balasubramanyam, K.; Altaf, M.; Varier, R.A.; Swaminathan, V.; Ravindran, A.; Sadhale, P.P. & Kundu, T.K. (2004a). Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. *Journal of Biological Chemistry*, Vol. 279, No. 32, (August 2004), pp. 33716-33726, ISSN 0021-9258.

Balasubramanyam, K.; Swaminathan, V.; Ranganathan, A. & Kundu, T.K. (2003). Small molecule modulators of histone acetyltransferase p300. *Journal of Biological Chemistry*, Vol. 278, No. 21, (May 2003), pp. 19134-19140, ISSN 0021-9258.

Balasubramanyam, K.; Varier, R.A.; Altaf, M.; Swaminathan, V.; Siddappa, N.B.; Ranga, U. & Kundu, T.K. (2004b). Curcumin, a novel p300/CREB-binding protein-specific inhibitor of acetyltransferase, represses the acetylation of histone/nonhistone proteins and histone acetyltransferasedependent chromatin transcription. *Journal of Biological Chemistry*, Vol. 279, No. 49, (December 2004), pp. 51163-51171, ISSN 0021-9258.

Banerjee, S.; Li, Y.; Wang, Z. & Sarkar, F.H. (2008). Muti-targeted therapy of cancer by genistein. *Cancer Letters*, Vol. 269, No. 2, (October 2008), pp. 226-242, ISSN 0304-3835.

Bartsch, H.; Nair, J. & Owen, R.W. (1999). Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. *Carcinogenesis*, Vol. 26, No. 12, (December 1999), pp. 2209–2218, ISSN 0143-3334.

Béliveau, R. (2007). Role of nutrition in preventing cancer. *Canadian Family Physician,* Vol. 53, (November 2007), pp. 1905-1911, ISSN 0008-350X.

Bellamy, W.; Takase, M.; Wakabayashi, H.; Kawase, K. & Tomita, M. (1992). Antibacterial spectrum of lactoferricin-B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *The Journal of Applied Bacteriology*, Vol. 73, No. 6, (December 1992), pp. 472-479, ISSN 0021-8847.

Bernard-Gallon, D.J.; Satih, S.; Chalabi, N.; Rabiau, N.; Bosviel, R.; Fontana, L. & Bignon, Y.J. (2010). Phytoestrogens regulate the expression of genes involved in different biological processes in BRCA2 knocked down MCF-7, MDA-MB-231 and MCF-10a cell lines. *Oncology Reports*, Vol. 23, No. 3, (March 2010), pp. 647-653, ISSN 1021-335X.

Bertone-Johnson, E.R. (2009). Vitamin D and Breast Cancer. *Annals of Epidemiology*, Vol. 19, No. 7, (July 2009), pp. 462-467, ISSN 1047-2797.

Bhutia, S.K. & Maiti, T.K. (2008). Targeting tumors with peptides from natural sources. *Trends in Biotechnology*, Vol. 26, No. 4, (April 2008), pp. 210-217, ISSN 0167-7799.

Blanckaert, V.; Ulmann, L.; Mimouni, V.; Antol, J.; Brancquart, L. & Chénais, B. (2010). Docosahexaenoic acid intake decreases proliferation, increases apoptosis and decreases the invasive potential of the human breast carcinoma cell line MDA-MB-231. *International Journal of Oncology*, Vol. 36, No. 3, (March 2010), pp. 737-742, ISSN 1019-6439.

Bonanni, B.; Lazzeroni, M. & Veronesi, U. (2007). Synthetic retinoid fenretinide in breast cancer chemoprevention. *Expert Review of Anticancer Therapy*, Vol. 7, No. 4, (April 2007), pp. 423-432, ISSN 1473-7140.

Caëtano, B.; Le Corre, L.; Chalabi, N.; Delort, L.; Bignon, Y.J. & Bernard-Gallon, D.J. (2006). Soya phytonutrients act on a panel of genes implicated with BRCA1 and BRCA2 oncosuppressors in human breast cell lines. *British Journal of Nutrition*, Vol. 95, No. 2, (February 2006), pp. 406-413, ISSN 0007-1145.

Chen, J.; Stavro, P.M. & Thompson, L.U. (2002). Dietary flaxseed inhibits human breast cancer growth and metastasis and downregulates expression of insulin-like growth factor and epidermal growth factor receptor. *Nutrition and Cancer*, Vol. 43, No. 2, pp. 187-192, ISSN 0163-5581.

Chen, Y.W.; Huang, S.C.; Lin-Shiau, S.Y. & Lin, J.K. (2005). Bowman–Birk inhibitor abates proteasome function and suppresses the proliferation of MCF7 breast cancer cells through accumulation of MAP kinase phosphatase-1. *Carcinogenesis*, Vol. 26, No. 7, (July 2005), pp. 1296–1306, ISSN 0143-3334.

Cordes, T.; Diesing, D.; Becker, S.; Diedrich, K.; Reichrath, J. & Friedrich, M. (2006). Modulation of MAPK ERK1 and ERK2 in VDR-positive and -negative breast cancer cell lines. *Anticancer Research*, Vol. 26, No. 4A, (July-August 2006), pp. 2749-2753, ISSN 0250-7005.

Costa, I.; Solanas, M. & Escrich, E. (2002). Histopathologic characterization of mammary neoplastic lesions induced with 7,12-dimethylbenz(α)antracene in the rat. A comparative analysis with human breast tumours. *Archives of Pathology & Laboratory Medicine*, Vol. 126, No. 8, (August 2002), pp. 915–927, ISSN 0003-9985.

Cuzick, J. (2008). Chemoprevention of breast cancer. *Breast Cancer*, Vol. 15, No. 1, (January 2008), pp. 10–16, ISSN 1340-6868.

Dalvai, M. & Bystricky, K. (2010). The role of histone modifications and variants in regulating gene expression in breast cancer. *Journal of Mammary Gland Biology and Neoplasia*, Vol. 15, No. 1, (March 2010), pp. 19-33, ISSN 1083-3021.

Damiens, E.; El Yazidi, I.; Mazurier, J.; Duthille, I.; Spik, G. & Boilly-Marer, Y. (1999). Lactoferrin inhibits G1 cyclin-dependent kinases during growth arrest of human breast carcinoma cells. *Journal of Cellular Biochemistry*, Vol. 74, No. 3, (September 1999), pp. 486-498, ISSN 0730-2312.

de Kok, T.M.; van Breda, S.G. & Manson, M.M. (2008). Mechanisms of combined action of different chemopreventive dietary compounds. *European Journal Nutrition*, Vol. 47, Suppl. 2, (May 2008), pp. 51-59, ISSN 1436-6207.

de Lumen, B.O. (2005). Lunasin: A cancer preventive soy peptide. *Nutrition Reviews*, Vol. 63, No. 1, (January 2005), pp. 16-21, ISSN 0029-6643.

de Mejia, E.G. & Dia, V.P. (2010). The role of nutraceutical proteins and peptides in apoptosis, angiogenesis, and metastasis of cancer cells. *Cancer Metastasis Review*, Vol. 29, No. 3, (September 2010), pp. 511-528, ISSN 0167-7659.

de Mejia, E.G. & Prisecaru, V.I. (2005). Lectins as bioactive plant proteins: a potential in cancer treatment. *Critical Reviews in Food Science and Nutrition*, Vol. 45, No. 6, pp. 425-455, ISSN 1040-8398.

de Mejia, E.G.; Bradford, T. & Hasler, C. (2003). The anticarcinogenic potential of soybean lectin and lunasin. *Nutrition Reviews*, Vol. 61, No. 7, (July 2003), pp. 239-246, ISSN 0029-6643.

de Mejia, E.G.; Vasconez, M.; de Lumen, B.O. & Nelson, R. (2004). Lunasin concentration in different soybean genotypes, commercial soy protein, and isoflavone products. *Journal of Agricultural and Food Chemistry*, Vol. 52, No. 19, (September 2004), pp. 5882-5887, ISSN 0021-8561.

de Mejia, E.G. & Dia, V.P. (2009). Lunasin and lunasin-like peptides inhibit inflammation through suppression of NF-kB pathway in the macrophage. *Peptides*, Vol. 30, No. 12, (December 2009), pp. 2388-2398, ISSN 0196-9781.

Dia, V.P.; Torres, S.; de Lumen, B.O.; Erdman, J.W. & de Mejia, E.G. (2009a). Presence of Lunasin in Plasma of Men after Soy Protein Consumption. *Journal of Agricultural and Food Chemistry*, Vol. 57, No. 4, (February 2009), pp. 1260-1266, ISSN 0021-8561.

Dia, V.P.; Wang, W.; Oh, V.L.; de Lumen, B.O. & de Mejia, E.G. (2009b). Isolation, purification and characterisation of lunasin from defatted soybean flour and in vitro evaluation of its anti-inflammatory activity. *Food Chemistry*, Vol. 114, No. 1, (May 2009), pp. 108-115, ISSN 0308-8146.

Du, X.; Beloussow, K. & Shen, W.C. (2001). Bowman-Birk protease inhibitor and its palmitic acid conjugate prevent 7,12-dimethylbenz[a]anthracene-induced transformation in cultured mouse mammary glands. *Cancer Letters*, Vol. 164, No. 2, (March 2001), pp. 135-141, ISSN 0304-3835.

Duarte, D.C.; Nicolau, A.; Teixeira, J.A. & Rodrigues, L.R. (2011). The effect of bovine milk lactoferrin on human breast cancer cell lines. *Journal of Dairy Science*, Vol. 94, No. 1, (January 2011), pp. 66-76.

Dwarakanath, B.S.; Verma, A.; Bhatt, A.N.; Parmar, V.S. & Raj, H.G. (2008). Targeting protein acetylation for improving cancer therapy. *Indian Journal of Medicinal Research*, Vol. 128, No. 1, (July 2008), pp. 13-21, ISSN 0971-5916.

Elsheikh, S.E.; Green, A.R.; Rakha, E.A.; Powe, D.G.; Ahmed, R.A.; Collins, H.M.; Soria, D.; Garibaldi, J.M.; Paish, C.E.; Ammar, A.A.; Grainge, M.J.; Ball, G.R.; Abdelghany, M.K.; Martinez-Pomares, L.; Heery, D.M. & Ellis, I.O. (2009). Global histone modifications in breast cancer correlate with tumor phenotypes, prognostic factors, and patient outcome. *Cancer Research*, Vol. 69, No. 9, (May 2009), pp. 3802-3809, ISSN 0008-5472.

Escrich, E.; Ramirez-Tortosa, M.C.; Sanchez-Rovira, P.; Colomer, R.; Solanas, M. & Gaforio, J.J. (2006). Olive oil in cancer prevention and progression. *Nutrition Reviews*, Vol. 64, No. 10, (October 2006), pp. S40-S52, ISSN 0029-6643.

Escrich, E.; Moral, R.; Grau, L.; Costa I. & Solanas, M. (2007). Molecular mechanisms of the effects of olive oil and other dietary lipids on cancer. *Molecular Nutrition & Food Research*, Vol. 51, No. 10, (October 2007), pp. 1279-1292, ISSN 1613-4125.

Flanagan, L.; Packman, K.; Juba, B.; O'Neill, S.; Tenniswood, M. & Welsh, J. (2003). Efficacy of Vitamin D compounds to modulate estrogen receptor negative breast cancer growth and invasion. *Journal of Steroid Biochemistry and Molecular Biology*, Vol. 84, No. 2-3, (February 2003), pp. 181-192, ISSN 0960-0760.

Fraga, M.F.; Ballestar, E.; Villar-Garea, A.; Boix-Chornet, M.; Espada, J.; Schotta, G.; Bonaldi, T.; Haydon, C.; Ropero, S.; Petrie, K.; Iyer, N.G.; Perez-Rosado, A.; Calvo, E.; Lopez, J.A.; Cano, A.; Calasanz, M.J.; Colomer, D.; Piris, M.A.; Ahn, N.; Imhof, A.; Caldas, C.; Jenuwein, T. & Esteller, M. (2005). Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nature Genetics*, Vol. 37, No. 4, (April 2005), pp. 391-400, ISSN 1061-4036.

Furlong, S.J.; Mader, J.S. & Hoskin, D.W. (2006). Lactoferricin-induced apoptosis in estrogen-nonresponsive MDA-MB-435 breast cancer cells is enhanced by C6 ceramide or tamoxifen. *Oncology Reports*, Vol. 15, No. 5, (May 2006), pp. 1385-1390, ISSN 1021-335X.

Galvez, A.F. & de Lumen, B.O. (1999). A soybean cDNA encoding a chromatin binding peptide inhibits mitosis of mammalian cells. *Nature Biotechnology*, Vol. 17, No. 5, (May 1999), pp. 495-500, ISSN 1087-0156.

Galvez, A.F.; Chen, N.; Macasieb, J. & de Lumen, B.O. (2001). Chemopreventive property of a soybean peptide (Lunasin) that binds to deacetylated histones and inhibit

acetylation. *Cancer Research*, Vol. 61, No. 20, (October 2001), pp. 7473-7478, ISSN 0008-5472.

Galvez, A.F.; Revilleza, M.J.R. & de Lumen, B.O. (1997). A novel methionine-rich protein from soybean cotyledon: cloning and characterization of cDNA (accession No. AF005030). Plant Register #PGR97-103. *Plant Physiology*, Vol. 114, pp. 1567-1569.

Gaur, A. & Bhatia, A.L. (2009). Genistein: A multipurpose isoflavone. *International Journal of Green Pharmacy*, Vol. 3, No. 3, (July 2009), pp. 176-183.

Gayther, S.A.; Batley, S.J.; Linger, L.; Bannister, A.; Thorpe, K.; Chin, S.F.; Daigo, Y.; Russell, P.; Wilson, A.; Sowter, H.M.; Delhanty, J.D.A.; Ponder, B.A.J.; Kouzarides, T. & Caldas, C. (2000). Mutations truncating the EP300 acetylase in human cancers. *Nature Genetics*, Vol. 24, No. 3, (March 2000), pp. 300-303, ISSN 1061-4036.

Gediya, L.K.; Khandelwal, A.; Patel, J.; Belosay, A.; Sabnis, G.; Mehta, J.; Purushottamachar, P. & Njar, V.C. (2008). Design, synthesis, and evaluation of novel mutual prodrugs (hybrid drugs) of all-trans-retinoic acid and histone deacetylase inhibitors with enhanced anticancer activities in breast and prostate cancer cells in vitro. *Journal of Medicinal Chemistry*, Vol. 51, No. 13, (July 2008), pp. 3895-3904, ISSN 0022-2623.

Greenwald, P. & Dunn, B.K. (2009). Landmarks in the History of Cancer Epidemiology. *Cancer Research*, Vol. 69, No. 6, (March 2009), pp. 2151-2162, ISSN 0008-5472.

Gudas L.J.; Sporn M.B. & Roberts A.B. (1994) Cellular biology and biochemistry of the retinoids. In: *The Retinoids: Biology, Chemistry and Medicine*, M.B. Sporn, A.B. Roberts, & D.S. Goodman (Eds), pp. 443–520, Raven Press, New York, USA.

Hauner, H. & Hauner, D. (2010). The Impact of Nutrition on the Development and Prognosis of Breast Cancer. *Breast care*, Vol. 5, No. 6, (December 2010), pp. 377-381, ISSN 1661-3791.

Hernández-Ledesma, B.; Hsieh, C.-C. & de Lumen, B.O. (2009a). Lunasin and Bowman-Birk protease inhibitor (BBI) in US commercial soy foods. *Food Chemistry*, Vol. 115, No. 2, (July 2009), pp. 574-580, ISSN 0308-8146.

Hernández-Ledesma, B.; Hsieh, C.-C. & de Lumen, B.O. (2009b). Anti-inflammatory and antioxidant properties of peptide lunasin in RAW 264.7 macrophages. *Biochemical and Biophysical Research Communications*, Vol. 390, No. 3, (December 2009), pp. 803-808, ISSN 0006-291X.

Hernández-Ledesma, B.; Hsieh C.-C. & de Lumen, B.O. (2011). Relationship between lunasin's sequence and its inhibitory activity of histones H3 and H4 acetylation. *Molecular Nutrition and Food Research*, Vol. 55, No. 7, (Jul 2011), pp. 989-998, ISSN 1613-4125.

Howells, L.M.; Gallacher-Horley, B.; Houghton, C.E.; Manson, M.M. & Hudson, E.A. (2002). Indole-3-carbinol inhibits protein kinase B/Akt and induces apoptosis in the human breast tumor cell line MDA-MB468 but not in the nontumorigenic HBL100 line. *Molecular Cancer Therapy,* Vol. 1, No. 13, (November 2002), pp. 1161-1172, ISSN 1535-7163.

Hsieh, C.-C.; Hernández-Ledesma, B. & de Lumen, B.O. (2010a). Complementary roles in cancer prevention: protease inhibitor makes the cancer preventive peptide lunasin bioavailable. *PLoS ONE*, Vol. 5, (January 2010), e8890, ISSN 1932-6203.

Hsieh, C.-C.; Hernández-Ledesma, B. & de Lumen, B.O. (2010b). Lunasin, a novel seed peptide, sensitizes human breast cancer MDA-MB-231 cells to aspirin-arrested cell

cycle and induced-apoptosis. *Chemico-Biological Interactions*, Vol. 186, No. 2, (July 2010), pp. 127-134, ISSN 0009-2797.

Hsieh, C.-C.; Hernández-Ledesma, B.; de Lumen, B.O. (2010c). Soybean peptide lunasin suppresses in vitro and in vivo 7,12-dimethylbenz[a]anthracene-induced tumorigenesis. *Journal of Food Science*, Vol. 75, No. 9, (November 2010), pp. H311-H316. ISSN 0022-1147.

Hsieh, C.-C.; Hernández-Ledesma, B.; de Lumen, B.O. (2011a). Cell proliferation inhibitory and apoptosis inducing properties of anacardic acid and lunasin in human breast cancer MDA-MB-231 cells. *Food Chemistry*, Vol. 125, No. 2, (March 2011), pp. 630-636. ISSN 0308-8146.

Hsieh, C.-C.; Hernández-Ledesma, B. & de Lumen, B.O. (2011b). Lunasin-aspirin combination against NIH/3T3 cells transformation induced by chemical carcinogens. *Plant Foods for Human Nutrition*, Vol. 66, No. 2, (Jun 2011), pp. 107-113, ISSN 0921-9668.

Hurley, W.L.; Hegarty, H.M. & Metzler, J.T. (1994). In vitro inhibition of mammary cell growth by lactoferrin: a comparative study. *Life Science*, Vol. 55, No. 24, pp. 1955-1963, ISSN 0024-3205.

Im, J.Y.; Park, H.; Kang, K.W.; Choi, W.S. & Kim, H.S. (2008). Modulation of cell cycles and apoptosis by apicidin in estrogen receptor (ER)-positive and-negative human breast cancer cells. *Chemico-Biological Interactions*, Vol. 172, No. 3, (April 2008), pp. 235-244, ISSN 0009-2797.

Jeong, H.J.; Jeong, J.B.; Hsieh, C.-C., Hernández-Ledesma, B. & de Lumen, B.O. (2010a). Lunasin is prevalent in barley and is bioavailable and bioactive in *in vivo* and *in vitro* studies. *Nutrition and Cancer*, Vol. 62, No. 8, (November 2010), pp. 1113-1119, ISSN 0163-5581.

Jeong, H.J.; Jeong, J.B.; Kim, D.S. & de Lumen, B.O. (2007a). Inhibition of core histone acetylation by the cancer preventive peptide lunasin. *Journal of Agricultural and Food Chemistry*, Vol. 55, No. 3, (February 2007), pp. 632-637, ISSN 0021-8561.

Jeong, H.J.; Jeong, J.B.; Kim, D.S.; Park, J.H.; Lee, J.B.; Kweon, D.H.; Chung, G.Y.; Seo, E.W. & de Lumen, B.O. (2007b). The cancer preventive peptide lunasin from wheat inhibits core histone acetylation. *Cancer Letters*, Vol. 255, No. 1, (September 2007), pp. 42-48, ISSN 0304-3835.

Jeong, H.J.; Lam, Y. & de Lumen, B.O. (2002). Barley lunasin suppresses ras-induced colony formation and inhibits core histone acetylation in mammalian cells. *Journal of Agricultural and Food Chemistry*, Vol. 50, No. 21, (October 2002). pp. 5903-5908, ISSN 0021-8561.

Jeong, H.J.; Lee, J.R.; Jeong, J.B.; Park, J.H.; Cheong, Y.K. & de Lumen, B.O. (2009). The cancer preventive seed peptide lunasin from rye is bioavailable and bioactive. *Nutrition and Cancer*, Vol. 61, No. 5, pp. 680-686, ISSN 0163-5581.

Jeong, H.J.; Park, J.H.; Lam, Y. & de Lumen, B.O. (2003). Characterization of lunasin isolated from soybean. *Journal of Agricultural and Food Chemistry*, Vol. 51, No. 27, (December 2003), pp. 7901-7906, ISSN 0021-8561.

Jeong, J.B.; de Lumen, B.O. & Jeong, H.J. (2010b). Lunasin peptide purified from *Solanum nigrum* L. protects DNA from oxidative damage by suppressing the generation of hydroxyl radical via blocking fenton reaction. *Cancer Letters*, Vol. 293, No. 1, (July 2010), pp. 58-64, ISSN 0304-3835.

Jeong, J.B.; Jeong, H.J.; Park, J.H.; Lee, S.H.; Lee, J.R.; Lee, H.K.; Chung, G.Y.; Choi, J.D. & de Lumen, B.O. (2007c). Cancer-preventive peptide lunasin from *Solanum nigrum* L. inhibits acetylation of core histones H3 and H4 and phosphorylation of retinoblastoma protein (Rb). *Journal of Agricultural and Food Chemistry*, Vol. 55, No. 26, (December 2007), pp. 10707-10713, ISSN 0021-8561.

Joanitti, G.A.; Azevedo, R.B. & Freitas, S.M. (2010). Apoptosis and lysosome membrane permeabilization induction on breast cancer cells by an anticarcinogenic Bowman–Birk protease inhibitor from *Vigna unguiculata* seeds. *Cancer Letters*, Vol. 293, No. 1, (July 2010), pp. 73–81, ISSN 0304-3835.

Kaefer, C.M. & Milner, J.A. (2008). The role of herbs and spices in cancer prevention. *Journal Nutritional Biochemistry*, Vol. 19, No. 6, (June 2008), pp. 347-361, ISSN 0955-2863.

Kato, J.; Matsushime, H.; Hiebert, S.W.; Ewen, M.E. & Sherr, C.J. (1993). Direct binding of cyclin-D to the retinoblastoma gene-product (pRB) and pRBb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes & Development*, Vol. 7, No. 3, (March 1993), pp. 331-342, ISSN 0890-9369.

Kubo, I.; Ochi, M.; Vieira, P.C. & Komatsu, S. (1993). Antitumor agents from the cashew (*Anacardium occidentale*) apple juice. *Journal of Agricultural and Food Chemistry*, Vol. 41, No. 6, (June 1993), pp. 1012-1015, ISSN 0021-8561.

Kuper, H.; Adami, H.O. & Trichopoulos, D. (2000). Infections as a major preventable cause of human cancer. *Journal of International Medicine*, Vol. 248, No. 3 (September 2000), pp. 171-183, ISSN 0954-6820.

Laine, L. (2006). Review article: gastrointestinal bleedingwith low-dose aspirin: what's the risk? *Alimentary Pharmacology & Therapeutics*, Vol. 24, No. 6, (September 2006), pp. 897-908, ISSN 0269-2813.

Lam, Y.; Galvez, A.F. & de Lumen, B.O. (2003). Lunasin suppresses E1A-mediated transformation of mammalian cells but does not inhibit growth of immortalized and established cancer cell lines. *Nutrition and Cancer*, Vol. 47, No. 1, pp. 88-94, ISSN 0163-5581.

Lanas, A.; Bajador, E.; Serrano, P.; Fuentes, J.; Carreño, S. & Guardia, J. (2000). Nitrovasodilators, low-dose aspirin, other nonsteroidal antiinflammatory drugs, and the risk of upper gastrointestinal bleeding. *New England Journal of Medicine*, Vol. 343, No. 12, (September 2000), pp. 834-839, ISSN 0028-4793.

Larkins, T.L.; Nowell, M.; Singh, S. & Sanford, G.L. (2006). Inhibition of cyclooxygenase-2 decreases breast cancer cell motility, invasion and matrix metalloproteinase expression. *BMC Cancer*, Vol. 6, Art. No. 181, (July 2009), ISSN 1471-2407.

Lee, J.S.; Newman, R.A.; Lippman, S.M.; Huber, M.H.; Minor, T.; Raber, M.N.; Krakoff, I.H. & Hong, W.K. (1993). Phase I evaluation of all-transretinoic acid in adults with solid tumors. *Journal of Clinical Oncology*, Vol. 11, No. 5, (May 1993)), pp. 959-966, ISSN 0732-183X.

Lee-Huang, S.; Huang, P.L.; Sun, Y.; Chen, H.C.; Kung, H.F.; Huang, P.L. & Murphy, W.J. (2000). Inhibition of MDA-MB-231 human breast tumor xenografts and HER2 expression by anti-tumor agents GAP31 and MAP30. *Anticancer Research*, Vol. 20, No. 2A, (March-April 2000), pp. 653-659, ISSN 0250-7005.

Li, Y.W.; Ahmed, F.; Ali, S.; Philip, P.A.; Kucuk, O. & Sarkar, F.H. (2005). Inactivation of nuclear factor B by soy isoflavone genistein contributes to increased apoptosis

induced by chemotherapeutic agents in human cancer cells. *Cancer Research*, Vol. 65, No. 15, (August 2005), pp. 6934-6942, ISSN 0008-5472.

Li, Y. & Brown, P.H. (2007). Translational approaches for the prevention of estrogen receptor-negative breast cancer. *European Journal of Cancer Prevention*, Vol. 16, No. 3, (June 2007), pp. 203-15, ISSN 0959-8278.

Liao, C.H.; Pan, S.L.; Guh, J.H. & Teng, C.M. (2004). Genistein inversely affects tubulin-binding agent-induced apoptosis in human breast cancer cells. *Biochemical Pharmacology*, Vol. 67, No. 11, (June 2004), pp. 2031-2038, ISSN 0006-2952.

Lin, Y.J.; Hou, Y.C.; Lin, C.H.; Hsu, Y.A.; Sheu, J.J.; Lai, C.H.; Chen, B.H.; Lee Chao, P.D.; Wan, L. & Tsai, F.J. (2009). Puerariae radix isoflavones and their metabolites inhibit growth and induce apoptosis in breast cancer cells. *Biochemical and Biophysical Research Communications*, Vol. 378, No. 4, (January 2009), pp. 683-688, ISSN 0006-291X.

Lönnerdal, B. (2009). Nutritional roles of lactoferrin. *Current Opinion in Clinical Nutrition and Metabolic Care*, Vol. 12, No. 3, (May 2009), pp. 293-297, ISSN 1363-1950.

Losso, J.N. (2008). The biochemical and functional food properties of the Bowman-Birk Inhibitor. *Critical Reviews in Food Science & Nutrition*, Vol. 48, No. 1, (January 2008), pp. 94-118, ISSN 1040-8398.

Malkowicz, S.B.; McKenna, W.G.; Vaughn, D.J.; Wan, X.S.; Propert, K.J.; Rockwell, K.; Marks, S.H.F.; Wein, A.J. & Kennedy, A.R. (2001). Effects of Bowman–Birk inhibitor concentrate (BBIC) in patients with benign prostatic hyperplasia. *Prostate*, Vol. 48, No. 1, (June 2001), pp. 16–28, ISSN 0270-4137.

Mandal, C.C.; Ghosh-Choudhury, T.; Yoneda, T.; Choudhury, G.G. & Ghosh-Choudhury, N. (2010). Fish oil prevents breast cancer cell metastasis to bone. *Biochemical and Biophysical Research Communication*, Vol. 402, No. 4, (November 2010), pp. 602-607. ISSN 0006-291X.

Mangiapane, S.; Blettner, M. & Schlattmann, P. (2008). Aspirin use and breast cancer risk: a meta-analysis and meta-regression of observational studies from 2001 to 2005. *Pharmacoepidemiology and Drug Safety*, Vol. 12, No. 2, (February 2008), pp. 115-124, ISSN 1053-8569.

Matsuki, K.; Sasho, T.; Nakagawa, K.; Tahara, M.; Sugioka, K.; Ochiai, N.; Ogino, S.; Wada, Y. & Moriya, H. (2008). RGD peptide-induced cell death of chondrocytes and synovial cells. *Journal of Orthopaedic Science*, Vol. 13, No. 6, (November 2008), pp. 524-532, ISSN 0949-2658.

Messina, M. & Flickinger, B. (2002). Hypothesized anticancer effects of soy: evidence points to isoflavones as the primary anticarcinogens. *Pharmaceutical Biology*, Vol. 40, pp. S6–S23, ISSN 1388-0209.

Messina, M.; McCaskill-Stevens, W. & Lampe, J.W. (2006). Addressing the soy and breast cancer relationship: review, commentary, and workshop proceedings. *Journal of National Cancer Institute*, Vol. 98, No. 18, (September 2006), pp. 1275–1284, ISSN 0027-8874.

Mineva, N.D.; Wang, X.; Yang, S.; Ying, H.; Xiao, Z.X.; Holick, M.F. & Sonenshein, G.E. (2009). Inhibition of RelB by 1,25-dihydroxyvitamin D3 promotes sensitivity of breast cancer cells to radiation. *Journal of Cell Physiology*, Vol. 220, No. 3, (September 2009), pp. 593-599, ISSN 0021-9541.

Mohr, S.B.; Garland, C.F.; Gorham, E.D.; Grant, W.B. & Garland, F.C. (2008) Relationship between low ultraviolet B irradiance and higher breast cancer risk in 107 countries. *Breast Journal*, Vol. 14, No. 3, (May-June 2008), pp. 255–260, ISSN 1075-122X.

Nakagawa, S.; Fujii, T.; Yokoyama, G.; Kazanietz, M.G.; Yamana, H. & Shirouzu, K. (2003). Cell growth inhibition by all-trans retinoic acid in SKBR-3 breast cancer cells: involvement of protein kinase C alpha and extracellular signal-regulated kinase mitogen-activated protein kinase. *Molecular Carcinogenesis*, Vol. 38, No. 3, (November 2003), pp. 106-116, ISSN 0899-1987.

Okoh, V.; Deoraj, A. & Roy, D. (2011). Estrogen-induced reactive oxygen species-mediated signalings contribute to breast cancer. *Biochimica et Biophysica Acta*, Vol. 1815, No. 1, (January 2011), pp. 115-133.

Ooi, L.L.; Zhou, H.; Kalak, R.; Zheng, Y.; Conigrave, A.D.; Seibel, M.J. & Dunstan, C.R. (2010). Vitamin D deficiency promotes human breast cancer growth in a murine model of bone metastasis. *Cancer Research*, Vol. 70, No. 5, (March 2010), pp. 1835-1844, ISSN 0008-5472.

Park, J.H.; Jeong, H.J. & de Lumen, B.O. (2005). Contents and bioactivities of lunasin, Bowman-Birk inhibitor, and isoflavones in soybean seed. *Journal of Agricultural and Food Chemistry*, Vol. 53, No. 20, (October 2005), pp. 7686-7690, ISSN 0021-8561.

Park, J.H.; Jeong, H.J. & de Lumen, B.O. (2007). In vitro digestibility of the cancer-preventive soy peptides lunasin and BBI. *Journal of Agricultural and Food Chemistry*, Vol. 55, No. 26, (December 2007), pp. 10703-10706, ISSN 0021-8561.

Park, J.H.; Ryu, C.S.; Kim, H.N.; Na, Y.J.; Park, H.J. & Kim, H. (2004). A sialic acid-specific lectin from the mushroom Paecilomyces Japonica that exhibits hemagglutination activity and cytotoxicity. *Protein & Peptide Letters*, Vol. 11, No. 6, (December 2004), pp. 563-569, ISSN 0929-8665.

Park, K.; Choi, K.; Kim, H.; Kim, K.; Lee, M.H.; Lee, J.H. & Rim, J.C.K. (2009). Isoflavone-deprived soy peptide suppresses mammary tumorigenesis by inducing apoptosis. *Experimental and Molecular Medicine*, Vol. 41, No. 6, (June 2009), pp. 371-380, ISSN 1226-3613.

Park, O.J. & Surh, Y-H. (2004). Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies. *Toxicology Letters*, Vol. 150, No. 1, (April 2004), pp. 43-56, ISSN 0378-4274.

Paucar-Menacho, L.M.; Amaya-Farfan, J.; Berhow, M.A.; Mandarino, J.M.G.; de Mejia, E.G. & Chang, Y.K. (2010a). A high-protein soybean cultivar contains lower isoflavones and saponins but higher minerals and bioactive peptides than a low-protein cultivar. *Food Chemistry*, Vol. 120, No. 1, (May 2010), pp. 15-21, ISSN 0308-8146.

Paucar-Menacho, L.M.; Berhow, M.A.; Mandarino, J.M.G.; Chang, Y.K. & de Mejia, E.G. (2010b). Effect of time and temperature on bioactive compounds in germinated Brazilian soybean cultivar BRS 258. *Food Research International*, Vol. 43, No. 7, (August 2010), pp. 1856-1865, ISSN 0963-9969.

Paucar-Menacho, L.M.; Berhow, M.A.; Mandarino, J.M.G.; de Mejia, E.G. & Chang, Y.K. (2010c). Optimisation of germination time and temperature on the concentration of bioactive compounds in Brazilian soybean cultivar BRS 133 using response surface methodology. *Food Chemistry*, Vol. 119, No. 2, (March 2010), pp. 636-642, ISSN 0308-8146.

Phipps, S.M.; Love, W.K.; White, T.; Andrews, L.G. & Tollefsbol, T.O. (2009). Retinoid-induced histone deacetylation inhibits telomerase activity in estrogen receptor-negative breast cancer cells. *Anticancer Research*, Vol. 29, No. 12, (December 2009), pp. 4959-4964, ISSN 0250-7005.

Phromnoi, K.; Yodkeeree, S.; Anuchapreeda, S. & Limtrakul, P. (2009). Inhibition of MMP-3 activity and invasion of the MDA-MB-231 human invasive breast carcinoma cell line by bioflavonoids. *Acta Pharmacologica Sinica*, Vol. 30, No. 8, (August 2009), pp. 1169-1176, ISSN 1671-4083.

Prasain, J.K. & Barnes, S. (2007) Metabolism and bioavailability of flavonoids in chemoprevention: current analytical strategies and future prospectus. *Molecular Pharmaceutics*, Vol. 4, No. 6, (November-December 2007), pp. 846–864, ISSN 1543-8384.

Ramos, S. (2008). Cancer chemoprevention and chemotherapy: Dietary polyphenols and signalling pathways. *Molecular Nutrition & Food Research*, Vol. 52, No. 5, (May 2008), pp. 507-526, ISSN 1613-4125.

Ristimäki, A.; Sivula, A.; Lundin, J.; Lundin, M.; Salminen, T.; Haglund, C.; Joensuu, H. & Isola, J. (2002). Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. *Cancer Research*, Vol. 62, No. 3 (February 2002), pp. 632-635, ISSN 0008-5472.

Ruoslahti, E. & Pierschbacher, M.D. (1986). Arg-Gly-Asp: A versatile cell recognition signal. *Cell*, Vol. 44, No. 4, (February 1986), pp. 517-518, ISSN 0092-8674.

Shin, M.H.; Holmes, M.D.; Hankinson, S.E.; Wu, K.; Colditz, G.A. & Willett, W. (2002). Intake of dairy products, calcium, and vitamin D and risk of breast cancer. *Journal of the National Cancer Institute*, Vol. 94, No. 17, (September 2002), pp. 1301-1311, ISSN 0027-8874.

Silva-Sanchez, C.; de la Rosa, A.P.B.; Leon-Galvan, M.F.; de Lumen, B.O.; de Leon-Rodriguez, A. & de Mejia, E.G. (2008). Bioactive peptides in amaranth (*Amaranthus hypochondriacus*) seed. *Journal of Agricultural and Food Chemistry*, Vol. 56, No. 4, (February 2008), pp. 1233-1240, ISSN 0021-8561.

Singh-Ranger, G.; Salhab, M. & Mokbel, K. (2008). The role of cyclooxygenase-2 in breast cancer: Review. *Breast Cancer Research and Treatment*, Vol. 109, No. 2, (May 2008), pp. 189-198, ISSN 0167-6806.

Simeone, A.M. & Tari, A.M. (2004). How retinoids regulate breast cancer cell proliferation and apoptosis. *Cellular and Molecular Life Sciences*, Vol. 61, No. 12, (June 2004), pp. 1475-1484, ISSN 1420-682X.

Son, S.H.; Yu, E.; Ahn, Y.; Choi, E.K.; Lee, H. & Choi, J. (2007). Retinoic acid attenuates promyelocytic leukemia protein-induced cell death in breast cancer cells by activation of the ubiquitin-proteasome pathway. *Cancer Letters*, Vol. 247, No. 2, (March 2007), pp. 213-223, ISSN 0304-3835.

Spencer, L.; Mann, C.; Metcalfe, M.; Webb, M.; Pollard, C.; Spencer, D.; Berry, D.; Steward, W. & Dennison, A. (2009). The effect of omega-3 FAs on tumour angiogenesis and their therapeutic potential. *European Journal of Cancer*, Vol. 45, No. 12, (August 2009), pp. 2077-2086, ISSN 0959-8049.

Stearns, V.; Zhou, Q. & Davidson, N.E. (2007). Epigenetic regulation as a new target for breast cancer therapy. *Cancer Investigation*, Vol. 25, No. 8, (December 2007), pp. 659-665, ISSN 0735-7907.

Strahl, B.D. & Allis, C.D. (2000). The language of covalent histone modifications. *Nature*, Vol. 403, No. 6765, (January 2000), pp. 41-45, ISSN 0028-0836.

Sung, B.; Pandey, M.K.; Ahn, K.S.; Yi, T.F.; Chaturvedi, M.M.; Liu, M.Y. & Aggarwal, B.B. (2008). Anacardic acid (6-nonadecyl salicylic acid), an inhibitor of histone acetyltransferase, suppresses expression of nuclear factor-κB-regulated gene products involved in cell survival, proliferation, invasion, and inflammation through inhibition of the inhibitory subunit of nuclear factor-κBα kinase, leading to potentiation of apoptosis. *Blood*, Vol. 111, No. 10, (May 2008), pp. 4880-4891, ISSN 0006-4971.

Sutherland, R.L. & Musgrove, E.A. (2004). Cyclins and breast cancer. *Journal of Mammary Gland Biology and Neoplasia*, Vol. 9, No. 1, (January 2004), pp. 95-104, ISSN 1083-3021.

Thangapazham, R.L.; Passi, N. & Maheshwari, R.K. (2007a). Green tea polyphenol and epigallocatechin gallate induce apoptosis and inhibit invasion in human breast cancer cells. *Cancer Biology & Therapy*, Vol. 6, No. 12, (December 2007), pp. 1938-1943, ISSN 1538-4047.

Thangapazham, R.L.; Singh, A.K.; Sharma, A.; Warren, J.; Gaddipati, J.P. & Maheshwari, R.K. (2007b). Green tea polyphenols and its constituent epigallocatechin gallate inhibits proliferation of human breast cancer cells in vitro and in vivo. *Cancer Letters*, Vol. 245, No. 1-2, (January 2007), pp. 232-241, ISSN 0304-3835.

Toma, S.; Isnardi, L.; Raffo, P.; Dastoli, G.; De Francisci, E.; Riccardi, L.; Palumbo, R. & Bollag, W. (1997). Effects of all-trans-retinoic acid and 13-cis-retinoic acid on breast-cancer cell lines: growth inhibition and apoptosis induction. *International Journal of Cancer*, Vol. 70, No. 5, (March 1997), pp. 619-627, ISSN 0020-7136.

Uray, I.P. & Brown, P.H. (2006). Prevention of breast cancer: current state of the science and future opportunities. *Expert Opinion on Investigational Drugs*, Vol. 15, No. 12, (December 2006), pp. 1583-1600, ISSN 1354-3784.

Valentiner, U.; Fabian, S.; Schumacher, U. & Leathem, A.J. (2003). The influence of dietary lectins on the cell proliferation of human breast cancer cell lines in vitro. *Anticancer Research*, Vol. 23, No. 2B, (March-April 2003), pp. 1197-1206, ISSN 0250-7005.

van Breda, S.G.J.; de Kok, T.M.C.M. & van Delft, J.H.M. (2008). Mechanisms of colorectal and lung cancer prevention by vegetables: a genomic approach. *Journal Nutritional Biochemistry*, Vol. 19, No. 3, (March 2008), pp. 139-57, ISSN 0955-2863.

Vissac-Sabatier, C. ; Bignon, Y.J. & Bernard-Gallon, D.J. (2003). Effects of the phytoestrogens genistein and daidzein on BRCA2 tumor suppressor gene expression in breast cell lines. *Nutrition and Cancer*, Vol. 45, No. 2, pp. 247-255, ISSN 0163-5581.

Wang, W.; Dia, V.P.; Vasconez, M.; Nelson, R.L. & de Mejia E.G. (2008b). Analysis of soybean protein-derived peptides and the effect of cultivar, environmental conditions, and processing on lunasin concentration in soybean and soy products. *Journal of AOAC International*, Vol. 91, No. 4, (July-August 2008), pp. 936-946, ISSN 1060-3271.

Wu, A.H.; Yu, M.C.; Tseng, C.C.; Hankin, J, & Pike, M.C. (2003). Green tea and risk of breast cancer in Asian Americans. *International Journal of Cancer*, Vol. 106, No. 4, (September 2003), pp. 574-579, ISSN 0020-7136.

Yang, L.M.; Tin, U.C.; Wu, K. & Brown, P. (1999). Role of retinoid receptors in the prevention and treatment of breast cancer. *Journal of Mammary Gland Biological Neoplasia*, Vol. 4, No. 4, (October 1999), pp. 377–388, ISSN 1083-3021.

Ye, X. & Ng, T.B. (2009). A trypsin-chymotrypsin inhibitor with antiproliferative activity from small glossy black soybeans. *Planta Medica*, Vol. 75, No. 5, (April 2009), pp. 550-556, ISSN 0032-0943.

Zhang, L.; Wan, X.S.; Donahue, J.J.; Ware, J.H. & Kennedy, A.R. (1999). Effects of the Bowman-Birk inhibitor on clonogenic survival and cisplatin- or radiation-induced cytotoxicity in human breast, cervical, and head and neck cancer cells. *Nutrition and Cancer*, Vol. 33, No. 2, pp. 165-173, ISSN 0163-5581.

12

Experimental Therapeutics in Breast Cancer Cells

Weena Jiratchariyakul[1] and Tanawan Kummalue[2]
[1]*Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok*
[2]*Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok*
Thailand

1. Introduction

Many cancer patients have medicated themselves with traditional herbal remedies, which are mostly composed of a single or combined medicinal plants. The effectiveness of the remedies can be explained by the presence of the phytochemicals and the bioactivity. The investigators select the traditional-used and evidence-based herbs for the research and development. The investigation of the following Thai herbs and their reasonable utilization are presented.

2. The selected medicinal plants

2.1 Single plant

2.1.1 *Trichosanthes cucumerina* L.

It is a cucurbitaceous vine, grooved stem with scattered hairs and tendrils. The leaf is simple, alternate, five-lobed margin and 8-12 cm blade width. It is dioecious, the male flower is white and the female yellow. The fruit is round with pointed ends and green striped lengthwise, usually 3-4 cm wide and 5-6 cm long (Fig. 1).

It is distributed wildly in the tropical Asia, climbs over the big trees along the riverside and mixed forest. The fruit is very bitter and inedible. It is used for shampooing to relieve itching, dandruff and lice. The fruit and the aerial plant parts are the ingredients in traditional Thai herbal medicine for dizziness. The dried fruit is ground, mixed with tobacco and smoked to alleviate the asthma. The seed (20-30 seeds) is an emetic, antidysentery, febrifuge, emenagogue and anthelmintic. The root decoction is laxative, febrifuge and used to relieve headach and bronchitis. The vine is decocted and used as febrifuge, laxative and tonic.

The bitterness of *T. cucumerina* fruit arises from the presence of cucurbitacins, the highly oxygenated C30-triterpenes, which are frequently found in the cucurbitaceous plants. The fruit juice was extracted with diethyl ether. The extract was concentrated and the crystalline solid was produced. The marc was continuously extracted with petroleum ether, chloroform and methanol in a Soxhlet apparatus. We tested the biological activities of the fruit juice, crystalline solid (TC compound) and the methanol extract. The fruit juice which was prepared as lyophilized (spray dried) solid, did not inhibit the standard strains of the microorganism (*E. coli*, *S. aureus*, *B. subtitis*, *M. smegmatis* and *C. albicans*), whereas the

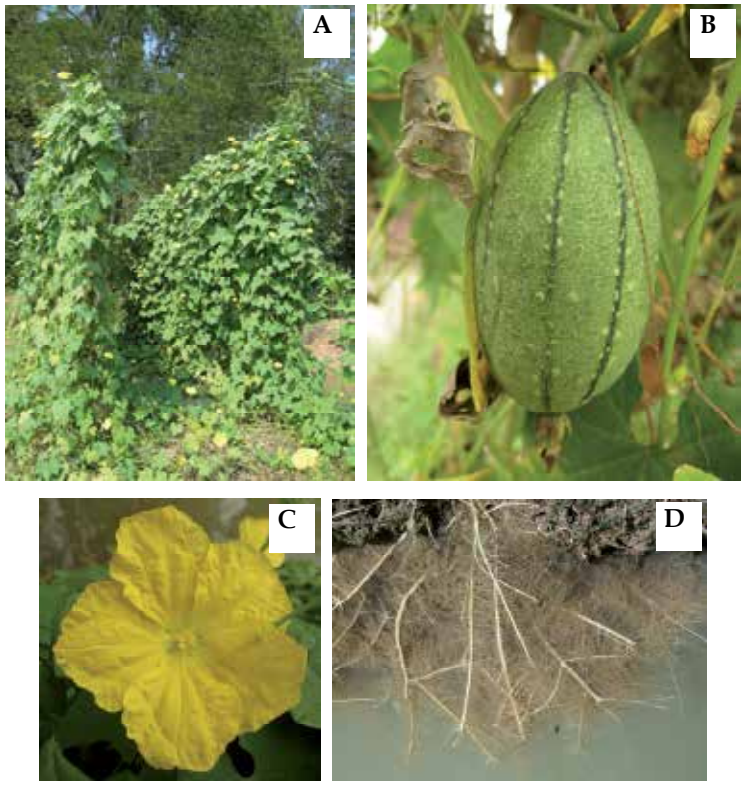


Fig. 1. *Trichosanthes cucumerina* L. : A whole plant; B fruit; C flower; D root.

methanol extract inhibited *S. aureus*, *M. smegmatis* and had a bactericidal action against *C. diptheriae* (Tiangda et al, 1986). TC compound comprised cucurbitacin B (Fig. 2) and dihydrocucurbitacin B, which had a strongly cytotoxic action, in vitro, against KB cell (human nasopharynx carcinoma cell) (Silapaarcha et al, 1981; Jiratchariyakul et al, 1992).
The MTT colorimetric assay for cytotoxicity against breast cancer cell line (SKBR3) of the TC compound, cucurbitacin B and dihydrocucurbitacin B resulted the ED_{50} of 0.48, 0.05 and 0.40 μg/mL, respectively (Jiratchariyakul et al, 1999). The lyophilized fruit juice of 5 mg/kg body weight was injected peritoneally to Swiss albino mice exhibited no sign of toxicity as in the control group. The LD_{50} of the juice was 13 mg/kg body weight, which was considered as moderate toxicity according to Casarett and Doull (Tiangda et al, 1986; Casarett & Doull, 1975).

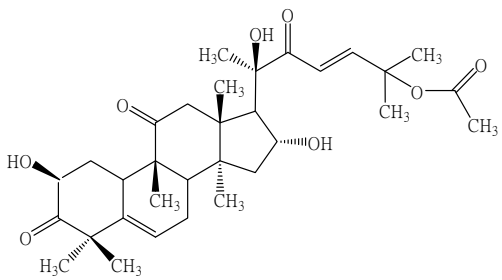


Fig. 2. Structure of cucurbitacin B.

The antiproriferative effect of the fruit juice and the isolated cucurbitacin against four breast cancer cell lines was further carried out. The cell lines included SKBR3, MCF7, T47D and MDA-MB435. SKBR3 is a human breast cancer cell line with overexpression of the HER2/neu receptor and absence of the ER receptor. In contrast, T47D and MCF7 are breast cancer cell lines that are ER receptor positive and HER2/neu negative, whereas the MDA-MB435 breast cancer cell line, both the ER receptor and HER2/neu expressions are absent (Table 1).

Breast cancer cell lines	ER receptor	HER2/neu	IC_{50} (μg/mL)		Doxorubicin (positive control)
			Lyophilized fruit juice	Cucurbitacin B	
MDA-MB435	Negative	Negative	155.77 ± 0.14	25.84 ± 0.83	0.48 ± 0.05
SKBR3	Negative	Overexpressed	130.52 ± 0.17	73.29 ± 0.88	0.65 ± 0.13
MCF7	Positive	Negative	374.77 ± 0.19	34.52 ± 0.11	0.62 ± 0.04
T47D	Positive	Negative	249.32 ± 0.40	60.26 ± 0.61	0.63 ± 0.04

Table 1. Cytotoxicity of the fruit juice of *T. cucumerina* (Kongtun et al., 2009).

The mechanism that cucurbitacin B inhibits the breast cancer cell can be explained through the increased telomerase expression, which is associated with the neoplastic growth. Over 90% of breast cancer has highly telomerase expression. We investigated the effect of cucurbitacin B on telomerase activity. Cucurbitacin B inhibited telomerase activity especially in the ER-negative breast cancer cell SKBR3 with IC_{50} of 3.29 μg/mL, wheras TC compound 4.6 μg/mL and the lyophilized fruit juice less than 10 μg/mL. The activity of human telomerase reverse transcriptase (hTERT) and the level of c-Myc protein were also decreased by cucurbitacin B. Thus cucurbitacin B possibly inhibited the breast cancer cell by reducing the telomerase activity via down regulating both hTERT and c-Myc expressions (Duangmano et al, 2010). Cucurbitacin B inhibited also lung and especially colon cancer cell lines (Kummalue et al, 2009). The recent research indicated the strong cancerostatic action of cucurbitacins B which worked through Jak/STAT-signal ways (Jak = Janus-kinase; STAT = signal transducer and activator of transcription). Cucurbitacin B specifically inhibited STAT 3 which involved in the formation of interleukin 6 (IL-6), an important mediator in immune system (Haensel & Sticher, 2007). Cucurbitacin B was possible to act as immunosuppressive agent according to its inhibition of PHA-activated PBMC (U-pratya et al, 2010).

2.1.2 *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy

M. loriformis is commelinaceous, and monocotyledonous, perennial herb, about 10 cm high. The leaves are simple, glabrous, and alternate. The leaf blade is linear about 1.5-2.0 cm wide, 15-20 cm long. The flowers are inflorescence, terminal and densely panicle. The pedicels slightly curved, translucent bracts about 4 mm, sepal ovate-elliptic, about 3 mm; petals blue or bluish violet (Fig.3). The plant is also used in traditional Chinese medicine as a remedy for detoxification and respiratory tract complaints (Book of traditional Chinese medicine in Sichuan, 1992).

For over 30 years pressed herb juice from *M. loriformis* has been used to support self-medication in Thailand by patients who suffer from different types of cancer. It is supposed to prolong the patient's life and reduce the side effects resulting from modern therapy (radiation and chemotherapy) (Report from the first seminar on herbs and cancer, 1988).

Fig. 3. *Murdannia loriformis* Rolla Rao et Kammathy.

The phytochemical work on this plant indicated the presence of cytotoxic glycosphingolipid namely 1β-*O*-D-glucopyranosyl-2-(2′-hydroxy-6′-ene-cosamide)-sphingosine (Fig. 4) which had moderate cytotoxicity with ED_{50} of 10.7, 15.6, 18.9 and 32.5 μmolL^{-1} against human breast, lung, colon and liver cancer cell lines, respectively, whereas the positive control (quercetin) had the ED_{50} of 23.2, 13.2, 24.8 and 59.6 μmolL^{-1}, respectively (Jiratchariyakul et at., 1997, 1998, 2006).

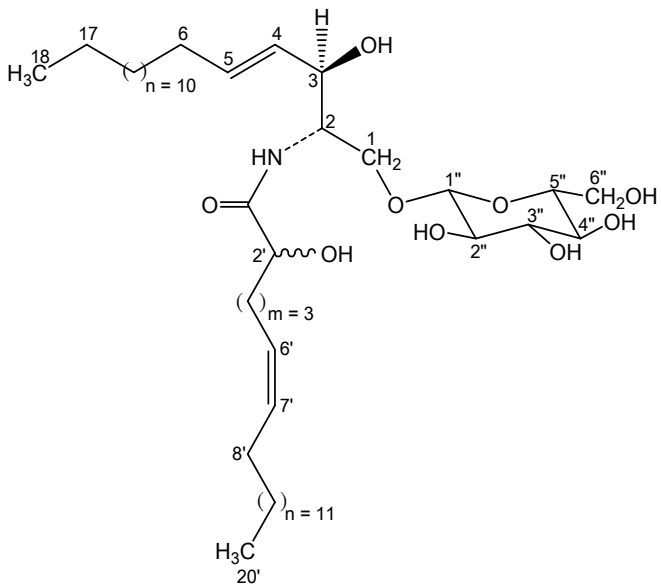


Fig. 4. Structure of 1β-*O*-D-glucopyranosyl-2-(2′-hydroxy-6′-ene-cosamide)-sphingosine, a glycosphingolipid from *M. loriformis*.

The investigation of the indirect (immune-mediated) cytotoxicity was also carried out. The pressed herb juice and the isolated cytotoxic glycosphingolipid were not toxic to the

peripheral blood mononuclear cells (PBMCs) in vitro. Both increased PBMCs proliferation in the presence of the mitogen PHA (phytohaemagglutinin); the glycosphingolipid had the stronger effect. The pressed juice slightly increased the CD3, 4 : CD3, 8 ratio in the 3 day culture and the glycosphingolipid increased this ratio at both the 3 and 7 day cultures. They were the indicative of an in vitro immunomodulatory effect of the pressed juice and the glycosphingolipid (Jiratchariyakul et al., 2006).
The pressed juice had LD_{50}, orally in rats, more than 120 g/kg body weight, and did not damage the growth, the blood chemistry and the pathology of organs. It was safe according to World Health Organization (Tappayuthpijarn et al., 1991; Intayot et al., 2002). The sub-chronic toxicity study of *M. loriformis* in rats for 3 months did not damage the growth, the blood chemistry and the pathology of organs (Tappayuthpijarn et al., 1991). The ethanol extract of this plant induced in vitro DT-diaphorase, a detoxifying enzyme (Vinitketkumnuen, 1996), and decreased in vivo the formation of aberrant crypt foci (ACF), which was related to the colon cancer (Intayot et al., 2002).

2.1.3 *Ficus hispida* L. f.

F. hispida is a traditional Thai plant from family Moraceae. It is a moderate sized tree and grows well in damp and shady place (Fig. 5). Almost all parts of this plant can be used in traditional medicine for the treatment of various ailments, for example, an anti-diarrhea, emetic, astringent, hepatoprotective, antitussive, antipyretic, antiinflammatory, depurative, vulnerary, hemostatic, antiulcer as well as in the treatment of anemia (Wuttidhammaved, 1997; Mandal and Ashok Kumar, 2002; Peraza-Sanchez et al., 2002).

Fig. 5. *Ficus hispida* L. f.

F. hispida, was investigated for antiproliferative activity on human breast cancer cell lines: SKBR3, MDA-MB435, T47D, and MCF7. Based on the results, the methanolic extract exhibited the most powerful activity on T47D breast cancer cell line with ED_{50} of 110.3±9.63 μg/mL as compared to DMSO, the negative control. Confirmation of the result using colony-forming assay (clonogenic assay) yielded the effect in a dose - dependent manner. The cell cycle analysis of this plant extract revealed non-specificity with prominent in apoptosis. Therefore, *F. hispida* might provide some benefits in the treatment of breast cancer. (Pratumvinit et al., 2009)

2.1.4 *Erycibe elliptilimba* Merr. & Chun.

Erycibe elliptilimba, belonging to the Convolvulaceae family, is a Thai medicinal plant that has long been used for many decades to relieve symptoms from fever caused by infection, inflammation and prescribed in the mixture of traditional medicine for treating of various malignancies (Wuttidhammaved, 1997; Sintusarn, 2002).

Fig. 6. *Erycibe elliptilimba* Merr. & Chun.

The ED_{50} values of the methanol fraction against SKBR3 and MDA-MB435 were 56.07 and 30.61 μg/mL, respectively. Doxorubicin as a positive control. This inhibitory growth activity of the treated cells acted in a dose-dependent manner which was also confirmed by the cell viability with the trypan blue exclusion assay. (Kummalue et al., 2007).

2.2 Herbal mixture

Two Thai remedies (TR1 and TR2) were tested for antiproliferative effect. TR1 was composed of following ingredients : *Albizia procera* (stem), *Diospyros mollis* (stem), *Ficus hispida* (stem), *Smilax glabra* (stem), *Smilax china* (stem) and cobra bone. TR2 was composed of *Gelonium multiflorum* (stem), *Erycibe elliptilimba* (stem), *Balanophora abbreviate* (stem), *Smilax glabra* (stem), *Smilax china* (stem) and *Millingtonia hortensis* (stem). TR1 and TR2 had no antiproliferative effect on breast, lung and colon cancer cell lines. However the combination of the remedies (either TR1 or TR2, 30 μg/mL) with doxorubicin (0.5 μg/mL) could significantly inhibit growth of lung cancer cell line (A549) at G2/M phase stronger than doxorubicin alone (Srisapoomi et al., 2008).

In addition two Thai medicinal plants, they were *P. indica and S. rarax*, were tested for antiproliferative effect. *P. indica* inhibited the leukemic cancer cells (NB4, HT93A) (U-pratya et al., 2008) and *S. rarax* inhibited the lung cancer cell (A549) (Kummalue et al., 2011). Both did not inhibit the breast cancer cell.

3. The herbal preparation

The usage of herbs nowadays has been developed according to the laboratory evidence. Herbs are prepared as drug materials, which are conformed to the following pattern (Dingermann, 2000).

- The nature (crude drug, tincture, pressed juice, fluidextract, dry extract, etc.) of the drug material (drug composition) must be specified.
- The quantity of the drug material in each dose of the solid dosage form (tablets) or in each package of the liquid dosage form must be specified.

  For example: 200 mg dry extract / tablet or
  100 mL fluidextract
- The drug extract ratio (DER), the amount (g) of crude drug to produce one g extract or drug material, and the extraction method must be specified.
- The solvent or solvent mixture used for the extraction must be specified.
- The indication must be conformed to the monograph established by the Ministry of the Public Health, or supported by the clinical evidence.
- The daily dose must be specified as the weight of the crude drug.

  For example :

labeled DER	=	4-6.7 : 1
recommended daily dose	=	500 mg extract
thus, the daily dose	=	2-3.35 g crude drug.

3.1 The preparation of *Trichosanthes cucumerina*

- Declaration

  The drug material comprises pressed juice from *T. cucumerina* fruit, which is standardized with cucurbitacin B, a specific marker compound.
- DER

  10.5:1

  One g pressed juice is prepared from 10.5 g fresh fruit.
- According to the preparation which is made from the pressed juice it is non-drug and can be considered as traditional remedy (Haensel & Hoelzl, 1996). The indication is supported by the laboratory evidence and the traditional use. It is supposed to be used as natural cytostatic agent.
- The daily dose

  10.5 g fresh fruit

3.2 The preparation of *Murdannia loriformis*

- Declaration

  The drug material comprises pressed juice from *M. loriformis* herb, which is standardized with glycosphingolipid namely 1β-*O*-D-glucopyranosyl-2-(2′-hydroxy-6′-ene-cosamide)-sphingosine, a specific marker compound.
- DER

  1.7:1

  One g pressed juice is prepared from 1.7 g fresh herb.

- According to the preparation made from the pressed juice it is non-drug and can be considered as traditional remedy (Haensel & Hoelzl, 1996). The indication can be supported by the traditional use over 30 years and the case reported by the physicians. To use as an adjunct to anticancer therapy in order to reduce the side effects and prevent the cancer metastasis.
- The daily dose

  100 g fresh herb (aerial plant parts)

4. Breast cancer cells and experimental procedure in antiproliferative activity background

Breast cancer is one of the leading causes of cancer death around the world. In Thailand, the incidence of breast cancer disease accounts for 20.5 per 100,000 female population (Chaiwerawatana, 2007). Though breast cancer treatment nowadays has dramatically improved due to new drug emerging such as monoclonal antibody, and tyrosine kinase inhibitor, the resistance to the drug itself, unfortunately, has significantly increased. Therefore, searching for the new drug to treat breast cancer is very essential. Based on this important issue, four specific breast cancer cell lines, i.e., SKBR3, MCF7, T47D, and MDA-MB435 have been used to evaluate the potential and promising chemotherapeutic agents from medicinal plants sources.

These four breast cancer cell lines, SKBR3, MCF7, T47D, and MDA-MB435, have different origins and properties which are considered as the very useful tools for investigating the medicinal plants effects. Actually, medicinal plants have long been prescribed for over centuries to treat various diseases including infections and malignancies such as breast cancer in many countries, for example, China, and Thailand. This traditional medicine nowadays plays an important role in healthcare system with approximately 80% of people around the world using it (Itharat and Ooraikul, 2007). Determination of the plants activities especially growth inhibition and their mechanisms for cancer therapy is, indeed, undergoing in much effort.

In this section, four breast cancer cell lines and their experiments on medicinal plants are demonstrated. These include antiproliferative activity and cytotoxicity, cell cycle study, apoptotic study, mechanisms of action in plants, and future direction for testing the drugs from plant sources. Most of the experiments are performed in the biosafety level 1 which is suitable enough for manipulating these breast cancer cell lines in vitro.

4.1 Breast cancer cell lines: Their origins and properties

Breast cancer cell lines can be categorized into three groups according to their phenotypes and invasiveness (Lacroix and Leclercq, 2004).

The first group is luminal epithelial-like cells. This group expresses high amount of typical luminal epithelial phenotype of breast cells such as estrogen receptor (ER), E-cadherin (gene CDH1), zonula occludens-1(TJP1), desmoplakin I/II (DSP), and desmosomal junctions. This group of breast cancer cells will grow as interconnected colonies of polygonal cells on plastic and as fused colonies in Matrigel, which is semisolid medium. This kind of cells are weakly invasive. BT-483, MCF7, T47D, and ZR-75 are all in this group.

The second group is called weakly luminal epithelial-like cells. This group of cells shows the expression closely to the first group with a reduced extent or at least some of those markers.

Cells are weakly invasive in vitro. In Matrigel, most of these cells grow as non-fused spheres. Moreover, on plastic, they will accumulate in clusters of loosely attached cells and rarely reach full confluency. Breast cancer cell lines in this group are BT-474, CAMA-1, MDA-MB134, MDA-MB361, MDA-MB453, MDA-MB468, and SKBR3.
The third group is mesenchymal-like or stromal-like cells. It does not express the markers found in the first and second group. In contrast, it exhibits a high level of vimentin (gene VIM) which is the marker of mesenchymal cells. These cells have fibroblastoid phenotype on plastic and grow as colonies with large stellate projections in Matrigel. They are highly invasive in vitro. MDA-MB435S, MDA-MB231, Hs578T, and BT-549 are classified in this group.
Herein, details of breast cancer cell lines which have been employed in our medicinal plant research are demonstrated.

4.1.1 SKBR3 breast cancer cell line

SKBR3 is a human breast carcinoma cell line established in late 1970s and derived from pleural effusion of Caucasian patient diagnosed as invasive ductal carcinoma (CLS, Germany). It is an adherent cell which is the usual character found in most breast cancer cell lines. Ultra structures of cells show microvilli and desmosomes, large lysosomes, and bundles of cytoplasmic fibrils. In nude mice, these cells can form poorly differentiated adenocarcinoma (http://www.cell-lines-service). In addition in nude mice, c-Jun, the ending molecule of signal transduction pathways, is demonstrated to have the critical role in the tumorigenesis and metastasis in SKBR3 breast cancer cells (Zhang et al, 2007).
Estrogen receptor (ER) is found to be absent or expressed at the very low level in SKBR3 cells and progesterone receptor is also absent in this type of cell (Lacroix and Leclercq, 2004). In contrast, Her2/neu receptor including leptin receptor are overexpressed in this cell line whereas insulin- like growth factor receptor- I is expressed at the lower level when compared with those in MCF7 cells (Ozbay and Nahta, 2008). Interestingly, recent report revealed that SKBR3 expressed NMDAR1 and NMDAR2 receptors, both of which are important calcium channels. These receptors are reported to be essential for the growth of human breast cancer xenografts in mice (North et al, 2010).
This SKBR3 cell line with high expression of HER2 protein has long been used in various research. Recently, it has been shown to be the suitable candidate for reference materials in quality control of HER2 testing together with MCF7 cell line. Based on this report, SKBR3 and MCF7 breast cancer cells provide valuable controls for quantitative measurement of HER2 amplification and production (Xiao et al, 2009). The photograph of SKRB3 cultured in the flask is shown in Figure 7.

4.1.2 MCF7 breast cancer cell line

MCF7 is a human breast carcinoma cell line derived from pleural effusion of Caucasian patient diagnosed with breast adenocarcinoma (CLS, Germany). It is a relatively resistant to cisplatin treatment (Westmose Yde and Issinger, 2006). Morphology of this cell line exhibits epithelial-like cell including the ability to process estradiol via cytoplasmic estrogen receptors and domes formation. This cell line has oncogene, wnt7h and can induce tumor in nude mice (http://www.cell-lines-service).
This MCF7 cell line expresses both estrogen and progesterone receptors whereas the expression of Her2/neu is absent (Lacroix and Leclercq, 2004). The cells can be suppressed by catechin hydrate, product from plant sources such as green tea, through TP53/caspase-

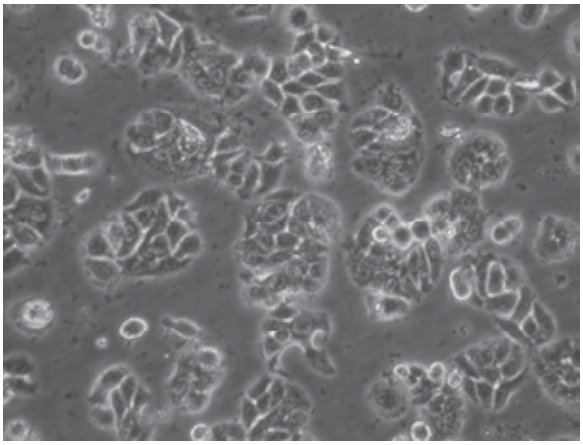


Fig. 7. SKBR3 cells grow in DMEM supplemented with 10% fetal bovine serum and 1% penicillin+ streptomycin. Cells show monolayer when grow in plastic with polygonal shaped. This cell line is categorized as weakly luminal epithelial-like cell.

mediated apoptosis (Alshatwi, 2010). Some vasoactive peptides such as endothelin 1 is found at low level in MCF7 while in SKBR3 is expressed at the higher level. This expression might correlate with high invasiveness phenotype in breast cancer (Hagemann et al, 2005).
MCF7 has been extensively used as the model for breast cancer and breast cancer therapy. However, different sources of MCF7 show the differences in response to 17beta-estradiol resulting from activation or inhibition of insulin-like growth factor I (IGF-1) (Hamelers et al, 2003). Therefore, the different responses of MCF7 should be realized due to the expression of IGF-1. As mentioned above, MCF7 is suitable to be candidate of reference materials in quality control for HER2 testing (Xiao et al, 2009).

4.1.3 T47D breast cancer cell line

T47D is a human breast carcinoma cell line derived from pleural effusion of an infiltrating ductal carcinoma of the breast (CLS, Germany). It contains cytoplasmic junctions, receptors to 17 beta estradiol including steroids and calcitonin (Moseley et al, 1983). Receptors for estrogen and progesterone are also present (Hevir et al, 2011). This cell line has tumorigenicity in nude mice and has three oncogenes, i.e., wnt3, wnt7h, and wnt7b (http://www.cell-lines-service).
By activation Janus kinase2/signal transducer and transcription 5 pathway, this cell line expresses ample growth hormone receptor and prolactin receptor (Xu et al, 2011). Both receptors are structurally similar cytokine receptor superfamily members. Direct protein identification by MALDI post-source decay (PSD) or MALDI collision-induced dissociation (CID) in T47D cells showed only histone H2B (Pevsner et al, 2007). Chemokine CXCL12 and its promoter activity are found to be increase in human T47D breast cancer cells (Chen et al, 2010). Indeed, T47D cell line, as well as MCF7, represents the good model of estrogen-dependent breast cancer with capacity of 17beta-estradiol local production (Hevir et al, 2011).

4.1.4 MDA-MB435 breast cancer cell line

MDA-MB435 is derived from the pleural effusion of metastatic ductal adenocarcinoma of the breast. Recent data have pointed out that MDA-MB435 cells are from melanoma

(Lacroix, 2009; Rae et al, 2004). This cell line is found to express certain genes commonly transcribed in melanocyte which are not seen in breast cancer cell lines such as DCP gene. Moreover, expression of melanocyte proteins tyrosinase and melan-A has also been demonstrated in MDA-MB435. However, some evidences have been presented that MDA-MB435 might be breast cancer cell line expressing a poorly differentiated aggressive breast tumor together with epithelial and melanocyte markers (Chambers, 2009; http://www.cell-lines-service).
Investigation on protein identification in MDA-MB435 cells was performed and found 30 important proteins expressing in this cell line (Chandramouli et al, 2009). For example, caspase 14 precursor, heat shock protein 60, cell adhesion proteins, and some immune response proteins such as complement C3 were also demonstrated.

4.2 Breast cancer cells: Experimental procedures in antiproliferative activity

Antiproliferative and cytotoxic activity are the basic and very common investigations used for determination in the efficacy of plant extracts for searching the novel drugs. The measurement of herb treated mammalian cells either surviving or proliferating cells could be achieved by several techniques. Counting cells that include or exclude a dye such as trypan blue dye with the use of a hemocytometer under a microscope or in an automated cell counter is one assay. Quantitation of suspension cells could be performed by counting them directly whereas adherent cells which grow in monolayer culture require proteolytic agents such as trypsin prior to quantitation. This method is, therefore, not only time consuming, labor intensive, but also dependent upon individual skills.
A number of various indirect methods to quantitate living or dead cell number has been developed. Based on the fact that active or living cells will increase or decrease some specific proteins or even nucleic acids that can indicate cellular proliferation or cytotoxicity. Measuring released 51Cr-labeled protein after cell lysis, and measuring incorporation of 3H-thymidine during cell proliferation have been employed (Mosmann, 1983). These traceable radioactive moieties are very efficient and specific. However, the radioactive methods could handle very limited numbers of samples at each time and also require long sample preparation procedure with inherent dangers and high cost (Haslam et al, 2000). Therefore, several non-radioactive methods have been developed instead of an old traditional method, the cell counting.
In this section, breast cancer cells and experimental procedures in antiproliferative activity, three non-radioactive assays, i.e., MTT assay, Lactate dehydrogenase (LDH) cytotoxicity assay, and clonogenic assay, commonly used have been demonstrated in details.

4.2.1 MTT assay

Principle & mechanism of action

This colorimetric assay was first developed and reported by Mosmann (Mosmann, 1983). Tetrazolium salt MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) is tested and used in this assay. These tetrazolium salts can measure the activity of several dehydrogenase enzymes especially mitochondrial enzyme succinate-dehydrogenase (Slater et al., 1963). The reaction is known to be occurred in the living cells by cleaving the tetrazolium ring in the metabolically active mitochondria, not in dead cells or erythrocytes. From the MTT pale yellow substrate, the dark blue (purple) formazan products are generated in the direct proportion to the living cell numbers. Notably, this MTT assay

cannot distinquish between the cytotoxic and cytostatic effect of the tested drug or herb (Plumb, 2004).

Interestingly, MTT solution at 1 mg/mL increases the formazan product whereas MTT solution at 1-2 mg/mL will slow down the production (Sylvester, 2011). This is the reason why MTT solution is usually used at the concentration of 1 mg/mL. From the kinetics of formazan production, incubation time over 4 hours will get the plateau production curve. Therefore, standard incubation time at 3-4 hours is usually enough for the production time (Denizot and Lang, 1986).

As reported, the blue formazan products are partially dissolved in the medium. To induce complete solubilization of these MTT formazan products, several organic solvents have been tested and used such as ethanol, dimethylsulfoxide (DMSO), and isopropanol which is reported to be the most suitable solvent. After getting the homogeneous solution, optical density (OD) will be measured by reading on a scanning multiwell spectrophotometer (ELISA reader). The absorption spectrum of MTT formazan crystal is pH dependent. At pH 10.5, the maximum absorption is 570 nm. whereas, at pH 7.0, the absorption peak will show at 500 and 570 nm. (Plumb, 2004). The use of flat-bottomed well will help in increasing the sensitivity due to the doubled light-path length in the final optical density reading (Denizot and Lang, 1986).

In general, the accurate and reliable results from the MTT assay depend upon various important parameters which need to be optimized first. The proper cell number for seeding should be characterized by using standard curve. Changing into fresh medium prior to adding MTT solution is also critical in getting the good yield because of reduction of MTT activity in nutrient-depleted medium. Time and dose of MTT solution in each experiment may be different for each cell or cell lines used (Sylvester, 2011). Importantly, chemotherapeutic agents and some drugs such as valproic acid can yield the lower results from the MTT assay (Ari et al, 2010; Ulukaya et al, 2008). The experimental procedure is as followed (Kummalue et al, 2005).

Experimental procedure:

- Breast cancer cells will be seeded at $1x10^4$ cells per well in 96 flat-bottomed well plate containing 100µl of the culture medium DMEM supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotic.
- After 24 hours, the cells will be treated with herbal extract at final concentrations from 1, 10, 100, and 500 µg/mL for 48 hours. These serial concentrations are used for determining the activity in crude extracts only.
- After 48 hours incubation, medium will be changed into fresh medium, and MTT 50 µl (stock concentration at 1mg/mL in PBS) will be added to each well.
- After 4 hours incubation, the MTT solution will then be removed by rapid flick-off of the medium and 100 µl of 100% DMSO will be added to solubilize the formazan products. The dark purple solution from formazan products can be seen as shown in Figure 8.
- Plate will be rotated on the platform rotator for 10 minutes before further measurement the O.D. by a microplate Elisa reader at 595 nm.
- Calculation of ED_{50} (50% of inhibition of cancer cell growth) will be performed by using the formula:

$$\text{percentage of viability} = \text{OD sample}/\text{OD control} \times 100 \quad (1)$$

$$\text{percentage of inhibition} = 100 - \% \text{ viability}$$

Special comments:

- For pure compounds, serial concentrations can be diluted to the final concentrations as low as 0.1 µg/mL. This is because pure compounds are active compounds which are purified from crude extracts.
- After adding MTT solution, remember to wrap plate with aluminium foil because MTT substrate is light sensitive.
- We usually perform the O.D. measurement at 595 nm. because of the limitation of our instrument.
- Rotate the cell culture plate at the rotator platform for 10 minutes is important to induce the homogeneous solution that will be easy for the O.D. measurement and to achieve the correct data.
- The rapid flick-off of the medium after the MTT incubation will be done only in adherent cell lines such as breast cancer cell lines. For suspension cells such as leukemic cell lines, K562, the 100 µl of the stop solution (10% SDS dissolved in 0.01 N HCl) will be added into each well and incubated overnight.
- If dilute the herbal extract with DMSO, it is better to keep the percentage of DMSO in the final concentration not more than 0.5% to avoid cell damage and false positive result. In our experience, 1% DMSO still can cause some effect on cell viability.
- Please remember to set the control wells (both positive and negative control). We usually use doxorubicin or vinca alkaloid for positive control. Only cells in the medium will be used as negative control.
- The number of the seeding cells used in our experiment is from the standard curve at the O.D 0.7-1.2.
- We always perform the MTT assay with three independent experiments.

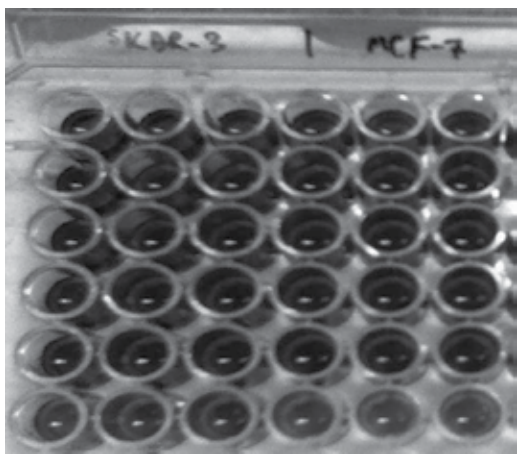


Fig. 8. The MTT assay showed the dark purple formazan products in the wells after dissolving the crystal with 100 µl of 100% DMSO.

4.2.2 Lactate dehydrogenase (LDH) cytotoxicity assay

Principle & mechanism of action

For MTT tetrazolium salt assay, the method measures the surviving cells after treatment with chemical substances. However, for the very low toxic reagents, calculation from the

data of control cells and treated cells from MTT assay results in low sensitivity. In this case, direct measurement of dead cells will give more accurate information (Sasaki et al, 1992). Therefore, development of assay using cytolytic enzymes releasing from dead cells has been initiated which can represent proportionally to the dead cell number (Sasaki et al, 1992). Detection of lactate dehydrogenase enzyme, one of the stable cytolytic enzyme found in animal cells, has been established for this purpose (Decker and Lohmann-Matthes, 1988).
Lactate dehydrogenase (LDH) enzyme has the function to catalyze lactate to pyruvate under anaerobic condition. It is a soluble enzyme in the cytoplasm. This enzyme will be released to the culture medium when the cell is ruptured or dead. Usually the assay will contain two steps. First, LDH releasing from the cells will oxidize lactate to generate NADH and H+. The second step, diaphorase in the reaction will use NADH and H+ to catalyze the tetrazolium salt such as WST-8, or INT to generate colored formazan which will absorbs strongly at wavelength 490-520 nm. The amount of the formazan production will correlate in the proportion to the damaged cells in the reaction (Haslam et al, 2000; Rodney et al, 1966). The experimental procedure is shown belowed (Kummalue et al, 2009; LDH-cytotoxicity assay kit with WST-8, MBL international corporation).

Experimental procedure:

- Breast cancer cells will be seeded at a density of 1 x 10^4 cells per well in 96 well plates flat-bottom in 100 µl of culture medium DMEM supplemented with 10% heat inactivated fetal bovine serum and 1% antibiotic.
- The next morning, cells will be treated with the herbal extract at various final concentrations from 1 to 500 µg/mL for 48 hours in the incubator. These concentrations are used to evaluate the ED50 for crude extracts only.
- Centrifuge the cells at 1500 rpm or 250 g for 10 minutes.
- Transfer 10 µl of culture medium into the new 96 well plate with corresponding well.
- Add 100 µl of LDH reaction mix (containing LDH and WST-8) to 10 µl of tested culture medium and incubated at 37 ºC with a 5% CO_2 incubator for an additional 30 minutes.
- The absorbance at 450 nm of the dissolved solution will be measured by using an Elisa plate reader.

Special comments:

All MTT, WST-8, XTT, and INT are commonly used tetrazolium salts. They have different chemical structures and properties such as solubility. These properties depend on the substituents on the tetrazole rings in the compounds (Zhivich et al, 1990).

4.2.3 Clonogenic assay

Principle & mechanism of action

This method for cell sensitivity assay is considered as the gold standard technique and has been used for a long time (Plumb, 2004). It is a well known method for testing the effects of drugs on the proliferation of treated cell lines. This assay can measure the ability of cells to proliferate and form colonies after treating cells with chemical substances or herbal extracts (cytostatic effect of drug) whereas cells that are killed by the drugs cannot grow and form colonies (cytotoxic effect of drug). In addition, clonogenic assay is reported to be able to detect cell survival as low as 1% (Plumb, 2004).
Staining technique is a very important step because the accuracy of counting colonies depends on the good visualization. Colony staining has been performed with several dye

solutions such as methylene blue, ethidium bromide, and crystal violet (Guda et al, 2007). Counting colony can be done by using manual counting with microscope or using the specific software (Niyazi et al, 2007). The experimental procedure is demonstrated belowed (Pratumvinit et al, 2009).

Experimental procedure:

- Breast cancer cell lines will be plated in 12 well plate at a density of $7x10^2$ cells per well.
- The next morning, cells will be treated with the herbal extract at the concentrations of 0, 50, 100, and 150 µg/mL.
- After 48 hours incubation, culture medium will be changed into fresh medium.
- Cells will be further cultured for 7-14 days until colonies are observed.
- Plates will be stained with 1 gm of crystal violet in 50% methanol for 30 minutes.
- Pour off the stain, carefully rinse with running water.
- Colonies will be counted by using ChemiDocXRS with specific software. Figure 9 showed the colonies detected in our experiment.

Special comments:

- The number of cells seeded in each well will be vary depending on the area of the well.
- The concentrations used in the experiment depend upon the ED_{50} value of each medicinal plant.
- Changing into fresh medium (after treated cells) before continue keeping cells in culture is very important for cell growth and colony formation.
- The time for culturing after exposure to the herbal extract depend upon the cell growth curve. For breast cancer cell lines, small colony can be detected after culturing cells for at least 7 days.
- Counting the colonies can be achieved by using only light microscope, no software use (but may be too difficult to count in some cases).
- We always perform the clonogenic assay with three independent experiments.

A B C

Fig. 9. Demonstration of colonies detected in the well. A. T47D breast cancer cells as a negative control. B. T47D treated with plant extract at 0.5 times ED_{50}. No colony was detected which was due to the cytotoxicity of the extract. C. T47D treated with 0.5% DMSO as a control because the extract was diluted with 0.5% DMSO. Colonies could be seen as dots at the bottom of the wells after staining with crystal violet in this experiment and counted by using the software.

4.3 Breast cancer cells and experimental procedures in mechanism of actions

This is usually the next step of investigation in searching for the new drug from plant sources. Plant extracts that exhibit the promising ED_{50} values, i.e., the plant extracts show some antiproliferative or cytotoxic activities on specific breast cancer cell lines, the extracts will be further studied to find the mechanism of actions in view of inhibition of cancer cells growth or even killing cells. Cell cycle analysis and apoptotic study are the most two common methods used in the field for searching the mechanism of actions in plants.

4.3.1 Breast cancer cells and experimental procedure in cell cycle study

Based on the knowledge of normal cell cycle, breast cancer cells actively synthesize DNA in the same process (Ross et al, 2003). Normally, the cell cycle phase contains G0, G1, S, G2, and M phase in order. Cells in the G0 phase or resting state contain diploid DNA which will enter the second step G1 (Gap 1). After this step, cells increase their DNA content to two folds with twice the diploid DNA content at the end. This accumulation state of DNA is S phase. Cells then enter the G2 phase (Gap 2) and finally go into the M phase, while M means the mitotic state. Finally, each mitotic cell will end up with 2 daughter cells.

In breast cancer cells, DNA ploidy has been reported to correlate with the prognosis in patients. The first retrospective report using flow cytometry to study DNA content as a predictor in breast cancer was done by Auer et al with pure diploid showing an excellent prognosis (Auer et al, 1980). However, the use of DNA ploidy as an prognostic indicator in clinical practice still remains in controversy (Ross et al, 2003). Estrogen has also been reported to induce breast cancer which might due to the inappropriate activation of cyclin dependent kinase and consequently abnormal transition through G1 phase (Foster et al, 2001).

The most popular technique used for demonstration DNA content in the cells is by flow cytometry (Nunez, 2001). Four distinct phases could be categorized, i.e., G1, G2, S, and M phases with G2 and M phase will represent at the same DNA content (so called G2/M phase). The staining techniques to distinquish these cell cycle phases are the measurement of the incorporation of bromodeoxyuridine (BrdU) and propidium iodide (Kummalue et al, 2002; Pratumvinit et al, 2009). Interference from RNA may result in misinterpretation as false positive. Therefore, the use of RNase should be added in the protocol. The experiment in breast cancer demonstrated here will share the experience in using the cell cycle kit which is much easier than the traditional technique. (The details of cell cycle study using BrdU incorporation and propidium iodide can be found in the references as mentioned in the text.) Belowed is the experimental procedure (Kummalue et al, 2007).

Experimental procedure:

- Breast cancer cells will be seeded at $1x10^6$ cells in 100 mm tissue culture dish and incubated overnight.
- The next morning, cells will be treated with the plant extract at the dosage ranging from 0.5 times ED_{50} to 2 times ED_{50}.
- After 48 hours incubation, cells will be harvested and incubated with reagents as described in the protocol of the CycleTEST™PLUS DNA reagent kit (Becton Dickinson, USA).
- Measurement of DNA content of cells will be performed within 3 hours by flow cytometry and analysed by CellQuest Software.

Special comments:

- The incubation time for cell cycle study depends on the time that is used for cell sensitivity assay.
- Dosage used in the experiment to investigate the effect of plant on cell cycle depends upon the ED_{50} of the plant extract.

4.3.2 Breast cancer cells and experimental procedure in apoptotic study

Apoptosis or programmed cell death is a very interesting investigation in drug discovery and development especially for cancer therapies. Searching for the anticancer agents that can induce apoptosis is necessary and needed for the effective treatment. For example, tamoxifen, which is prescribed as an adjuvant therapy in breast cancer, has been shown to induce apoptosis by down regulation of bcl-2 (Zhang et al, 1999). Therefore, most of medicinal plant researchers keep looking on finding these properties of plants. By the way, high cost and time consumption are unavoidable to face.

Apoptosis is a process that organisms, including human beings, use to tightly regulate the cell numbers and tissue size (Hengartner, 2000). It is caused by a group of cysteine proteases known as caspases. There are 2 major apoptotic pathways. One is via death receptor pathway which is triggered by death receptor superfamily such as CD95 and also tumor necrosis factor receptor I. This results in activation of caspase 8. The other pathway is mitochondrial pathway. This activates pro-apoptotic member of the Bcl-2 family and then pro caspase 9. Both pathways converge at the caspase 3 activation (Hengartner, 2000). To evaluate the apoptotic activity of plant extracts, caspase activity is usually investigated either by detection at the level of the enzyme caspase itself or by the level of its cleavage product, poly (ADP-ribose) polymerase-1 (PARP-1) (Los et al, 2002; Kummalue et al, 2011).

In summary, characterization of apoptosis is chromatin condensation, fragmentation of DNA (seen as ladder pattern in gel electrophoresis), and reduction of cell volume. Externalization of phosphatidylserine at the cell surface also plays an important role in macrophages recognition and consequently eradication of these apoptotic cells (Koopman et al, 1994). With these specific expression of phosphatidylserine, the new method was developed for detection of apoptotic cells by flow cytometry and has been frequently used nowadays. (Koopman et al, 1994). The experimental procedure is shown belowed. (Kummalue et al, 2011)

Experimental procedure:

- Cells will be seeded on 100 cm tissue culture dish at the density of $1x10^6$ cells.
- After 24 hours, cells will be treated with plant extracts at the various final concentrations ranging from 0.5 times ED_{50}, to 2 times ED_{50}.
- After incubating cells for various time points, cells will be harvested. The time points used to treat cells depend on the efficacy of the plant extract itself.
- Cells will be labeled with Annexin V-PI following the protocol of Annexin V-FITC Apoptosis Detection Kit from the manufacture and analysis by flow cytometry.

Special comments:

Detection of early and late apoptosis using annexin V depends upon the doses and time that treating the cells.

4.3.3 Breast cancer cells and specific mechanism of action of the plant extracts

Elucidation of the mechanism of action will greatly help in understanding the activity from plant sources on these breast cancer cell lines. Following the cell cycle and apoptotic studies as the basis, the mechanisms of these activities such as telomerase activity, antioxidant activity, and details of special signaling pathways are always considered for studying.

Telomerase is a eukaryotic ribonucleoprotein complex that helps in stabilizing telomere length in human stem cells, reproductive cells, and cancer cells (Shay and Bacchetti, 1997; Shay et al, 2001). The activity of telomerase increases in almost all human cancers except for the normal cells (Shay et al, 2001;Kim et al, 1994). Based on several studies, there are correlations between telomere shortening and growth failure of human cells (Harley et al, 1990; Shay et al, 2001). Therefore, human cancer cells show to have short telomeres with high telomerase, which is in contrast to normal human cells. Notably, cancer cells need to maintain telomeres for their immortalization. Based on this correlation, investigations of human cancer on telomerase activity have been done for over decades due to the potential development in drug targeting cancers.

Human telomerase consists of a catalytic protein component (hTERT) and integral RNA which are essential for the function of telomerase (Shay et al, 2001). hTERT is reported to be the critical for the production of telomerase (Nakamura et al, 1997). Investigation on telomerase activity can usually perform by detection the hTERT expression using RT-PCR. RNA extraction from treated breast cancer cells will be done before further processing to quantitate the level of transcripts. The method can be undertaken by real time RT-PCR and specific software for interpretation (Duangmano et al, 2010).

5. Breast cancer cells in medicinal plants research: Future direction in drug testing

Generating of induced pluripotent stem cells (iPS) for the first time in the world by Professor Dr. Shinya Yamanaka in Year 2006 has very high impact on several fields such as stem cell therapies, regenerative medicine, replacement therapy, and also drug screening and toxicity (Inoue and Yamanaka, 2011). Researchers worldwide are very interested in this iPS technology because of the high possibility in its usefulness and application to human diseases.

Induced pluripotent stem cells or iPS has first been generated from mouse tip tail fibroblasts which were somatic cells (Takahashi and Yamanaka, 2006). The process involved in transduction of fibroblasts with retrovirus carrying 4 transcription factors, i.e., Sox2, Klf4, c-Myc, and Oct3/4. These four factors are now known as Yamanaka's four factors. iPS cells exhibit several features characteristics of embryonic stem cells such as positive for alkaline phosphatase, forming teratoma in mice, and expression of specific embryonic antigen (Park et al, 2008). Nowadays, iPSs have been generated from various kinds of animals cells such as monkey, mouse, etc., including normal human cells, human cancer cell lines, and patients' cells to generate the patient-specific iPSs (Chun et al, 2010; Raya et al, 2009).

In drug screening and toxicity testing, human iPSs offer the high values in this field. For example, breast cancer patients' fibroblasts can be induced with transcription factors either 2 or 4 factors to create patient-specific iPS. Based on the fact that the iPS cells are generated from patients' own somatic cells, therefore, these specific iPS cells can be used to identify and test with the novel drug developing for cancer patients including the toxic effects of the drugs themselves (Chun et al, 2010). Interestingly, the advantage of iPS is the generation of

the library for human cancer that will represent the genetic and epigenetic variation of the population (Chun et al, 2010). In addition, since iPSs grow in culture like cell lines, therefore, this technology can provide the unlimited sources of cells for desired situation.

6. Discussion and conclusion

The investigation of the traditional used and evidence-based medicinal plants provides the chance to discover natural anticancer agents. The breast cancer cell lines with specific properties and the progress in antiproliferative and cytotoxic experiments are the important tools for the investigation of the medicinal plants. Clear differences in specific properties including receptors of these 4 breast cancer cell lines have been demonstrated so far. Comparing specific receptors, i.e., estrogen receptor, progesterone receptor, and Her2/neu in these 4 breast cancer cell lines: SKBR3, MCF7, T47D, and MDA-MB435 are demonstrated in Table 2. These 4 breast cancer cell lines are classified in different groups and express different receptors. Therefore, treatment of plant extracts on these 4 breast cancer cell lines can help, at least, in the decision whether or not the extracts will exert activities in breast cancer with different subtypes. However, the established cell lines from patients do not always represent the genotypes of parental tumor tissues (Tsuji et al, 2010). Therefore, testing in cell lines may not yield the same results when performing in vivo though most of these cells deregulate in signaling along the EgfR-MAPK pathway (Heiser et al, 2009).

Characters	**SKBR3**	**MCF7**	**T47D**	**MDA-MB435**
Category	Second group	First group	First group	Third group
Estrogen receptor	-	+	+	-
Progesterone receptor	-	+	+	-
Her-2/neu	overexpression	-	-	-

(-) = not expressed; (+) = expressed

Table 2. Characters of receptors on four breast cancer cell lines: SKBR3, MCF7, T47D, and MDA-MB435 (http://www.cell-lines-service; Lacroix and Leclercq, 2004; Hevir et al, 2011).

Searching for the novel chemotherapeutic agents from plant sources usually involves with the initial process of antiproliferative activity. These three non-radioactive assays are commonly used nowadays in the determination of antiproliferative and cytotoxic activities. Several aspects from them are summarized in Table 3, i.e., sensitivity, cost and time consuming for each assay. Notably, each assay has its own advantages and disadvantages which should be carefully considered.

Prediction of toxicology and therapeutic responses induced by the novel drugs can be approached by iPS technology as mentioned aboved. Lots of current disease specific based patients iPS have been reported since then (Chun et al, 2010). This application as a personalized approach leads to the specialized model in vitro. However, the safety and risk in applying to the clinical trials should be aggressively considered because of the techniques in generation of iPS with viral gene transfer.

The elucidation of the action mechanism provides the concrete evidence of the study plant. The utilization of herbs as natural anticancer agents can solve the problem of the

	MTT assay	LDH assay with WST-8	Clonogenic assay
Sensitivity	++ (high sensitivity for antiproliferative activity)	+++ (high sensitivity for cytotoxic activity)	+ (low sensitivity and high false negative result due to small colony formation)
Cost consuming	++ (high cost reagent)	++ (high cost reagent)	+ (low cost staining)
Time consuming	++ (moderate term culture)	+ (short term culture)	+++ (long term culture and time spending in counting)

+++ represent the highest scores
+ represent the lowest scores

Table 3. Comparison of three non-radioactive methods for assaying the antiproliferative activities (Kosaka et al, 1996; Kawada et al, 2002; Fotakis and Timbrell, 2006; Miyamoto et al, 2002).

unaccessible anticancer drugs. We investigated thoroughly two Thai herbs and discovered the possible benefit to the breast cancer patients. *T. cucumerina* fruit juice exerted strongly antiproliferative effect. The juice itself can be considered as a natural cytostatic agent because of its potent effect. According to the presence of cucurbitacins, the juice irritates the mucous membrane of the gastrointestinal tract. The juice produced toxicity to the central nervous system and the respiratory tract, the usage of the herbal juice should be under the supervision of the physician.

For the part of *M. loriformis* herb juice, it can be used as an adjunct to the modern therapy according to the moderate cytotoxicity, immunomodulatory effect and safety. The patient cases, which were reported by the physicians, have supported the use of herbal juice to reduce the side effects from the modern therapy. Several active compounds in the pressed juice worked together to exert the activities, which were expected to have broad spectrum and lowered toxicity. The appropriate daily doses of cytotoxic effect from *T. cucumerina* and immunomodulatory effect from *M. loriformis* require further clinical investigation.

F. hispida and *E. elliptilimba* are interesting herbs for further investigation. It was also noticeable that herbal remedy could potentiate the effect of anticancer drug.

7. References

Alshatwi AA. (2010) Catechin hydrate suppresses MCF7 proliferation through TP53/caspase-mediated apoptosis. J Exp Clin Cancer Res Vol. 29 pp. 167-75.

Ari F, Ikitimur E, Ulukaya E. (2010) The ATP assay, but not the MTT assay, detects further cytotoxicity of the combination of anthracycline-based therapy with histone deacetylase inhibitor (valproic acid) in breast cancer cells. Turk J Biochem Vol. 35 pp. 293-9.

Auer GU, Capersson TO, Wallgren AS. (1980) DNA content and survival in mammary carcinoma. Vol. 2 pp. 161-5.

Book of traditional Chinese Medicine in Sichuan. (1992) pp. 181. (chinese).

Casarett LJ and Doull J. (1975) Toxicology: The basic science of poisons. New York: MacMillan Publishing Co., pp. 24.

Chaiwerawatana A. (2007) Chapter 13. Cancer in Thailand Vol. IV, 1998-2000. Edited by Khuhaprema T, Srivatanakul P, Sriplung H, Wiangnon S, Sumitsawan Y, Attasara P. Bangkok Medical Publisher, Bangkok, Thailand. pp. 48-50.

Chambers A. (2009) MDA-MB435 and M14 cell line: Identical but not M14 melanoma Cancer Res Vol. 69 pp. 5292-3.

Chandramouli KH, Agrawal P, Thimmaiah KH. (2009) Protein identification in sub proteome fractions of breast cancer cells by OFFGEL-IEF and iTRAQ labeling. Current Proteomic Vol. 6 pp. 43-50.

Chen L, Xu S, Zeng X, Li J, Yin W, Chen Y, et al. (2010) c-myb activated CXCL12 transcription in T47D and MCF7 breast cancer cells. Acta Biochem Biophys Sin Vol. 42, pp. 1-7.

Chun YS, Chaudhari P, Jang YY. (2010) Application of patient-specific induced pluripotent stem cells; focused on disease modeling, drug screening and therapeutic potential for liver disease. Int J Biol Sci Vol.6, No.7, pp. 96-805.

Decker T, Lohmann-Matthes ML. (1988) A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunolo Methods Vol. 15 pp. 61-9.

Denizot F, Lang R. (1986) Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods Vol. 89 pp. 271-7.

Dingermann T. (2000) (unter Mitarbeit der Expertenkommission BARMER/BPI). Transporenzkriterien fuer pflanzliche, homoeopathische und anthroposophische Arzueimittch Skarger. Freiburg. pp 3-4.

Duangmano S, Dakeng S, Jiratchariyakul W, Suksamrarn A, Smith DR and Patmasiriwat P. (2010) Antiproliferative effects of cucurbitacin B in breast cancer cells: down-regulate c-Myc/hTERT/telomerase pathway and obstruct the cell cycle. Int. J. Mol. Sci. Vol. 11 No. 12 pp. 5323-5338.

Foster JS, Henley DC, Ahamed S, Wimalasena J. (2001) Estrogens and cell cycle regulation in breast cancer. Trends Endocrinol Metabol Vol.12 pp. 320-7.

Fotakis G, Timbrell JA. (2006) In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicology Vol.160. pp. 171-7.

Guda K, Natale L, Markowitz S. (2007) An improved method for staining cell colonies in clonogenic assays. Cytotechnology Vol.54 pp. 85-8.

Haensel R, Hoelzl J. (1996) Lehrbuch der pharmazeutischen biologie. Spinger-Verlag. Heidelberg. pp. 516-7.

Haensel R, Sticher O. (2007) Pharmakognosie – Phytopharmazie. 8th rev.ed. Springer, Heidelberg : Springer-Medizin Verlag pp. 928-30.

Hagemann T, Binder C, Binder L, Pukrop T, Trumper L, Grimshaw M. (2005) Expression of endothelins and their receptors promotes an invasive phenotype of breast tumor cells but is insufficient to induce invasion on benigh cells. DNA Cell Biol Vol.24 pp.766-76.

Hamelers I, van Schaik R, Sussenbach J, Steenbergh P. (2003) 17 beta-estradiol responsiveness of MCF7 laboratory strains is dependent on an autocrine signal activating the IGF type I receptor. Cancer Cell Inter 3:10. Published online 2003 July 11. doi:10.1186/1475-2867-3-10.

Harley CB, Fletcher AB, Greider CW. (1990) Telomeres shorten during aging. Nature Vol. 345 pp. 458-60.

Haslam G, Wyatt G, Kitos PA. (2000) Estimating the number of viable animal cells in multi-well cultures based on their lactate dehydrogenase activities. Cytotechnology Vol. 32 pp. 63-75.

Heiser LM, Wang NJ, Talcott CL, Laderoute KR, Knapp M, Guan Y, et al. (2009) Integrated analysis of breast cancer cell lines reveals unique signaling pathway. Genome Biol Vol. 10 No. 3: R31. Published online 2009 March 25. doi:10.1186/gb-2009-10-3-r31.

Hengartner M. (2000) The biochemistry of apoptosis. Nature Vol.407 pp.770-6.

Hevir N, Trost N, Debeljak N, Rizner L. (2011) Expression of estrogen and progesterone receptors and estrogen metabolizing enzymes in different breast cancer cell lines. Chemico biological Interaction 2011 online doi:10.1016/j.cbi.2010.12.013.

Inoue H, Yamanaka S. (2011) The use of induced pluripotent stem cells in drug development. Nature 23 March, 2011. Doi:10.1038/clpt.2011.38.

Intayot Y, Kinouchi T, Kataoka K, et al. (2002) Antimutagenicity of *Murdannia loriformis* in the Salmonella mutation assay and its inhibitory effects on azoxymethane-induced DNA methylation and aberrant crypt focus formation in male F344 rats. J Med Invest Vol. 49 pp. 25-34.

Itharat A, Ooraiku B. (2007). Chapter 13 Research on Thai medicinal plants for cancer treatment, In: Advance Medicinal Plant Research Acharaya SN, Thomas JE, Trivandrum, pp.287-317,

Jiratchariyakul W and Frahm AW. (1992) Cucurbitacin B and dihydrocucurbitacin B from *Trichosanthes cucumerina* L. Mahidol Univ J Pharm Sci Vol. 19 pp, 5-12.

Jiratchariyakul W, Moogkarndi P, Theppeang K, Sethjintanin D, Jarikasem S and Frahm AW. (1999) Cytotoxic principles from *Trichosanthes cucumerina* L. Thai J Phytopharmacy Vol. 6, No. 2 pp. 1-9.

Jiratchariyakul W, Moongkarndi P, Okabe H, Frahm AW. (1997) Investigation of anticancer components from *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy. In : Proceeding of the first Pharma Indochina conference on Pharmaceutical Sciences. Pharmacy in Harmony. Faculty of Pharmacy, Mahidol Univ. Bangkok, Thailand, 20-23 May 1pp. 171-92.

Jiratchariyakul W, Okabe H, Moongkarndi P, et al. (1998) Cytotoxic glycosphingolipid from *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy. Thai Journal of Phytopharmacy Vol. 5 No. 1 pp. 10-20.,

Jiratchariyakul W, Vongsakul M, Sunthornsuk L, Moongkarndi P, Narintorn A, Somanabandhu A, Okabe H, Frahm A.W. (2006) Immunomodulatory Effect and Quantitation of a Cytotoxic Glycosphingolipid from *Murdannia loriformis*. J Nat Med Vol. 60 pp. 210-216.

Kawada K, Yonei T, Ueoka H, Kiura K, Tabata M, Takigawa N, et al. (2002) Comparison of hemosensitivity tests: Clonogenic assay versus MTT assay. Acta Med Okayama Vol. 56 pp. 129-34.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. (1994) Specific association of human telomerase activity with immortal cells and cancer. Science Vol. 266 pp. 2011-15.

Kongtun S, Jiratchariyakul W, Kummalue T, Tan-ariya P, Kunnachak S, Frahm AW. (2009) Cytotoxic properties of root extract and fruit juice of *Trichosanthes cucumerina*. Planta Med Vol. 75 pp. 839-842.

Koopman G, Reutelingsperger CPM, Kuijten GAM, Keehnen RMJ, Pals ST, Oers V. (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood Vol. 84 pp. 1415-20.

Kosaka T, Fukaya K, Tsuboi S, Pu H, Ohno T, Tsuji T, et al. (1996) Comparison of various methods of assaying the cytotoxic effects of ethanol on human hepatoblastoma cells (HUH-6 line). Acta Med Okayama Vol. 50 pp.151-6.

Kummalue T, Jiratchariyakul W, Srisapoomi T, Sukpanichnant S, Hara T, Tani K. (2009) Antiproliferative effect of cucurbitacin B extracted from *Trichosanthes cucumerina* L. on human cancer cell Lines. Siriraj Med J Vol. 61 No. 2 pp. 75-7.

Kummalue T, Lou J, Friedman A.D. (2002) Multimerization via Its Myosin domain facilitates nuclear localization and Inhibition of CBF activities by the CBFbeta- SMMHC myeloid leukemia oncoprotein. Mol. Cell. Biol. Vol. 22 No.23 pp. 8278-91.

Kummalue T, O-charoenrat P, Jiratchariyakul W, Chanchai M, Iemsri S. (2005) Antiproliferative activities of three Thai medicinal plants on human cancer cells. Siriraj Med J Vol. 57 pp. 491-5.

Kummalue T, O-charoenrat P, Jiratchariyakul W, Chanchai M, Pattanapanyasat K, Sukapirom K, Iemsri S. (2007) Antiproliferative effect of *Erycibe elliptilimba* on human breast cancer cell lines. J Ethnopharmacol Vol. 110 No. 3 pp. 439-43.

Kummalue T, Sujiwattanarat P, Jiratchariyakul W. (2011) Apoptotic inducibility of *Sapindus rarax* water extract on A549 human lung cancer cell line. J Med Plants Res Vol. 5(7), pp. 1087-94.

Lacroix M, Leclercq G. (2004) Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat Vol. 83 pp. 249-89.

Lacroix M. (2009) MDA-MB435 cells are from melanoma, not from breast cancer. Cancer Chemother Pharmacol Vol. 63 pp. 567.

Los M, Mozoluk M, Ferrari D, Stepczynska A, Stroh C, Renz A, et al. (2002) Activation and caspase-mediated inhibition of PARP a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling. Molecular biol Cell 13:978-8.

Mandal SC, Ashok CK (2002). Studies on anti-diarrhoeal activity of *Ficus hispida*. Leaf extract in rats. Fitoterapia Vol. 73 pp. 663-7.

Miyamoto T, Min W, Lillehoj HS. (2002) Lymphocyte proliferation response during Eimeria tenella infection assessed by a new, reliable, nonradioactive colorimetric assay. Avian Dis vol.46 pp. 10-6.

Moseley JM, Findlay DM, Gorman JJ, Michelangeli VP, Martin TJ. (1983) The calcitonin receptor on T47D breast cancer cells. Biochem Vol. 212 pp.609-16.

Mosmann T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods Vol. 65 pp.55-63.

Nakamura TM, Morin GB, Chapman KB, Weinrich SL, Andrews WH, Linger J, et al. (1997) Telomerase catalytic subunit homologs from fission yeast and humans. Science Vol.277 pp. 955-9.

Niyazi M, Niyazi I, Belka C. (2007) Counting colonies of clonogenic assays by using densitometric software. Rad Oncol 2:4 Published online 2007 January 9. doi:10.1186/1748-717x-2-4.

North WG, Gao G, Memoli VA, Pang RH, Lynch L. (2010) Breast cancer expresses functional NMDA receptors. Breast Cancer Res Treat Vol. 122 pp. 307-14.

Nunez R. (2001) DNA measurement and cell cycle analysis by flow cytometry. Curr Issues Mol Biol Vol.3 pp.67-70.

Ozbay T, Nahta R. (2008) A novel unidirectional cross-talk from the insulin-like growth actor –I receptor to leptin receptor in human breast cancer cells. Mol Cancer Res Vol. 6 pp. 1052-8.

Park I, Zhao R, West J, Yabuuchi A, Huo H, Ince T, et al. (2008) Reprogramming of human somatic cells to pluripotency with defined factors. Nature Vol. 451 pp. 141-7.

Peraza-Sanchez, SR, Chai HB, Shin YG, Santisuk T, Reutrakul V, Farnsworth NR, Cordell GA, Pezzuto JM, Kinghorn AD (2002). Constituents of the leaves and twigs of *Ficus hispida*. Planta Med. Vol. 68 pp. 186-8.

Pevsner P, Naftolin F, Hillman D, Miller D, Fadiel A, Kogus A, et al. (2007) Direct identification of proteins from T47D cells and murine brain tissue by matrix-assisted laser desorption/ionization post-source decay/collision-induced dissociation. Rapid Commun Mass Spectrom Vol. 21 pp. 429-36.

Plumb Jane A. (2004) Cell sensitivity assays: The MTT assay. Methods Mol Med 88:165-9.

Pratumvinit B, Srisapoomi T, Worawattananon P, Opartkiattikul N, Jiratchariyakul W and Kummalue T. (2009) In vitro antineoplastic effect of *Ficus hispida* L. plant against breast cancer cell lines. Journal of Medicinal Plants Research Vol. 3 No. 4 pp. 255–261.

Rae JM, Ramus SJ, Waltham M, Armes JE, Campbell IG, Clarke R, et al. (2004) Common origins of MDA-MB435 cells from various sources with those shown to have melanoma properties. Clin Exp Met Vol. 21 pp. 543-52.

Raya A, Rodriguez-Piza I, Guenechea G, Vassena R, Navarro S, Barrero MJ, et al. (2009) Disease-corrected haematopoietic progenitors from Fanconi anemia induced pluripotent stem cells. Nature Vol. 460 pp. 53-60.

Report from the first seminar on herbs and cancer : *Murdannia loroformis*, held by National Cancer Institute of Thailand (1988) RamaVI Rd., Bangkok, June.

Rodney D, Capps II, Batsakis J, Briere RO, Calam R. (1966) An automated colorimetric (Tetrazolium salt) assay for serum lactate dehydrogenase. Clin Chem Vol. 12 pp. 406-13.

Ross JS, Linette GP, Stec J, Ross M, Anwar S, boguniewicz A. (2003) DNA ploidy and cell cycle analysis in breast cancer. Am J Clin Pathol 120 (Suppl 1): S72-S84.

Sasaki T, Kawai K, Saijo-Kurita K, Ohno T. (1992) Detergent cytotoxicity: Simplified assay of cytolysis by measuring LDH activity. Toxic In Vitro Vol. 6 pp. 451-7.

Shay JW, Bacchetti S. (1997) A survey of telomerase activity in human cancer. Eur J Cancer Vol. 33 pp. 787-91.

Shay JW, Zou Y, Hiyama E, Wright W. (2001) Telomerase and cancer. Hum Mol Gen Vol. 10 pp. 677-85.

Silapaarcha W, Picha P, Lurwongrattana O, Kittiwongsunthorn W, Ungsunthornsarit P. (1981) Investigation of the triterpenes in cucurbitaceae prevalent in Thailand. Mahidol University journal Pharmaceutical Science Vol. 8, No. 1 pp. 5-8.

Sintusarn, U. (2002) Natural Product of Chao-Krom-Po, 2nd ed. Thammasarn Printing, Bangkok, p. 190.

Slater TF, Sawyer B., Strauli UD. (1963) Studies on succinate tetrazolium reductase systems. III points of coupling of four different tetrazolium salts. Biochem Biophys Acta Vol. 77 pp. 383-93.

Srisapoomi T, Jiratchariyakul W, O-partkiattikul N, Kummalue T. (2008) Effects of two Thai herbal remedies on the sensitivity of chemotherapeutic agents in human cancer cells. Asian Journal of Traditional Medicine Vol. 3 No. 4 pp. 144-152.

Sylvester PW. (2011) Optimization of tetrazolium dye (MTT) colorimetric assay for cellular growth and viability. Methods Mol Biol Vol. 716 pp. 157-68.

Takahashi K, Yamanaka S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell Vol. 126 pp. 663-76.

Tappayuthpijarn P, Sattaboos P, Pidetcha P. (1991) Subchronic toxicity of *Murdannia loriformis*. Siriraj Hosp Gaz Vol. 43 pp. 529-533.

Tappayuthpijarn P, Wamanutajinda V, Pidetcha P. (1991) Acute toxicity of *Murdannia loriformis*. Siriraj Hosp Gaz Vol. 43 pp. 458-462.

Tiangda C, Silapaarcha W, wiwat C, Picha P. (1986) Chemical composition, acute toxicity and pharmacological screening of *Trichosanthes cucumerina* L., *Proceeding of the 11th Asian Congress of Pharmaceutical Sciences* pp. 320-324, Bangkok, Thailand,

Tsuji K, Kawauchi S, Saito S, Furuya T, Ikemoto K, Nakao M, et al. (2010) Breast cancer cell lines carry cell line-specific genomic alterations that are distinct from aberrations in breast cancer tissues: Comparison of the CGH profiles between cancer cell lines and primary cancer tissues. BMC Cancer 10:15 Published online 2010 January 14. doi:10.1186/1471-2407-10-15.

Ulukaya E, Ozdikicioglu F, Oral AY, Demirci M. (2008) The MTT assay yields a relatively lower result of growth inhibition than the ATP assay depending on the chemotherapeutic drugs tested. Toxicol In Vitro Vol. 22 No.1 pp. 232-9.

U-pratya Y, Jiratchariyakul W, Kummalue T. (2008) Anti-proliferative effects of *Pouzolzia indica* on acute promyelocytic cell lines: NB4 and HT93A. Asian Journal of Traditional Medicines Vol. 3 No. 4 pp. 124-133.

U-pratya Y, Lueangamornnara U, Jiratchariyakul W and Kummalue T. (2010) Immunosuppressive effects of Cucurbitacin B on human peripheral blood lymphocytes. Journal of Medicinal Plants Research Vol. 4 No. 22 pp. 2340–2347.

Vinitketkumnuen U, Charoenkunathum W, Kongtawelert P, Lertprasertsuk R, Picha P, Matsushima T. (1996) DT-diaphorase inducer activity of antimutagenic Thai medicinal plant, *Murdannia loriformis*. J. Herbs & Spices and Medicinal Plants. Vol. 4 pp. 45-51.

Westmose Yde C, Issinger OG. (2006) Enhancing cisplatin sensitivity in MCF7 human breast cancer cells by down regulation of Bcl-2 and cyclin D1. Inter J Oncol 29:1397-1404.

Wuttidhammaved,W. (1997) Thai Traditional Medicine, revised ed. O-dien Store Printing, Bangkok, p. 339.

Xiao Y, Gao X, Maragh S, Telford WG, Tona A. (2009) Cell lines as candidate reference materials for quality control of ERBB2 amplification and expression assays in breast cancer. Clin Chem Vol. 55 pp.7.

Xu J, Zhang Y, Berry PA, Jiang J, Lobie PE, Langenheim JF, et al. (2011) Growth hormone signaling in human T47D breast cancer cells: Potential role for a growth hormone receptor-prolactin receptor complex. Mol Endocrinol Vol. 25 pp. 597-610.

Zhang GJ, Kimijima I, Onda M, Kanno M, Sato H, Watanabe T, et al. (1999) Tamoxifen-induced apoptosis in breast cancer cells relates to down-regulation of bcl-2, but not bax and bcl-XL, without alteration of p53 protein levels. Clin Cancer Res Vol. 5 pp. 2971-7.

Zhang Y, Pu X, Shi M, Chen L, Qian L, Song Y, et al. (2007) c-Jun, a crucial molecule in metastasis of breast cancer and potential target for biotherapy. Oncology Reports Vol. 18 pp. 1207-12.

Zhivich AB, Koldobskii GI, Ostrovskii VA. (1990) Tetrazolium salts (Review) Chemistry of Heterocyclic compounds Vol. 26 pp. 1319-28.

13

Red American Ginseng and Breast Cancer

Chong-Zhi Wang, Guang-Jian Du and Chun-Su Yuan
Tang Center for Herbal Medicine Research and Department of Anesthesia & Critical Care, The Pritzker School of Medicine, University of Chicago, Chicago, U.S.A.

1. Introduction

Breast cancer is the most frequently diagnosed form of cancer among women. Breast cancer is the leading cause of cancer death in nearly all developed countries, showing an increased incidence over the last decades, and the expected number of new U.S. patients in 2010 would be 209,060, and the death number will be 40,230 [1]. The clinical management of breast cancer invariably involves diverse conventional modalities, including surgery, chemotherapy, and radiation [2]. The complex characteristics of breast cancer may also require some alternative management to improve the therapeutic efficacy of conventional treatment and the quality of life for cancer patients [3].

American ginseng (*Panax quinquefolius* L.) is a commonly used herb in the United States which belongs to the genus *Panax* L. in family Araliaceae [4]. *Panax* is a small genus, and nearly all species in this genus are important herbal medicines, especially Asian ginseng (*Panax ginseng* C. A. Meyer) [5]. Since Asian ginseng has been used as herbal medicine for a long history in oriental countries, many studies have been conducted on its constituents and its pharmacological effects [6]. Asian ginseng has many reported health benefits including anticancer activities [7-9]. In the 1990s, a case-control study on over a thousand Korean subjects showed that long-term ginseng consumption was associated with a decreased risk for many different cancers compared with those who did not consume ginseng [10,11]. In contrast to many studies on Asian ginseng's anticancer effects, investigation of American ginseng is limited [7], and its mechanisms of action are largely unknown.

American ginseng extracts were found to inhibit the growth of breast cancer cells[12]. We previously investigated the effects of several herbal extracts on reducing chemotherapeutic side effects and found that American ginseng can attenuate cisplatin-induced nausea and vomiting in a rat model, while not affecting its anticancer properties in human cancer cells[13]. In addition, the extract from American ginseng enhanced the antiproliferation effect of cisplatin on human breast cancer cells, suggesting that it possesses its own anticancer activity[13].

In Asia, ginseng root is air-dried into white ginseng or steamed at 100 °C to give red ginseng. It is believed that red ginseng is more pharmacologically effective than white ginseng [14]. The differences in the biological effects of white and red Asian ginseng are attributed to the significant changes in ginsenosides from the steaming treatment [15]. In this study, we treated American ginseng root at various temperatures and heating times. After heat-processing, the root of American ginseng, like Asian ginseng, changes from white to red; steamed *P. quinquefolius* root is therefore referred to as red American ginseng.

Currently, the most popular herbal products are in extract forms. New regulations released by FDA on alternative complementary supplements require the botanical extracts to be standardized [16]. The contents of ginsenosides in the extracts were not assayed before. We treated American ginseng root at various temperatures and heating times. The contents of representative ginsenosides in unsteamed and steamed American ginseng root extracts were determined using HPLC. In addition to the cell line MCF-7, we also used the MDA-MB-230 human breast cancer cells to evaluate the chemopreventive activities of unsteamed and steamed American ginseng root extracts and four ginsenosides. To observe the anticancer mechanisms, the effects of American ginseng extracts and single compounds on apoptosis and cell cycle were evaluated.

2. Materials and methods

2.1 Chemicals

All solvents were of high-performance liquid chromatography (HPLC) grade from Fisher Scientific (Norcross, GA). Milli Q water was supplied by a water purification system (US Filter, Palm Desert, CA). Standards for ginsenosides Rb_1, Rb_2, Rc, Rd, Re, and Rg_1 were obtained from Indofine Chemical Company (Somerville, NJ); ginsenosides Rb3, Rg2, Rg3, Rh1, Rh2 and 20*(R)*-ginsenoside Rg2 (20*R*-Rg2) were obtained from the Delta Information Center for Natural Organic Compounds (Xuancheng, Anhui, China). All standards were of biochemical-reagent grade and at least 95% pure as confirmed by HPLC. All the plastic materials were purchased from Falcon Labware (Franklin Lakes, NJ). Trypsin, RPMI 1640 medium, fetal bovine serum (FBS), and penicillin/streptomycin solution (200×) were obtained from Mediatech, Inc. (Herndon, VA). A CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit was obtained from Promega (Madison, WI). Annexin V-FITC, cyclin A-FITC and cyclin D1-FITC were obtained from BD Biosciences (San Diego, CA).

2.2 Plant materials, processing and extraction

American ginseng roots (*Panax quinquefolius* L.) were obtained from Roland Ginseng, LLC (Wausau, WI). The voucher samples were deposited at the Tang Center for Herbal Medicine Research at University of Chicago (Chicago, IL). For the heat-processing of American ginseng, the roots were steamed at 100 °C and 120 °C for 1 h or at 120 °C for 0.5, 1, 2, 3, and 4 h.

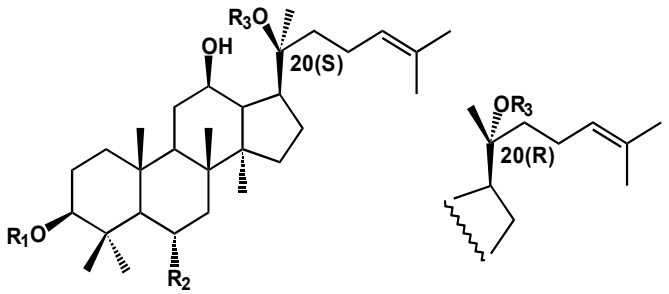
For the HPLC analysis, dried American ginseng sample (0.5 g) was extracted with methanol in a Soxhlet extractor for 8 h. The extract was concentrated, transferred into a 25-ml volumetric flask and diluted to the desired volume with methanol. One millimeter of the solution was purified by solid-phase extraction [17]. Purified solutions were stored at 4 °C until HPLC analysis.

For the *in vitro* anti-cancer studies, the process for the extraction of American ginseng samples was as follows: A root sample, unsteamed or steamed at various temperatures and times, was ground to powder and passed through a 40 mesh screen. Then 25 g of powder was extracted with 500 ml of 70% ethanol for 4 h; the water bath was maintained at 90 °C. When cooled, the solution was filtered with P8 filter paper (Fisher Scientific, Pittsburgh, PA) and the filtrate was collected. The residue was extracted with 500 ml of 70% ethanol once more and then filtered while the solution was cooled. The filtrates were combined and the solvent was evaporated under vacuum. The dried extract was dissolved in 100 ml water and then extracted with water-saturated *n*-butanol. The *n*-butanol phase was evaporated under vacuum and then lyophilized.

The root of *P. quinquefolius* L. was collected from Roland Ginseng, LLC (Wausau, WI, USA).

2.3 HPLC analysis

The HPLC system was a Waters 2960 instrument (Milford, MA) with a quaternary pump, an automatic injector, a photodiode array detector (Model 996), and Waters Millennium 32 software for peak identification and integration. The separation was carried out on a 250×3.2 mm i.d., 5μ, Ultrasphere C18 column (Alltech, Deerfield, IL) with a 7.5×3.2 mm i.d. guard column. For HPLC analysis, a 20-μL sample was injected into the column and eluted at room temperature with a constant flow rate of 1.0 ml/min. For the mobile phase, acetonitrile (solvent A) and water (solvent B) were used. Gradient elution started with 18% solvent A and 82% solvent B. Elution was changed to 21% A for 20 min, then to 26% A for 3 min and held for 19 min. It was then changed to 36% A for 13 min, to 50% A for 9 min, to 95% A for 2 min, and held for 3 min. Lastly elution was changed to 18% A for 3 min and held for 8 min. The detection wavelength was set to 202 nm. All tested solutions were filtered through Millex 0.2-μm nylon membrane syringe filters (Millipore Co., Bedford, MA) before use. The linearity of this method was assayed by analyzing standard solutions in the range of 2-400 μg/ml for the 12 ginsenosides. Calibration curves were constructed from the measured peak areas and the related amount of ginsenosides. Ginsenosides Rb1, Rb2, Rb3, Rc, Rd, Re, Rg1, Rg2, 20*R*-Rg2, Rg3, Rh1 and Rh2 (Figure **1**) in extract samples were identified by comparison of their retention times with those obtained from the chromatograms of mixed ginsenoside standards. The contents of saponins in each sample were calculated using standard curves of ginsenosides.



Name	R1	R2	R3
Ginsenoside Rb1	-glc^{2}- ^{1}glc	-H	20(S), -glc^{6}- ^{1}glc
Ginsenoside Rb2	-glc^{2}- ^{1}glc	-H	20(S), -glc^{6}- ^{1}ara(pyr)
Ginsenoside Rb3	-glc^{2}- ^{1}glc	-H	20(S), -glc^{6}- ^{1}xyl
Ginsenoside Rc	-glc^{2}- ^{1}glc	-H	20(S), -glc^{6}- ^{1}ara(fur)
Ginsenoside Rd	-glc^{2}- ^{1}glc	-H	20(S), -glc
Ginsenoside Re	-H	-O-glc^{2}- ^{1}rha	20(S), -glc
Ginsenoside Rg1	-H	-O-glc	20(S), -glc
Ginsenoside Rg2	-H	-O-glc^{2}- ^{1}rha	20(S), -H
20R-ginsenoside Rg2	-H	-O-glc^{2}- ^{1}rha	20(R), -H
Ginsenoside Rg3	-glc^{2}- ^{1}glc	-H	20(S), -H
Ginsenoside Rh1	-H	-O-glc	20(S), -H
Ginsenoside Rh2	-glc	-H	20(S), -H

Fig. 1. Chemical structures of assayed ginsenosides in American ginseng.

Ginsenoside Rg3 increased significantly from 0.5 to 3 h of steaming. Ginsenoside Rh2 is also an active anticancer saponin, however, after steaming treatment, the content of Rh2 was very low (Table **1**).

Ginsenoside	Asian Ginseng	Red Asian Ginseng	American Ginseng	Steaming Time (Red American Ginseng)				
				0.5 h	1 h	2 h	3 h	4 h
Rg1	0.279	0.276	0.133	0.098	0.075	0.017	ND	ND
Re	0.234	0.188	1.750	1.347	0.968	0.221	0.032	ND
Rh1	ND	0.015	ND	0.008	0.017	0.040	0.042	0.049
Rg2	0.031	0.034	0.015	0.154	0.266	0.405	0.444	0.363
20*R*-Rg2	ND	0.022	ND	0.086	0.155	0.388	0.425	0.422
Rb1	0.545	0.462	4.940	3.918	3.252	1.743	0.735	0.177
Rc	0.523	0.294	0.387	0.325	0.278	0.141	0.087	0.018
Rb2	0.443	0.200	0.043	0.042	0.036	0.026	0.018	ND
Rb3	0.067	0.032	0.063	0.064	0.050	0.035	0.017	ND
Rd	0.257	0.073	0.604	0.510	0.460	0.335	0.206	0.097
Rg3	0.004	0.033	0.003	0.143	0.271	0.664	1.053	1.225
Rh2	ND	0.009	0.007	0.007	0.018	0.047	0.057	0.066
Total	**2.38**	**1.64**	**7.95**	**6.70**	**5.85**	**4.05**	**3.11**	**2.42**

n = 3; ND, not detected; values are expressed as percentage of dry weight; RSD (relative standard derivation) are less than 15%.

Table 1. Ginsenoside contents in Asian ginseng, red Asian ginseng and American ginseng which were unsteamed or steamed at 120°C for 0.5-4 h.

2.4 Cell culture

The human breast cancer cell lines MCF-7 and MDA-MB-231 were purchased from American Type Culture Collection, ATCC (Manassas, VA, USA) and grown in RPMI 1640 medium supplemented with 10% FBS and 50 IU penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

2.5 Cell proliferation analysis

Cells were seeded in 96-well plates. After 1 d, various concentrations of extracts/ginsenosides were added to the wells. The final concentration of ethanol was 0.5%. Controls were exposed to culture medium containing 0.5% ethanol without drugs. All experiments were performed in triplicate and repeated 3 times. Cell proliferation was evaluated using an MTS assay according to the manufacturer's instructions. Briefly, at the end of the drug exposure period, the medium was replaced with 100 µl of fresh medium, 20 µl of MTS reagent (CellTiter 96 Aqueous Solution) in each well, and the plate was returned to the incubator for 1-2 h. A 60-µl aliquot of medium from each well was transferred to an ELISA 96-well plate and its absorbance at 490 nm was recorded [4,18]. Results were expressed as percentage of control (ethanol vehicles set at 100%).

2.6 Apoptosis assay

Cells were seeded in 24-well tissue culture plates. After culturing for 1 day, the medium was changed and extracts/ginsenosides were added. After treated for 48 h, cells floating in the medium were collected. The adherent cells were detached with 0.05% trypsin. Then culture medium containing 10% FBS (and floating cells) was added to inactivate trypsin. After being pipetted gently, the cells were centrifuged for 5 min at 1500 g. The supernatant was removed and cells were stained with annexin V-FITC and propidium iodide according to the manufacturer's instructions. Untreated cells were used as control for double staining. Cells were analyzed immediately after staining using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR). For each measurement, at least 20,000 cells were counted.

2.7 Cyclin A and cyclin D1 assay

Cells were seeded in 24-well tissue culture plates. On the second day, the medium was changed and cells were treated with extracts. Cells were incubated for 48 h before the cells were harvested. The cells were fixed gently by putting 80% ethanol in freezer for 2 h and were then treated with 0.25% Triton X-100 for 5 min on ice bath. Cells were resuspended in 300 ul of PBS containing 40 μg/ml propidium iodide and 0.1 mg/ml RNase. Then, 20 μl of cyclin A-FITC or cyclin D1-FITC was added to the cell suspension. Then the cells were incubated in a dark room for 20 min at room temperature, and analyzed with a FACScan flow cytometer. For each measurement, at least 20,000 cells were counted.

2.8 Cell cycle assay

After cells were treated with extracts/ginsenosides, the cells were harvested in similar manner to the method used in the cyclins assay. Then, the cells were stained with propidium iodide, and analyzed with a FACScan flow cytometer. For each measurement, at least 20,000 cells were counted.

2.9 Statistical analysis

Data are presented as mean ± standard deviation (SD) with n = 3. A one-way ANOVA determined whether the results had statistical significance. In some cases, Student's t-test was used for comparing two groups. The level of statistical significance was set at $P<0.05$.

3. Results

3.1 Saponin composition changes in extracts during heat-processing

Twelve ginsenosides (Figure **1**) were determined in American ginseng using high performance liquid chromatography (HPLC). All the assayed ginsenosides were dammarane glycosides [17,19]. The influence of steaming at 100°C and 120°C on the ginsenoside content of American ginseng was tested. Compared with unsteamed American ginseng, the roots treated at 100°C for 1 h decreased total ginsenoside content slightly, from 7.95% to 7.32%. For the main saponin contents, ginsenoside Rb1 was changed from 4.940% to 4.463%, and Re was from 1.756% to 1.630%; ginsenoside Rg3 increased from 0.003% to 0.048%. Steaming at 120°C for 1 h decreased the total ginsenoside content to 5.85%, as follows: Rb1, 3.252%; Re, 0.968%; and Rg3, 0.271%. Ginsenoside Rg3 increased significantly at 120°C.

Chromatograms of unsteamed and steamed American ginseng roots for 2 and 4 h at 120°C are shown in Figure **2**. The peak areas of Rb1, Rd and Re decreased during the steaming process. On the other hand, ginsenoside Rg3, which is a trace saponin in unsteamed root [17], was augmented during the steaming process (Figure **3**). The content of the 12 ginsenosides in steamed American ginseng roots is shown in Table **1**. During the steaming process, Rg1, Re, Rb1, Rc, Rb2, Rb3, and Rd decreased; Rh1, Rg2, 20R-Rg2, Rg3 and Rh2 increased.

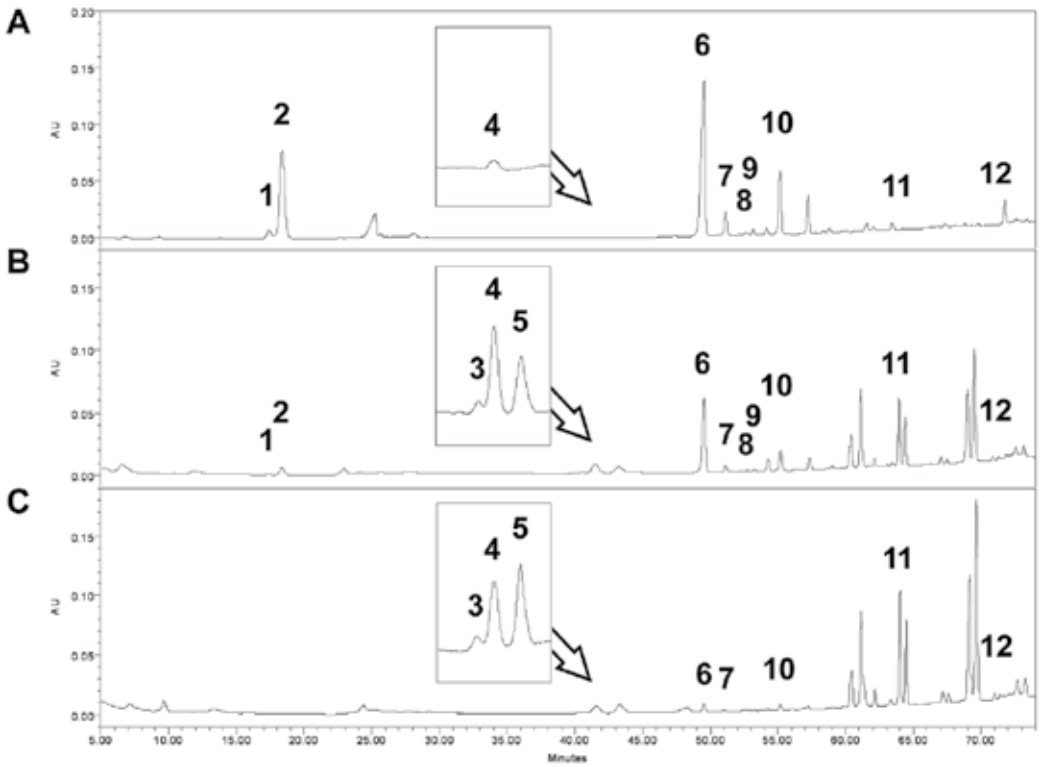


Fig. 2. HPLC analysis of ginsenosides in unsteamed and steamed American ginseng roots. Chromatograms of unsteamed (**A**), or steamed at 120°C for 2 h (**B**) and 4 h (**C**) are shown. Ginsenoside peaks: (**1**) Rg1, (**2**) Re, (**3**) Rh1, (**4**) Rg2, (**5**) 20R-Rg2, (**6**) Rb1, (**7**) Rc, (**8**) Rb2, (**9**) Rb3, (**10**) Rd, (**11**) Rg3, (**12**) Rh2. Peak numbers are not shown if saponins were not detected.

The chemical constituents of white ginseng and red ginseng (*P. ginseng* C. A. Meyer) were also determined. Data shown in Table **1** indicated that for the Asian ginseng, after steaming at 100°C, the contents of the main ginsenosides (Rg1, Re, Rb1, Rb2, Rc and Rd) were decreased. Ginsenoside Rg3 increased from 0.004% to 0.033%. The changes of ginsenoside contents are similar to that of American ginseng steamed at 100°C.

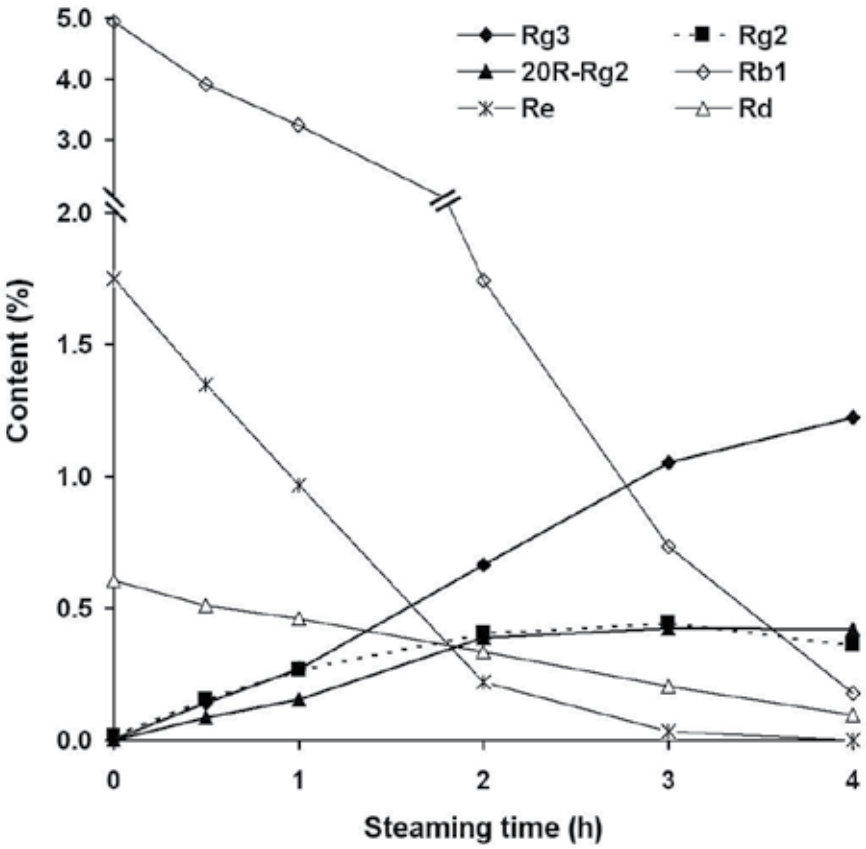


Fig. 3. Saponin content in American ginseng roots steamed at 120 °C for 0-4 h. The content of six major ginsenosides was changed during the steaming process.

3.2 Antiproliferative effects of extracts and ginsenosides on human breast cancer cells

Using two human breast cancer cell lines MCF-7 and MDA-MB-231, the antiproliferative effect of unsteamed and steamed American ginseng root extracts were evaluated. For the MCF-7 cells, at 0.25 mg/ml, unsteamed extract inhibited cell growth by 17.0%. After steaming for 1 h, the extract inhibited cell growth by 60.0% (P<0.01 vs. unsteamed extract). Moreover, after cells treated with 2 h steamed extract, cell growth was inhibited absolutely. At 0.5 mg/ml, extracts from 1 h and 2 h steamed roots inhibited cell growth over 99.6% (Figure **4A**). For the MDA-MB-231 cells, at 0.25 mg/ml, extracts from unsteamed and 1 h steamed roots did not show a antiproliferative effect, while extract from 2 h steamed roots showed a very strong effect, as cell growth was inhibited by 99.2% (Figure **4B**). These results suggested that steaming on American ginseng roots increased antiproliferative effects on human breast cancer cells, and the effects of extract from root steamed for 2 h were more potent than that of root steamed for 1 h.

Four representative ginsenosides were used to test for antiproliferative effects on breast cancer cells. Among them, two ginsenosides (Rb1 and Re) were major constituents in unsteamed American ginseng roots, while two other ginsenosides (Rg2 and Rg3) were main

constituents in steamed roots. At 30-300 μM, after 72 h treatment, ginsenosides Rb1, Re and Rg2 did not show antiproliferative effects on MCF-7 and MDA-MB-231 cells. Ginsenoside Rg3 showed positive antiproliferative effect on both the cancer cell lines. At 100 μM, ginsenoside Rg3 inhibited cell growth by 49.8% on MCF-7 cells, and by 27.3% on MDA-MB-231 cells (both $P<0.01$ vs. untreated control). At 300 μM, ginsenoside Rg3 almost inhibited cell growth on both the cell lines absolutely (Figure **4C** and **4D**).

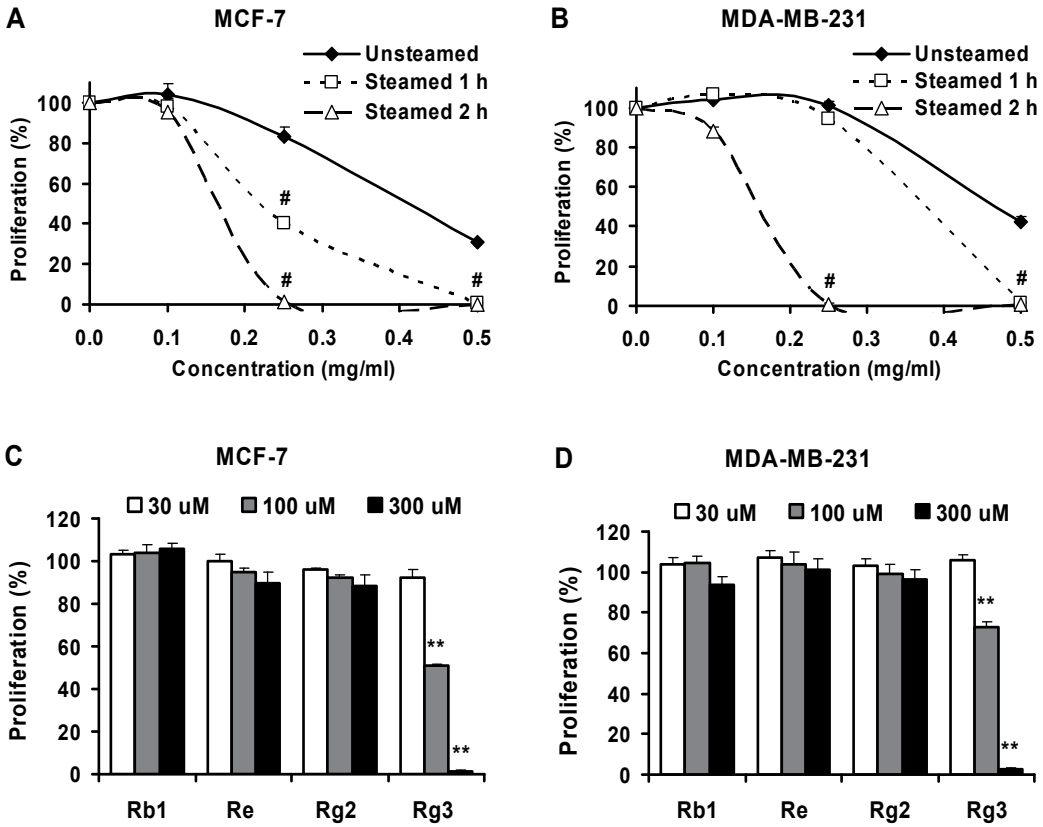


Fig. 4. Effects of American ginseng extracts and ginsenosides on proliferation of MCF-7 (A, C) and MDA-MB-231 (B, D) human breast cancer cells. Cells were treated with extracts (A, B) or ginsenosides (C, D) for 72 h, and then assayed by MTS method. #, $P<0.01$ vs. unsteamed extract; **, $P<0.01$ vs. control.

3.3 Apoptotic effect of extracts and ginsenosides on MCF-7 cells

Apoptosis and necrosis were evaluated in MCF-7 cells using annexin V-FITC, which detects translocation of phosphatidylinositol from the inner to outer cell membrane during early apoptosis, and propidium iodide, which can enter the cell in late apoptosis or necrosis. As shown in Figure **5**, compared to untreated control (early apoptosis 5.7%, late apoptosis/necrosis 6.1%), after treatment with 0.25 mg/ml for 48 h, unsteamed extract increased early apoptosis to 10.3%, but did not influence late apoptosis/necrosis (5.2%). After treatment with extract from 2 h steamed roots, early apoptosis increased slightly

(7.6%), and late apoptosis/necrosis increased significantly to 37.2%. Since early apoptosis is considered real apoptosis, steamed extract did not show obviously activity on early apoptosis. For the viable cells, the control was 88.1%, unsteamed extract was 83.8%, while 2 h steamed extract was 41.5%. Steamed extract significantly decrease the viable cells.

The effects of induction of apoptosis of representative ginsenosides on MCF-7 cells were also determined. Compared to control (5.5%), after treatment with 100-300 μM of ginsenosides Rb1 and Rg3 for 48 h, the percentage of early apoptotic cells were not increased (less than 6.2% and 5.8%, respectively). At the concentration of 300 μM, ginsenoside Re and Rg2 increased the cell percentage of early apoptosis to 13.0% and 9.1%, respectively. Consider both the apoptosis effects of steamed extract and its major ginsenosides Rb1 and Rg3, the MCF-7 cell growth inhibition activity of steamed extract may not be caused by the induction of apoptosis.

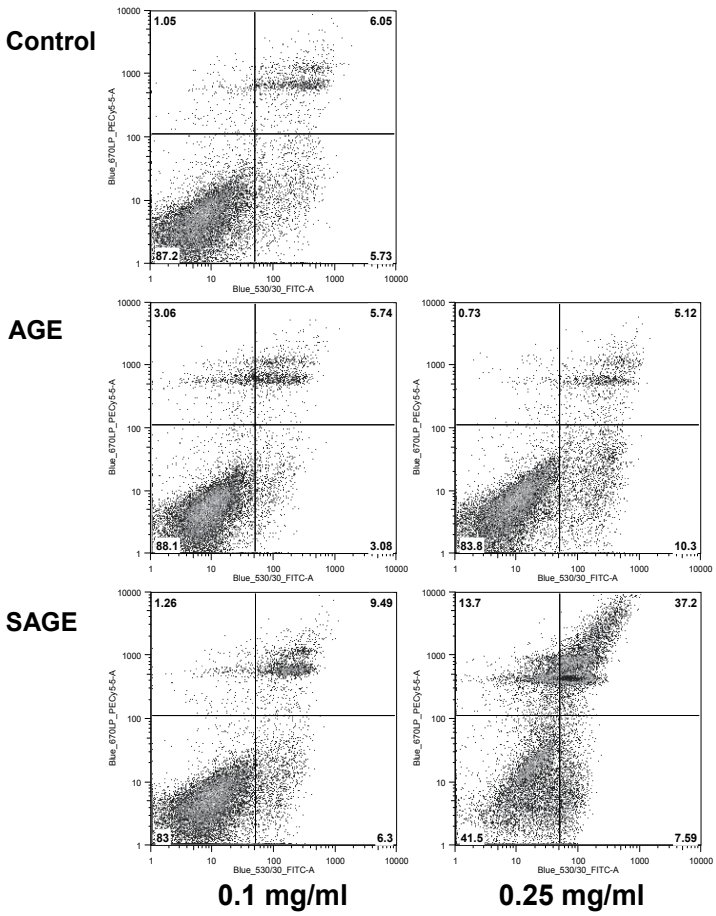


Fig. 5. Apoptosis assay using flow cytometry after annexin V-FITC/propidium iodide (PI) staining. MCF-7 cells were treated with 0.1 and 0.25 mg/ml of unsteamed and 2 h steamed extract for 48 h. Viable cells are in lower left quadrant, early apoptotic cells are in lower right quadrant, late apoptotic or necrotic cells are in upper right quadrant, and non-viable cells which underwent necrosis are in upper left quadrant.

3.4 Effect of extracts on the expression of cyclins on MCF-7 cells

The protein expression of cyclin A and cyclin D1 were evaluated by flow cytometry after staining with cyclin A-FITC and cyclin D1-FITC. For the percentage of cyclin A positive cells, the untreated control was 56.0%. After treatment with 0.1 and 0.25 mg/ml unsteamed extract for 48 h, the cyclin A positive cells were increased to 62.5% and 65.8%, while treated with 0.1 and 0.2 mg/ml 2 h steamed extract, cyclin A positive cells were decreased to 48.4% and 14.5%, respectively (Figure **6A**). For cyclin D1, unsteamed extract did not influence the expression of cyclin D1, while 2 h steamed extract decreased the expression of cyclin D1 significantly (Figure **6B**). Cyclin A and cyclin D1 are important cell cycle regulation proteins. Steamed extract decreased the expression of both cyclins, suggesting that the inhibition of cell growth of steamed extract may caused by the influences of cell cycle.

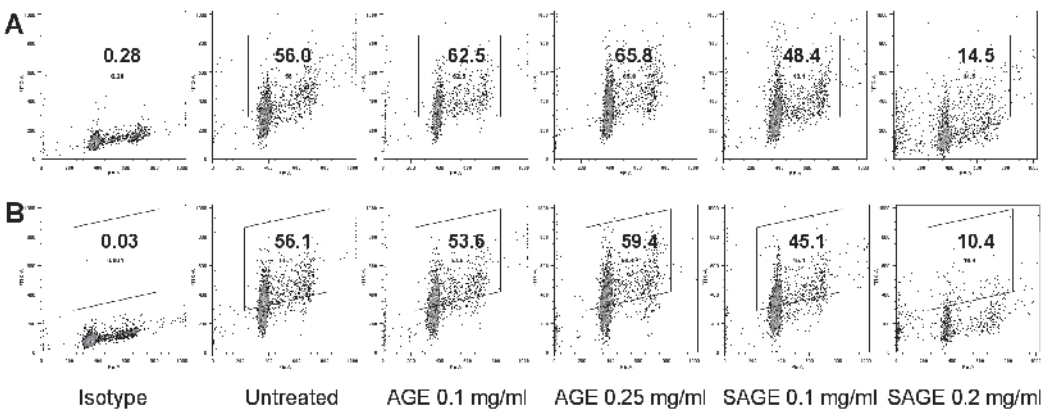


Fig. 6. Cyclins A and D1 analysis of MCF-7 cells using flow cytometry. After treated for 48 h, MCF-7 cells were stained with cyclin A-FITC/PI (A) and cyclin D1/PI (B). Isotype: untreated cells were stained with isotype antibody/PI. The percentage of cyclin A (A) and cyclin D1 (B) positive cells is shown in the gate.

3.5 Effects of extracts and ginsenosides on MCF-7 cell cycle

After treatment with unsteamed and steamed extract for 48 h, cells were stained with propidium iodide (PI) and then assayed by flow cytometry. Unsteamed extract did not influence the cell cycle profile (Figure **7**). Compared to untreated control (G1, 68.2%), 0.2 mg/ml 2 h steamed extract increased the percentage of cells in G1 phase to 83.6%. For the single compounds, ginsenosides Rb1, Re and Rg2 almost did not influence the cell cycle. Ginsenoside Rg3, a previously recognized anticancer compound, increased the G1 fraction to 87.4% (Figure **7**). Data from this study suggests that both the 2 h steamed extract and ginsenoside Rg3 arrested MCF-7 cells in G1 phase.

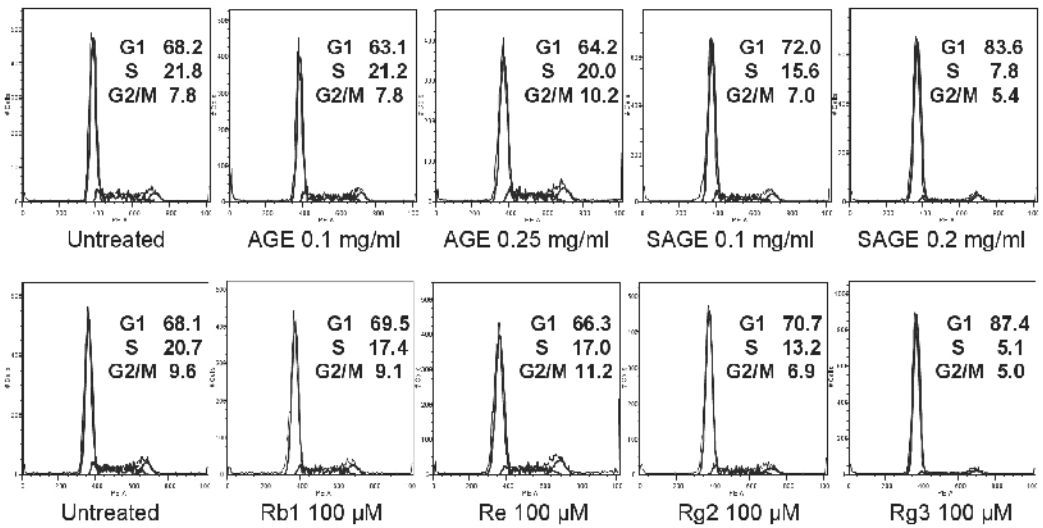


Fig. 7. Effects of extracts and ginsenosides on cell cycle. After MCF-7 cells were treated with extracts or ginsenosides for 48 h, the cells were stained with PI and assayed using flow cytometry. The percentage of cells in G1, S and G2/M phases are indicated.

4. Discussion

Herbal medicines are comprised of a complicated mixture of biologically active compounds. The concentrations of these compounds may vary significantly depending on many intrinsic and extrinsic factors such as genetics, season, geographical distribution, plant growth, and production and extract processes [20]. Thus, herbal medicine identification and analysis are very important issues in the quality assurance of herbal products. Since American ginseng from Wisconsin is a reliable ginseng source [21], in this study, the American ginseng used was from Roland Ginseng, LLC (Wausau, WI). The plant material was identified according to the United States Pharmacopoeia NF 21, monograph: American ginseng (*Panax quinquefolius* L.). The contents of major ginsenosides in different American ginseng extracts were determined using HPLC, which means that our American ginseng extracts were standardized and the quantitative assay data are critical references for future studies.

Heat-processing on American ginseng changes the constituent profiles. We have reported the steaming temperatures and times on the changes of ginsenosides in the crude herb [22]. In this study, the contents of main ginsenosides in the extracts were evaluated. In unsteamed extract, the content of ginsenoside Rb1 was 34.2%, and the summary of six ginsenosides were 63.9%. The content of total ginsenoside in extract was very high, suggesting that the extraction and purification method used in this study are reasonable.

Ginsenoside Rg3, a previously recognized anticancer compound [7], was detected in the unsteamed and steamed extracts. In unsteamed extract, Rg3 was only a trace saponin (0.06%). After 2 h steaming, the content of Rg3 was increased to 5.9%, becoming a main constituent in the extract. Therefore, steaming on American ginseng increased Rg3 content significantly.

Pharmacological studies showed that heat-processing on American ginseng increases the antiproliferative effect significantly. The biological effects of extracts were based on their

chemical constituents. We consequently evaluated the antiproliferative activities of representative ginsenosides on human breast cancer cells. Using both cancer cell lines (MCF-7 and MDA-MB-231), ginsenosides Rb1 and Re, taken from unsteamed extract almost had no effect at the concentration range of 30-300 uM. Ginsenoside Rg3, which was taken from steamed extract, showed strong antiproliferative activity. The increase of antiproliferative effect of steam-processing is based on the increase of anticancer constituents.

Apoptosis is considered an important pathway in the inhibition of cancer cells of many anticancer agents [23,24]. In this study, we assayed the induction of apoptosis by American ginseng extracts and single compounds. However, the apoptotic induction activities on MCF-7 cells were not confirmed by current data. The antiproliferative effect of red American ginseng extract on human breast cancer cells may be caused by other mechanisms.

Recent studies found that overexpression of cyclin D1 promotes tumor cell growth, and confers resistance of chemotherapy [23,24]. Cyclins assay found that American ginseng extracts regulated the expression of cyclin A and cyclin D1 on MCF-7 cells. Red American ginseng extract decreased the expression of cyclin A and cyclin D1 significantly. Since cyclins are important regulation proteins in cell cycle, we assayed the influence of extract on cell cycle. Data showed that red American ginseng extract arrested cells in G1 phase, and decrease the percentage of cells in S and G2/M phases. We subsequently studied the influence of representative saponins on the cell cycle. Treatment with 100 μM of ginsenosides Rb1, Re and Rg2 for 48 h, did not influence the cell cycle profile. Ginsenoside Rg3, a main constituent in red American ginseng, arrested cells in G1 phase. This result is similar to that of red American ginseng extract.

At the concentration of 0.25 mg/ml, 2 h steamed extract inhibited cell growth absolutely. The 0.25 mg/ml 2 h steamed extract contains 18.6 μM of ginsenoside Rg3. However, even 30 μM of ginsenoside Rg3 did not show significant antiproliferative activity. From the HPLC chromatogram of 2 h steamed extract, several peaks of unidentified compounds can be found. We expected that other more potent compounds existed in red American ginseng extract, and these compounds have similar effects to Rg3 on cell cycle. This should be focus of future studies.

In summary, analytical and pharmacological data obtained from our observations suggest that steamed American ginseng root significantly augments the content of ginsenoside Rg3 and other unidentified active compounds, which is responsible for the increase in anticancer activity. Red American ginseng, which is the heat-processed root of *P. quinquefolius*, may be a potent anti-breast cancer herbal medicine.

5. Acknowledgements

This work was supported in part by the NIH/NCCAM grants P01 AT004418 and K01 AT005362.

6. References

[1] Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* Sep-Oct 2010;60(5):277-300.

[2] Lee MC, Newman LA. Management of patients with locally advanced breast cancer. *Surg Clin North Am.* 2007;87:379-398.

[3] Gerber B, Scholz C, Reimer T, Briese V, Janni W. Complementary and alternative therapeutic approaches in patients with early breast cancer: a systematic review. *Breast Cancer Res Treat.* Feb 2006;95(3):199-209.
[4] Wang CZ, Luo X, Zhang B, et al. Notoginseng enhances anti-cancer effect of 5-fluorouracil on human colorectal cancer cells. *Cancer Chemother Pharmacol.* Jun 2007;60(1):69-79.
[5] Ang-Lee MK, Moss J, Yuan CS. Herbal medicines and perioperative care. *Jama.* Jul 11 2001;286(2):208-216.
[6] Attele AS, Wu JA, Yuan CS. Ginseng pharmacology: multiple constituents and multiple actions. *Biochem Pharmacol.* Dec 1 1999;58(11):1685-1693.
[7] Helms S. Cancer prevention and therapeutics: Panax ginseng. *Altern Med Rev.* Sep 2004;9(3):259-274.
[8] Yoo HH, Yokozawa T, Satoh A, Kang KS, Kim HY. Effects of ginseng on the proliferation of human lung fibroblasts. *Am J Chin Med.* 2006;34(1):137-146.
[9] Koo HN, Jeong HJ, Choi IY, et al. Mountain grown ginseng induces apoptosis in HL-60 cells and its mechanism have little relation with TNF-alpha production. *Am J Chin Med.* 2007;35(1):169-182.
[10] Yun TK, Choi SY. Preventive effect of ginseng intake against various human cancers: a case-control study on 1987 pairs. *Cancer Epidemiol Biomarkers Prev.* Jun 1995;4(4):401-408.
[11] Yun TK, Choi SY. Non-organ specific cancer prevention of ginseng: a prospective study in Korea. *Int J Epidemiol.* Jun 1998;27(3):359-364.
[12] Corbit R, Ebbs S, King ML, Murphy LL. The influence of lead and arsenite on the inhibition of human breast cancer MCF-7 cell proliferation by American ginseng root (Panax quinquefolius L.). *Life Sci.* Feb 16 2006;78(12):1336-1340.
[13] Aung HH, Mehendale SR, Wang CZ, Xie JT, McEntee E, Yuan CS. Cisplatin's tumoricidal effect on human breast carcinoma MCF-7 cells was not attenuated by American ginseng. *Cancer Chemother Pharmacol.* Feb 2007;59(3):369-374.
[14] Takaku T, Kameda K, Matsuura Y, Sekiya K, Okuda H. Studies on insulin-like substances in Korean red ginseng. *Planta Med.* Feb 1990;56(1):27-30.
[15] Kim WY, Kim JM, Han SB, et al. Steaming of ginseng at high temperature enhances biological activity. *J Nat Prod.* Dec 2000;63(12):1702-1704.
[16] Rapaka RS, Coates PM. Dietary supplements and related products: a brief summary. *Life Sci.* Mar 27 2006;78(18):2026-2032.
[17] Wang CZ, Wu JA, McEntee E, Yuan CS. Saponins composition in American ginseng leaf and berry assayed by high-performance liquid chromatography. *J Agr Food Chem.* Mar 22 2006;54(6):2261-2266.
[18] DiPaola RS, Kuczynski WI, Onodera K, et al. Evidence for a functional kit receptor in melanoma, breast, and lung carcinoma cells. *Cancer Gene Ther.* May-Jun 1997;4(3):176-182.
[19] Qi LW, Wang CZ, Yuan CS. American ginseng: potential structure-function relationship in cancer chemoprevention. *Biochem Pharmacol.* Oct 1 2010;80(7):947-954.
[20] Fong HH, Pauli GF, Bolton JL, et al. Evidence-based herbal medicine: Challenges in efficacy and safety assessments. In: Leung P-C, Fong, H., Xue, C.C., ed. *Current*

Review of Chinese Medicine, Annals of Traditional Chinese Medicine, Vol. 2. Singapore: World Scientific; 2006.

[21] Assinewe VA, Baum BR, Gagnon D, Arnason JT. Phytochemistry of wild populations of Panax quinquefolius L. (North American ginseng). *J Agric Food Chem.* Jul 30 2003;51(16):4549-4553.

[22] Wang CZ, Aung HH, Ni M, et al. Red American ginseng: ginsenoside constituents and antiproliferative activities of heat-processed Panax quinquefolius roots. *Planta Med.* Jun 2007;73(7):669-674.

[23] Reed JC, Pellecchia M. Apoptosis-based therapies for hematologic malignancies. *Blood.* Jul 15 2005;106(2):408-418.

[24] Wu WY, Guo HZ, Qu GQ, Han J, Guo DA. Mechanisms of pseudolaric Acid B-induced apoptosis in bel-7402 cell lines. *Am J Chin Med.* 2006;34(5):887-899.

14

Synthesis and *In Vitro* Screening of Novel Heterocyclic Compounds as Potential Breast Cancer Agents

Narsimha Reddy Penthala[1], Thirupathi Reddy Yerramreddy[2], Nikhil Reddy Madadi[1], Vijayakumar Sonar[2] and Peter A. Crooks[1]
[1]*Department of Pharmaceutical Sciences, College of Pharmacy, University of Arkansas for Medical Sciences, Little Rock, Arkansas*
[2]*Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington*
USA

1. Introduction

Breast cancer is one of the most common non-cutaneous type of cancer in women in worldwide and a leading cause of cancer-related deaths, and is increasing year by year in almost every areas of the globe. Breast cancer is commonly classified into the following two major types: (1) non-invasive breast cancer (cancer cells are confined within the duct and lobules) and (2) invasive breast cancer (cancer cells invade through the walls of the duct or lobules and infiltrate the surrounding tissues). Various kinds of treatments are available for breast cancer, such as chemotherapy, radiotherapy and hormone therapy (Ragaz, 2009). Many indole derivatives are reported as potent breast cancer agents, such as aplysinopsin analogs and indole-3-carbinols. Aplysinopsins are indole-derived marine natural products. The parent aplysinopsin was isolated for the first time (Kazlauskas, et al, 1977) as the major metabolite of eight Indo-Pacific sponge species, which are representatives of the genus *Thorecta*. The *N*-1-unsubstituted aplysinopsins have generated considerable interest due to their potentially useful medicinal properties (Dobroslawa, et al, 2009). Aplysinopsin has been reported as a potent cytotoxic agent against the Kβ-cell line and methyl-aplysinopsins against L-1210 and Kβ-cell lines has been reported as potent cytotoxic agents (Hollenbeak & Schmitz, 1977), and the anticancer activities of aplysinopsin and methyl-aplysinopsins against both 1210- and Kβ-cells has also been reported (Kondo *et al*. 1994). Indole-3-carbinol, a phytochemical derived from cruciferous vegetables such as broccoli and Brussel sprouts, exhibits potent antiproliferative effects against human breast cancer cells and has been shown to decrease metastatic spread of tumors in experimental animals (Brew, et al., 2010). From the above observations and as part of a program for the development of small molecules as potential anticancer treatments (Thirupathi Reddy, et al., 2010 and Narsimha Reddy et al., 2010), we initiated a drug discovery program to identify novel benzyl-aplysinopsin analogs as potent breast cancer agents.

Combretastins are plant products from the South African tree *Combretum caffrum*. This compound was found to inhibit tubulin polymerization, and competitively inhibit the

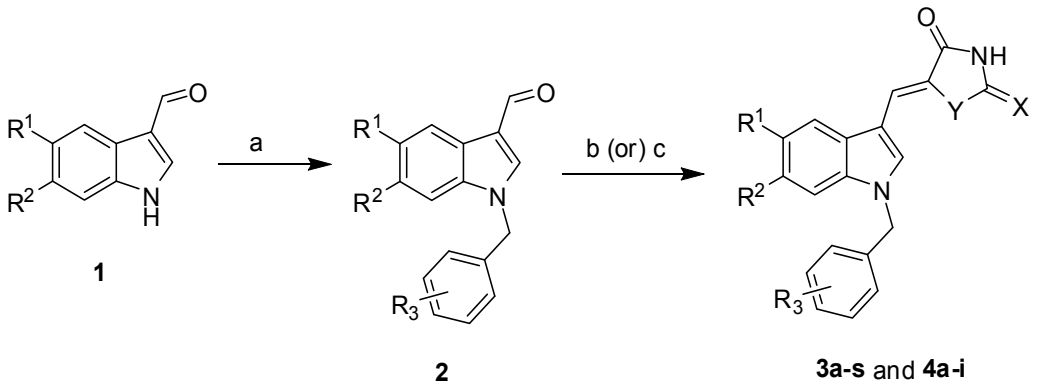
binding of radiolabeled colchicines to tubulin. Investigation of combretastatins revealed that combrestastatin A-4 was active against multidrug resistant (MDR) cancer cell lines (McGown, et al., 1990; Lin, et al., 1988 and Cushman, et al 1991). Combretastatin (A-4), as well as its trans-isomer and a number of related substances, has been found to cause mitotic arrest in cells in culture at cytotoxic concentrations. *trans*-Tetrahydroxystilbene and a number of other related substances were also reported to be cytotoxic agents (Lin, et al 1988). Also, many natural products possessing a trimethoxybenzene ring, e.g., colchicines, and podophyllotoxins, were found to be potent cytotoxic agents that exert their antitumor property by their antitubulin character. In view of the known antitubulin activity of combretstatin, a large number of its derivatives have been synthesized and evaluated for antitubulin activity. Some of the structural modifications carried out on these molecules are emerging as potent breast cancer agents. These observations encouraged us to also investigate combretastatin analogs as potent breast cancer agents.

Both the naturally occurring stilbene derivatives i.e. resveratrol (*trans*-3,5,4′-trihydroxystilbene) and structurally related combretastatin analogs have been reported as cytotoxic agents. Resveratrol is one of the phytoalexins widely distributed in plants products such as grapes, berries, peanuts, and red wine. Resveratrol has recently been reported as a potential chemotherapeutic agent due to its striking inhibitory effects on cellular events associated with cancer initiation, promotion, and progression (Jang, et al., 1997). In addition, there are several reports on the anti-cancer activity of resveratrol in animal models (Baur, et al., 2006). Resveratrol interacts with multiple molecular targets *in vitro*, and exhibits cytotoxic effects against breast, skin, gastric, colon, esophageal, prostate, and pancreatic cancer cells, and leukemia cells (Baur, et al., 2006). Based on the anticancer activity of resveratrol analogs we also focused on the synthesis several resveratrol analogs as potent breast cancer agents.

2.1 Synthesis and evaluation of *in vitro* anticancer activity of (*Z*)-5-((*N*-benzyl-1*H*-indol-3-yl) methylene) imidazolidine-2,4-diones and (*Z*)-5-((1-benzyl-1H-indol-3-yl) methylene)thiazolidine-2,4-diones

A series of novel substituted (*Z*)-5-((*N*-benzyl-1*H*-indol-3-yl)methylene)imidazolidine-2,4-diones and (*Z*)-5-((1-benzyl-1*H*-indol-3-yl)methylene)thiazolidine-2,4-diones that incorporate a variety of substituents in both the indole and *N*-benzyl moieties, and that are considered structurally related to the natural product, aplysinopsin, were synthesized utilizing aldol condensation chemistry under both microwave irradiation and conventional heating methodologies (**Scheme 1**). A library of simple and substituted *N*-benzylindole-3-carboxaldehydes were synthesized in 85-90% yield by reacting the corresponding indole-3-carboxaldehydes with various substituted benzyl halides under phase-transfer catalytic (PTC) conditions using triethylbenzyl ammonium chloride (TEBA) and 50% w/v aqueous NaOH solution in dichloromethane (**Scheme 1**). Aldol condensation of the appropriate *N*-benzylindole-3-carboxaldehyde with hydantoin or 2-iminothiazolidin-4-one, in the presence of CH_3COOH and ammonium acetate and utilizing both microwave irradiation and conventional heating methods (**Scheme 1**), afforded a series of novel simple and substituted (*Z*)-5-((*N*-benzyl-1*H*-indol-3-yl)methylene)imidazolidine-2,4-diones (**3a-s**) and (*Z*)-5-((1-benzyl-1*H*-indol-3-yl) methylene) thiazolidine-2,4-diones (**4a-i**) (Table 1). Microwave irradiation using an open vessel in a domestic microwave oven (1100 W; Kenmore) at atmospheric pressure afforded product yields in the range of 85–93% in 30–60s. All the synthesized compounds were fully characterized by ^{1}H NMR, ^{13}C NMR. The resulting analogs were evaluated for their anti-proliferative activity against a panel of human MCF-7, MDA-MB-

231/ATCC, HS-578T, BT-549, T-47D, MDA-MB-468 breast cancer cell lines (**Tables 1-3**). Compound (*Z*)-methyl-1-(4-cyanobenzyl)-3-((2,5-dioxoimidazolidin-4-ylidene) methyl)-1*H*-indole-6-carboxylate (**3n**) exhibited GI_{50} values of 970nM against HS 578T breast cancer cells, and afforded GI_{50} values ranging from 0.97μM-4.1μM against HS 578T, MCF-7, MDA-MB-231/ATCC, BT-549, T-47D, MDA-MB-468 (**Table 3**) (Narsimha Reddy et al, 2011). Two other analogs, (*Z*)-5-((1-(2-bromobenzyl)-1*H*-indol-3-yl)methylene) imidazolidine-2,4-dione (**3f**) and (*Z*)-5-((1-benzyl-5-methyl-1*H*-indol-3-yl)methylene) imidazolidine-2,4-dione (**3j**) exhibited IC_{50} values of 4.4μM and 5.2μM against MCF-7 cells (**Table 2**) (Narsimha Reddy et al, 2011). From the series of 2-iminothiazolidin-4-ones the compound *(Z)*-5-((5-chloro-1-(4-fluorobenzyl)-1*H*-indol-3-yl)methylene)-2-iminothiazolidin-4 one (**4c**) exhibited growth inhibition (GI_{50}= 3.7μM) against cultured HS 578T cancer cells (**Table 3**).



Scheme 1. Reagents and conditions: (a) appropriate benzylhalide, aqueous NaOH solution, triethylbenzylammonium chloride, DCM, RT; (b) Method A: hydantoin (or), 2-iminothiazolidin-4 one, ammonium acetate in acetic acid, microwave irradiation, 40-50 seconds, 87-95% yield; (c) Method-B: hydantoin (or) 2-iminothiazolidin-4 one, ammonium acetate in acetic acid, 120-125 ^{0}C, 8-12 hours, 74-85% yield.

S. No.	R^1	R^2	R^3	X	Y
3a	H	H	H	O	NH
3b	H	H	*p*-CN	O	NH
3c	H	H	*p*-NO_2	O	NH
3d	H	H	*p*-Cl	O	NH
3e	H	H	*p*-$COOCH_3$	O	NH
3f	H	H	*o*-Br	O	NH
3g	H	H	*p*-F	O	NH
3h	Cl	H	H	O	NH
3i	Br	H	*p*-OCH_3	O	NH
3j	CH_3	H	H	O	NH

S. No.	R^1	R^2	R^3	X	Y
3k	Br	H	H	O	NH
3l	OCH_3	H	H	O	NH
3m	H	H	*p*-CH_3	O	NH
3n	H	$COOCH_3$	*p*-CN	O	NH
3o	$COOCH_3$	H	*p*-CN	O	NH
3p	H	H	*p*-CN	O	NH
3q	H	H	*H*	O	NH
3r	H	Cl	H	O	NH
3s	H	OCH_3	H	O	NH
4a	H	H	*o*-F	NH	S
4b	Cl	H	*o*-F	NH	S
4c	Cl	H	*p*-F	NH	S
4d	Br	H	*o-F*	NH	S
4e	Br	H	*p*-F	NH	S
4f	H	$COOCH_3$	*p*-CN	NH	S
4g	H	$COOCH_3$	*p*-$COOCH_3$	NH	S
4h	$COOCH_3$	H	*p*-CN	NH	S
4i	$COOCH_3$	H	*p*-$COOCH_3$	NH	S

Table 1. List of *N*-benzyl aplysinopsin analogs synthesized (**3a-s and 4a-i**).

Entry	Breast cancer cell lines	
	MCF-7 (IC_{50})	MDA-231 (IC_{50})
3a	27.3	15.7
3b	14.1	17.6
3c	10.9	23.3
3d	21.4	25.3
3e	15.4	18.4
3f	4.4	21.8
3g	22.3	32.8
3h	9.2	18.0
3i	16.4	23.2
3j	5.2	20.1
3k	7.6	12.4
3l	7.2	7.0
3m	20.2	22.1

Table 2. IC_{50} values for simple and substituted *N*-benzyl aplysinopsin analogs (**3a-m).**

Breast Cancer Panel/cell line	**3n**		**4c**	
	GI_{50}	LC_{50}	GI_{50}	LC_{50}
MCF7	3.55	>100	>100	>100
MDA-MB-231/ATCC	3.32	>100	6.45	>100
HS 578T	0.97	>100	3.67	>100
BT-549	1.24	>100	98.8	>100
T-47D	1.50	>100	>100	>100
MDA-MB-468	4.14	>100	>100	>100

[a]GI_{50}: 50% Growth inhibition, concentration of drug resulting in a 50% reduction in net protein increase compared with control cells. [b]LC_{50}: Lethal concentration, concentration of drug lethal to 50% of cells

Table 3. IC_{50} values for compounds **3n** and **4c.**

2.2 Synthesis and *in vitro* cytotoxicity evaluation of (*Z*)-2-amino-5-(1-benzyl-1*H*-indol-3-yl) methylene-1-methyl-1*H*-imidazol-4(5*H*)-ones

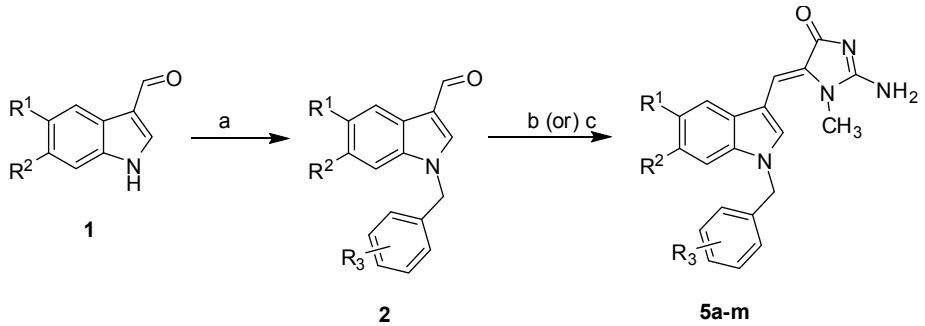
Another series of substituted (*Z*)-2-amino-5-(1-benzyl-1*H*-indol-3-yl)methylene-1-methyl-1*H*-imidazol-4(5*H*)-ones were synthesized (**Scheme 2**) utilizing similar aldol condensation chemistry. Simple and substituted *N*-benzylindole-3-carboxaldehydes were synthesized in 85-90% yield by reacting the corresponding indole-3-carboxaldehydes with various substituted benzyl halides under phase-transfer catalytic (PTC) conditions using triethylbenzyl ammonium chloride (TEBA) and 50% w/v aqueous NaOH solution in dichloromethane. Aldol condensation of the appropriate *N*-benzylindole-3-carboxaldehyde with creatinine, in the presence of CH_3COOH and sodium acetate and utilizing both microwave irradiation and conventional heating methods (**Scheme 2**), afforded a series of novel simple and substituted (*Z*)-2-amino-5-(1-benzyl-1*H*-indol-3-yl)methylene-1-methyl-1*H*-imidazol-4(5*H*)-ones.

Evaluation of analogs these analogs for *in vitro* cytotoxicity activity against MCF-7, MDA-MB-231/ATCC, HS-578T, BT-549, T-47D, MDA-MB-468 human breast cancer cell lines is shown in **Table 4** (Narsimha Reddy et al, 2010). From this series, compounds, (*Z*)-2-amino-5-((1-(2-bromobenzyl)-1*H*-indol-3-yl)methylene)-1-methyl-1*H*-imidazol-4-(5H)-one (**5e**) and (*Z*)-methyl 4-((3-((2-amino-1-methyl-4-oxo-1*H*-imidazol-5(4H)-ylidene)methyl)-1*H*-indol-1-yl)methyl)benzoate (**5f**) showed good growth inhibition with GI_{50} values ranging from 1.5μM-4μM, and LC_{50} values ranging from 5.7 μM->100 μM (**Table 4**).

Breast Cancer Panel/ cell lines	Compound **5e** Molar concentrations		Compound **5f** Molar concentrations	
	GI_{50}	LC_{50}	GI_{50}	LC_{50}
MCF7	2.45×10^{-6}	7.78×10^{-5}	2.25×10^{-6}	4.81×10^{-5}
MDA-MB-231/ATCC	2.01×10^{-6}	1.43×10^{-5}	4.07×10^{-6}	4.67×10^{-5}
HS 578T	2.17×10^{-6}	$>1.0 \times 10^{-4}$	3.97×10^{-6}	$>1.00 \times 10^{-4}$
BT-549	1.73×10^{-6}	$>1.0 \times 10^{-4}$	2.05×10^{-6}	$>1.00 \times 10^{-4}$
T-47D	1.50×10^{-6}	5.74×10^{-6}	1.68×10^{-6}	1.31×10^{-5}
MDA-MB-468	1.63×10^{-6}	6.78×10^{-6}	1.69×10^{-6}	6.90×10^{-6}

[a]GI_{50}: 50% Growth inhibition, concentration of drug resulting in a 50% reduction in net protein increase compared with control cells. [b]LC_{50}: Lethal concentration, concentration of drug lethal to 50% of cells

Table 4. IC_{50} values for compounds **3e** and **3f.**



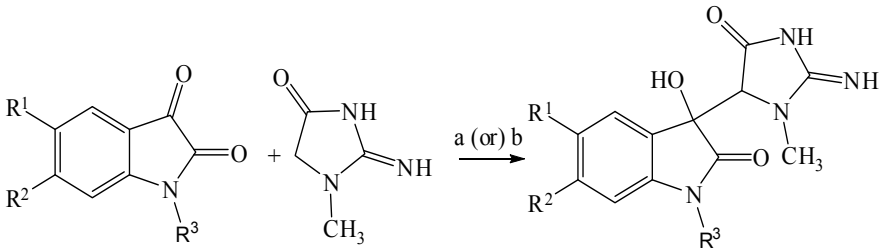
5a	R^1=R^2 = R^3= H	**5g**	R^1=H, R^2=H, R^3=*p*-F
5b	R^1=H, R^2=H, R^3=*p*-CN	**5h**	R^1=Cl, R^2=R^3=H
5c	R^1=H, R^2=H, R^3=*p*-NO_2	**5i**	R^1=Br, R^2=H, R^3=OCH_3
5d	R^1=H, R^2=H, R^3=*p*-Cl	**5j**	R^1=CH_3, R^2=R^3=H
5e	R^1=H, R^2=H, R^3= *o*-Br	**5k**	R^1=Br, R^2=R^3=H
5f	R^1= H, R^2=H, R^3=*p*-$COOCH_3$	**5l**	R^1=OCH_3, R^2= R^3=H
		5m	R^1=H, R^2=H, R^3=*p*-CH_3

Scheme 2. Synthesis of (*Z*)-2-amino-5-(1-benzyl-1*H*-indol-3-yl)methylene-1-methyl-1*H*-imidazol-4(5*H*)-one analogs: Reagents and conditions (a) appropriate benzyl halide, aqueous NaOH solution, triethylbenzyl ammonium chloride, DCM, RT; (b) Creatinine (1.1 mol. eq), NaOAc (1.2 mol. eq), AcOH, MWI, 30-60 sec; (c) Creatinine (1.1 mol. eq), NaOAc (1.2 mol. eq), AcOH, reflux, 7-10 h.

2.3 Synthesis and *invitro* cytotoxicity evaluation of *N*-alkyl-3-hydroxy-3-(2-imino-3-methyl-5-oxo-imidazolidin-4-yl)indolin-2-one

A third series of *N*-alkyl-3-hydroxy-3-(2-imino-3-methyl-5-oxo-imidazolidin-4-yl)indolin-2-one analogs that incorporated a variety of substituents in both the isatin phenyl and *N*-benzyl moieties were also synthesized. These novel analogs (**8a-w**) were prepared by condensation of the appropriate substituted *N*-alkyl isatin with creatinine, in the presence of sodium acetate and acetic acid via both microwave irradiation and conventional heating methodologies (**Scheme 3)** (Narsimha Reddy et al, 2010). Of these two methods, microwave irradiation was found to be advantageous over conventional heating, since the product yields were 83-94% for the former method, but only 70-83% for the latter method. In addition, the time course of the reaction was very fast using microwave irradiation (20-40 sec) compared to 6-8 h for conventional heating. The simple and *N*-alkyl substituted isatins (**6a-j**) were all prepared utilizing literature methods (Macpherson, et al., 2007; Jacobs, et al., 1948 and Shindikar, et al., 2006). All the synthesized compounds were characterized by ^{1}H-NMR and ^{13}C-NMR spectrometry. The geometry of the hydroxyl position in the representative compounds **8a, 8b** and **8t** was established as *trans* to the 4′-methyne hydrogen from X-ray crystallographic data (Narsimha Reddy et al, 2009). From the X-ray diffraction and ^{1}H-NMR data, analogs **8a-8w** were mixtures of *RR* and *SS* isomers. This is consistent with the mechanism of the aldol condensation reaction of **6** with **7**, which proceeds via the formation of the *E*-enolate, as per the Zimmerman–Traxler model, which favors *anti* products, and is predicted to lead to the formation of equimolar *RR* and *SS* enantiomers. We also determined from the crystal

structures of **8a, 8b** and **8t** that the 3-hydroxy group was *trans* to the 4′-methyne hydrogen, which may explain the inability of these analogs to undergo facile dehydration. The cytotoxicity data on these analogs is provided in **Table 5** (Narsimha Reddy et al, 2010). Two analogs, 3-hydroxy-3-(2-imino-3-methyl-5-oxoimidazolidin-4-yl)-1-(4-methoxy benzyl)indolin-2-one (**8n**) and 5-chloro-3-hydroxy-3-(2-imino-3-methyl-5-oxoimidazolidin-4-yl)-1-(4-methoxybenzyl)indolin-2-one (**8o**), showed growth inhibition with GI_{50} values ranging from 2μM-60μM. Substitution of a methoxy group at the 4th position of *N*-benzyl group (**8n**) increases the activity over the breast cancer cells. Further the substitution of chloro group at 5th position of *N*-benzyl *p*-methoxy isatin (**8n**) increased the activity.



Scheme 3. Reagents and conditions: (a) Method A: sodium acetate in acetic acid, microwave irradiation, 20-40 seconds, 83-94% yield; (b) Method-B: sodium acetate in acetic acid, 115-120 ^{0}C, 6-8 hours, 70-83% yield.

Compound	R^1	R^2	R^3
8a	H	H	H
8b	F	H	H
8c	Cl	H	H
8d	Br	H	H
8e	Br	Br	H
8f	NO_2	H	H
8g	H	H	-CH_3
8h	F	H	-CH_3
8i	Cl	H	-CH_3
8j	Br	H	-CH_3
8k	H	H	-Bz
8l	Cl	H	-Bz
8m	Br	H	-Bz
8n	H	H	4-OCH_3 Bz
8o	Cl	H	4-OCH_3 Bz
8p	Br	H	4-OCH_3 Bz
8q	H	H	4-Cl Bz
8r	H	H	4-$COOCH_3$Bz
8s	H	H	4-CN Bz
8t	H	H	-C_6H_5

Compound	R^1	R^2	R^3
8u	H	H	$-COCH_3$
8v	Cl	H	$-COCH_3$
8w	H	H	$-SO_2C_6H_5$

Breast Cancer Panel/cell lines	Compound **8o** Molar concentrations		Compound **8n** Molar concentrations	
	GI_{50}	LC_{50}	GI_{50}	LC_{50}
MCF7	3.22	>100	6.93	>100
MDA-MB-231/ATCC	8.55	>100	19.4	>100
HS 578T	2.04	>100	60.3	>100
BT-549	2.98	54.9	8.45	97.0
T-47D	13.5	>100	32.0	>100
MDA-MB-468	2.29	>100	11.6	>100

[a]GI_{50}: 50% Growth inhibition, concentration of drug resulting in a 50% reduction in net protein increase compared with control cells. [b]LC_{50}: Lethal concentration, concentration of drug lethal to 50% of cells

Table 5. IC_{50} values for compounds **8o** and **8n.**

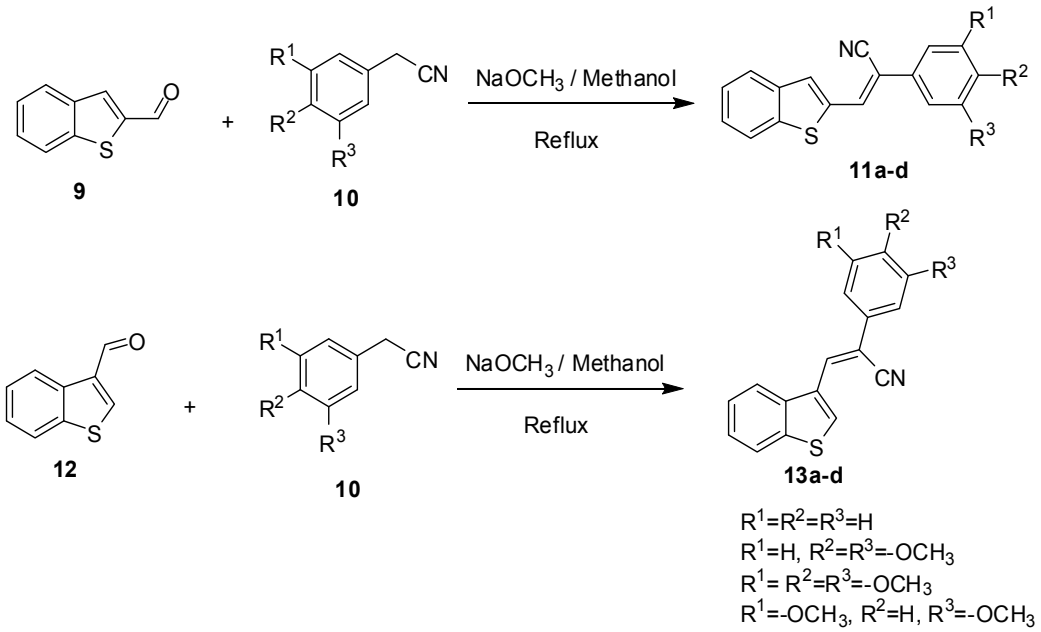
2.4 Synthesis and *in vitro* cytotoxicity evaluation of benzo[*b*]thiophene phenyl acrylontriles as novel combretastatin analogs

A series of combretastatin analogs were prepared via the reaction of benzo[b]thiophene-2-carbaldehyde or benzo[b]thiophene-3-carbaldehyde with simple and substituted benzyl cyanides in 5% sodium methoxide methanol (**Scheme 4**) and evaluated for their *in vitro* cytotoxicity against a panel of human MCF-7, MDA-MB-231/ATCC, HS-578T, BT-549, T-47D, breast cancer cell lines (**Table 6**). Two analogs, (*Z*)-3-(benzo[*b*]thiophen-3-yl)-2-(3,4-dimethoxy phenyl)acrylonitrile (**11b**), and (*Z*)-3-(benzo[*b*]thiophen-3-yl)-2-(3,4,5-trimethoxyphenyl) acrylonitrile (**11c**) showed very potent growth inhibitory properties against four breast cancer cell lines utilized (MCF7, MDA-MB-231/ATCC, HS 578T, BT-549), with GI_{50} values ranging from 28nm-269nm. benzo[b]thiophene-2-carbaldehyde series of compounds (11a-d) generally exhibits greater potency than the benzo[b]thiophene-3-carbaldehyde series of compounds (13a-d) over all the breast cancer cell lines screened.

Breast Cancer Panel/cell line	Compound **11b** Molar concentrations		Compound **11c** Molar concentrations	
	GI_{50}	LC_{50}	GI_{50}	LC_{50}
MCF7	$47.8x10^{-9}$	>100.0	$40.2 x10^{-9}$	>100.0
MDA-MB-231/ATCC	$269 x10^{-9}$	>100.0	$33.0 x10^{-9}$	>100.0
HS 578T	$30.0 x10^{-9}$	>100.0	$42.7 x10^{-9}$	>100.0
BT-549	$28.3 x10^{-9}$	>100.0	$36.6 x10^{-9}$	>100.0
T-47D	>100.0	>100.0	$27.2 x10^{-6}$	>100.0

[a]GI_{50}: 50% Growth inhibition, concentration of drug resulting in a 50% reduction in net protein increase compared with control cells. [b]LC_{50}: Lethal concentration, concentration of drug lethal to 50% of cells

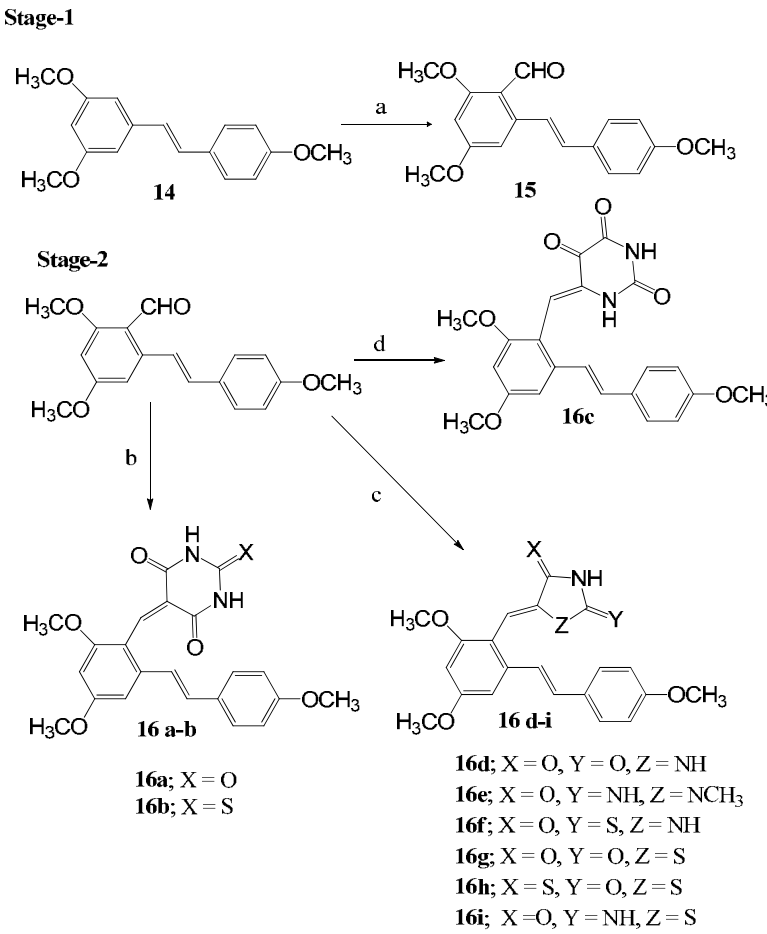
Table 6. IC_{50} values for compounds **11b** and **11c.**



Scheme 4. Synthesis of simple and substituted benzo[*b*]thiophene phenyl acrylontriles (**11a-d** and **13a-d**).

2.5 Synthesis and *in vitro* cytotoxicity evaluation of resveratrol analogs

A series of novel resveratrol analogs were synthesized (**Scheme 5**) and evaluated for their anti-proliferative activity against MCF-7 and MDA-231 breast cancer cells (**Table 7**). The initial step in the synthesis of resveratrol analogs **16a-i** is the synthesis of the common intermediate *trans*-2-formyl-3,4',5-trimethoxystilbene (**15**), which was prepared via formylation of (*E*)-1,3-dimethoxy-5-(4-methoxystyryl)benzene (**14**) with a slight excess of phosphorous oxychloride ($POCl_3$) in dimethyl formamide (DMF) at 0 ^oC in 69% yield (Xian, et al, 2007). The novel resveratrol analogs **16a-i** were then prepared by aldol condensation of resveratrol-2-carboxaldehyde with the appropriate active methylene compound, utilizing a variety of reaction conditions, i.e., ammonium acetate in acetic acid under microwave irradiation (MWI), by refluxing the reactants in ethanol, or by stirring the reaction at ambient temperature in methanol. The synthetic routes to the resveratrol analogs **16a-i** are illustrated in **Scheme 5**. Compounds **16a-i** were fully characterized by ^1H-NMR and ^{13}C-NMR spectrometry. The geometry of the double bond was established as *Z*, based on NMR spectrometric data. The X-ray crystallographic data of the representative compound **16e** confirmed the *Z*-geometry in this analog (Madadi, et al, 2010). The most potent compound, (*Z*)-5-(2,4-dimethoxy-6-(4-methoxystyryl)benzylidene)-2-imino-1-methylimidazolidin-4-one (**16e**), had IC_{50} values of 0.99μM against MDA-231 cancer cell lines. Compound (*Z*)-6-(2,4-dimethoxy-6-(4-methoxystyryl)benzylidene)-dihydropyrimidine-2,4,5(3H)-trione (**16c**) had an IC_{50} value of 1.28μM against the MCF-7 cell line.



Scheme 5. Synthesis of resveratrol analogs **16a-i**; reagents and conditions: (a) $POCl_3$, DMF, 0 ^oC, 69% yield; (b) barbituric acid or thiobarbituric acid, methanol, RT, 6 hrs, 95-96% yield; (c) five membered active methylene compound, NH_4OAc, AcOH, MWI, 1-2 min, 94-97% yield; (d) isobarbituric acid, ethanol, reflux, 4 hrs, 90% yield.

Entry	Breast cancer cell lines	
	MCF-7 (IC_{50})	MDA-231 (IC_{50})
16a	11.8	5.57
16b	>40	7.59
16c	1.28	12.3
16d	7.93	4.24
16e	14.8	0.99
16f	2.40	10.3
16g	3.96	10.1
16h	1.99	4.09

Table 7. IC_{50} values (μM) for resveratrol analogs **16a-h**.

The resveratrol analog, (*E*)-5-(2,4-dimethoxy-6-(4-methoxystyryl)benzylidene)-2-iminothiazolidin-4-one (**16i**) was also evaluated for its *in vitro* cytotoxicity against a panel of human MCF-7, MDA-MB-231/ATCC, HS-578T, BT-549, T-47D, breast cancer cell lines (**Table 8**). This compound showed growth inhibition (GI_{50} values ranging from 1.4μM-3.9μM) and cytotoxicity (LC_{50} values ranging from 7.1μM-65.7μM) amongst four out of five of the breast cancer cell lines utilized.

Breast Cancer Panel/cell line	GI_{50}	LC_{50}
MCF7	1.44	7.89
MDA-MB-231/ATCC	2.76	65.7
HS 578T	2.55	>100
BT-549	3.91	49.8
T-47D	1.66	7.26
MDA-MB-468	1.66	7.10

[a]GI_{50}: 50% Growth inhibition, concentration of drug resulting in a 50% reduction in net protein increase compared with control cells. [b]LC_{50}: Lethal concentration, concentration of drug lethal to 50% of cells

Table 8. Growth inhibition **(**GI_{50}/ μM)[a] and cytotoxicity (LC_{50}/ μM)[b] data of (*Z*)-2-amino-5-(2,4-dimethoxy-6-(4-methoxystyryl) benzylidene)thiazol-4(5*H*)-one (**16i**).

3. Conclusion

In conclusion, novel *N*-benzyl aplysinopsin, combretastatin and resveratrol analogs have been synthesized and evaluated for their anticancer activity against a number of breast cell lines. In the N-benzyl aplysinopsin series, the analog (*Z*)-methyl-1-(4-cyanobenzyl)-3-((2,5-dioxoimidazolidin-4-ylidene)methyl)-1*H*-indole-6-carboxylate (**3n**) emerged as promising lead compound for further structural optimization studies. The novel combretastatin analogs, (*Z*)-3-(benzo[*b*]thiophen-3-yl)-2-(3,4-dimethoxyphenyl)acrylonitrile (**11b**), and (*Z*)-3-(benzo[*b*]thiophen-3-yl)-2-(3,4,5-trimethoxy phenyl)acrylonitrile (**11c**) were shown to be potent cytotoxic agents against breast cancer cell lines in culture and worthy of further evaluation in animal models of breast cancer. From the library of novel resveratrol analogs, the creatinine analog, (*Z*)-5-(2, 4-dimethoxy-6-(4-methoxystyryl)benzylidene)-2-imino-1-methylimidazolidin-4-one (**16e**) was considered as a lead analog for subsequent structural optimization as a potential agent for the treatment of breast cancer.

4. References

Baur, J. A.; Sinclair, D. A. (2006), *Nat. Rev. Drug Discov*. 5(6), 493-506.

Brew, C. T.; Aronchik, I.; Kosco, K.; McCammon, J.; Bjeldanes, L. F.; Firestone, G. L. (2009), *Int J.Cancer*. 124(10), 2294-302.

Cushman, M.; Nagarathnam, D.; Gopal, D.; Chakraborti, A. K.; Lin, C. M.; Hamel, E. (1991), *J. Med. Chem.*, *34*, 2579.

Dobroslawa Bialonska.; Jordan K. Zjawiony (2009), *Marine Drugs*, 7, 166-183.

Hollenbeak, K. H;. Schmitz, F (1997). J. *Lloydia*, 40, 479.

Jacobs, T. L.; Winstein, S.; Linden, G. B.; Robson, J. H.; Levy, E. F.; Seymour, D. (1948), *Organic Synthesis*, *28*, 70-72.

Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W.; Fong, H. H.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M (1997). *Science*, 275, 218.

Kazlauskas, R.; Murphy, P. T.; Quinn, Wells, R. J (1997), *Tetrahedron letters*, 18, 61-64.

Kondo, K.; Nishi, J.; Ishibashi, M.; Kobayashi, J (1994), *J. Nat. Prod.* 57, 1008-1011.

Lin, C. M.; Singh, S. B.; Chu, P. S.; Dempcy, R. O.;Schmidt, J. M.; Pettit, G. R.; Hamel, E. (1988), *Mol. Pharmacol. 34*, 200.

Madadi, N. R.; Reddy, Y. R.; Reddy, N. R.; Parkin, S.; Crooks, P. A (2010), *Acta Crystallography , Section E*. E66(7), o1792.

McGown, A. T.; Fox, B. W. (1990), *Cancer Chemother. Pharmacol. 26*, 79.

Macpherson, L. J.; Dubin, A. E.; Evans, M. J.; Marr, F.; Schultz, P. G.; Cravatt, B. F.; Patapoutian, A. (2007), *Nature* (London, United Kingdom), *445*(7127), 541-545.

Narsimha Reddy, Penthala.; Thirupathi Reddy, Yerramreddy.; Peter A. Crooks (2010). *Bio.org & Med Chem Lett*, 20(2), 591-593.

Narsimha Reddy, Penthala.; Thirupathi Reddy, Yerramreddy.; Nikhil Reddy, Madadi.; Peter A. Crooks (2010), *Bio.org & Med Chem Lett*, *20,* (15), 4468-4471.

Narsimha Reddy, Penthala.; Thirupathi Reddy, Yerramreddy.; Peter A. Crooks (2011), *Bio.org & Med Chem Lett*, 21(5), 1411-1413.

Narsimha Reddy, P.; Thirupathi Reddy, Y.; Parkin, S.; Crooks, P. A (2009), *Acta Crystallographica*, E65(11), o2909-o2910.

Narsimha Reddy, P.; Thirupathi Reddy, Y.; Parkin, S.; Crooks, P. A (2009), *Acta Crystallographica*, E65(10), o2439-o2440.

Narsimha Reddy, P.; Thirupathi Reddy, Y.; Parkin, S.; Crooks, P. A (2009), *Acta Crystallographica*, E65(3), o552.

Ragaz, J (2009), *Breast Cancer Res.,* 11, Suppl 3: S14.

Shindikar, A. V.; Khan, F.; Viswanathan, C. L. (2006), *Euro. J. Med. Chem*., *41*(6), 786-792.

Thirupathi Reddy, Y.; Narsimha Reddy, P.; Srinivas, K.; Chendil Damodaran.; Peter A. Crooks (2010). *Bio.org & Med. Chem*., 18, (10), 3570-3574.

Xian, F. H.; Ban, F. R.; Xiao, T. W.; Chen, X.; Hui, M. G.; Hai, L. Z.; Ren, X. T (2007). *Euro. J. Med. Chem*. 42, 263-267.

15

The Beneficial Effects of Nutritional Compounds on Breast Cancer Metastasis

Jeffrey D. Altenburg and Rafat A. Siddiqui
Indiana University Health, Methodist Hospital, Methodist Research Institute
United States of America

1. Introduction

Metastasis, a process of cell migration from an existing cancer site to other anatomical sites, is the leading cause of death among women with breast cancer. There are numerous important signaling mediators that facilitate the migration of tumor cells from the area of origin to outlying tissues. While metastasis may be directed to several tissues, including the brain and the lungs, the primary sight of breast cancer metastasis is the bone (Mundy, 2002; Nguyen et al., 2009).

There have been extensive molecular studies in the cancer field that have resulted in a rich array of molecules that contribute to the process of metastasis. A summary of the process with some key metastatic factors may be seen in figure 1. Different proteins are required for such activities as cell migration, adhesion, angiogenesis, and extracellular matrix (ECM) degradation that allows invasion into the surrounding tissues and establishment of new tumors. Additionally, some proteins, such as vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) contribute to metastasis by helping the cells to sustain themselves independently through angiogenesis once they have migrated to the target tissues (Brown et al., 1995; Kolch et al., 1995; Perrotte et al., 1999; Toi et al., 1995).

There have been numerous reports of nutritional compounds that show promise against metastasis. The advantages of using nutritional compounds to treat breast cancer include the lack of adverse side effects and the significantly reduced expense, compared to synthetic drugs. For example, the omega-3 polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the primary fatty acids found in fish oil. Clinical and epidemiological studies have shown beneficial effects of DHA and EPA on a variety of diseases, including cancer and atherosclerosis (Altenburg & Siddiqui, 2010; Bang & Dyerberg, 1972; Blanckaert et al., 2010; Connolly et al., 1999; Wu et al., 2005). It is also important to note that many drugs used in the treatment of cancer and other diseases were originally discovered as components of nutritional compounds that were further improved upon through isolation of the active compound and modification of the original compound to develop more potent treatments. The purpose of this review is to examine and update the progress of nutritional compounds and their effects on breast cancer metastasis. We will also review the various mechanisms involved in metastasis and how the reported nutritional compounds affect these mechanisms. While this review will focus on key factors that are both important for metastasis and are regarded as targets for most bioactive nutritional

compounds, it is important to note that there are many other important signaling molecules as part of the signaling cascades or independent that contribute to metastasis.

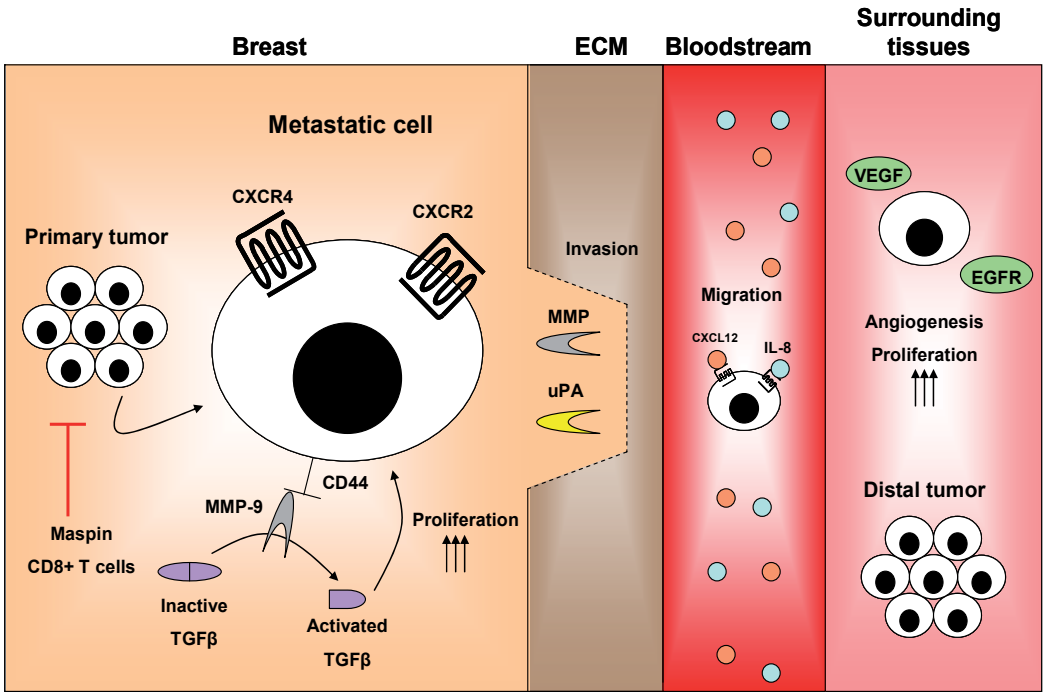


Fig. 1. The process of breast cancer metastasis. Breast cancer cells from the primary tumor site overexpress pro-metastatic proteins, including CXCR4, CXCR2, matrix metalloproteinases (MMP), CD44, and urokinase-like plasminogen activator (uPA). MMPs and uPA degrade the extracellular matrix (ECM), which allows metastatic cells to invade into the blood stream or surrounding tissues. Chemokines in the blood stream or surrounding tissues direct the migrating cells by signaling through their receptors. Cells that have successfully migrated to the surrounding tissues may undergo angiogenesis through vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) signaling to develop new distal tumors in the invaded tissues. MMP-9 is also known to tether to CD44 and activate tumor growth factor beta (TGFβ), which increases proliferation of the metastatic cells. Maspin and CD8+ cytotoxic T lymphocytes are important for tumor suppression and upregulated by some nutritional compounds.

2. Factors promoting metastasis

Cancer cell metastasis is a dynamic process that requires contribution from a large number of factors. In addition to the movement of the tumor cells from the origin to distal sites, the newly migrated cells must be allowed to proliferate and establish new tumors. It is important to note that while we have covered several of the factors in this review, there are an extensive number of other proteins and factors that are involved in the process either directly or indirectly. Here we will review some of the key factors that have also been shown to be inhibited by various nutritional compounds. It still remains to be seen whether other

still undiscovered nutritional factors affect the key modulators of metastasis. It is also very likely that other novel proteins will be discovered that contribute to metastasis.

2.1 Chemokines and chemokine receptors

Chemokines are small secreted chemotactic cytokines that contribute to the migration of select leukocyte subsets in response to inflammation or pathogen invasion. CXCR4 is a chemokine receptor that is primarily expressed on the cell surface and is overexpressed in some metastatic cancer cells. The only known natural ligand for CXCR4 is the small molecule secreted chemokine protein CXCL12 (Bleul et al., 1996; Oberlin et al., 1996). The primary function for CXCR4 is cellular migration toward large gradients of CXCL12. Metastatic breast cancer cells express large levels of CXCR4 on the surface, and as a result migrate toward tissues with abundant CXCL12 (Muller et al., 2001). Because CXCL12 is expressed in many tissues (Yu et al., 2006), CXCR4 is a key factor in metastasis of numerous cancers including breast (Chen, Y. et al., 2003; Helbig et al., 2003; Kang et al., 2005). Successful signaling through the CXCL12/CXCR4 axis requires cholesterol-rich lipid raft domains that facilitate receptor dimerization (Vila-Coro et al., 1999; Wang et al., 2006). CXCL12 signals through the Akt1 pathway and through nuclear factor kappa B (NFκB) to induce migration (Helbig et al., 2003; Zheng et al., 2008). While many nutritional compounds reviewed in this article have not been reported to effect CXCR4 expression or signaling, many have inhibitory effects on NFκB, suggesting that they may interfere with migration through this mechanism. Interleukin-8 (IL-8), also known as CXCL1, and CXCL2 are two other example of chemokines that have been implicated in breast cancer metastasis (Kang et al., 2003; Kluger et al., 2005; Minn et al., 2005). Both CXCL1 and CXCL2 interact with CXCR2 to induce cell migration.

2.2 Matrix metalloproteinases

Matrix metalloproteinases (MMP) are secreted from cells to degrade the extracellular matrix (ECM). There are currently 23 known members of the MMP family (Quesada et al., 2009). MMP-2 and MMP-9 have been strongly implicated in breast cancer metastasis as degradation of the ECM allows cells to gain access to the surrounding target tissues (Egeblad & Werb, 2002; Forget et al., 1999). Additionally, cleavage of the ECM reveals the presence of other prometastatic molecules that are normally hidden from the cancer cells. MMPs may also cleave precursors of prometastatic proteins to result in active proteins (Noe et al., 2001). E-cadherin is an example of a prometastatic protein that is activated by MMP-3 and MMP9. Expression of the MMPs is driven in part by signaling through the NFκB and AP-1 pathways (Sato & Seiki, 1993; Sen et al., 2010). Therefore, nutritional compounds that have bioactive components that inhibit NFκB may down-regulate expression of the key MMPs for metastasis. The folk medicine capillarisin from Artemisia capillaries is an example of a compound that inhibits MMP-9 expression through blocking NFκB (Lee et al., 2008). It is very possible if not likely that capillarisin will inhibit the expression of other pro-metastatic proteins driven by NFκB, such as CXCR4; however, investigations have not progressed in this area. There are other proteinases, such as urokinase-like plasminogen activator (uPA), that function in a similar manner to the MMPs (Blasi & Carmeliet, 2002). As an alternative mechanism, MMP-9 is known to interact with the cell surface receptor CD44 to facilitate the activation of tumor growth factor β and promote metastasis (Yu & Stamenkovic, 2000).

2.3 Nuclear factor kappa B

Nuclear factor kappa B (NFκB) is a transcription factor that is the end result of a large number of signaling cascades including those responsible for pro-metastatic protein expression (Helbig et al., 2003; Sato & Seiki, 1993; Sen et al., 2010). Under normal circumstances, NFκB is kept inactive by the inhibitors of κB (IκB) (Perkins, 2007). As part of many signaling cascades, the IκB is degraded through phosphorylation by IκB kinase (IKK) allowing activation of NFκB. The active NFκB binds to promoter regions of these proteins and facilitates the initial transcription. Additionally, protein functions, such as those of the CXCL12/CXCR4 signaling axis are driven by NFκB (Rehman & Wang, 2008). As a result, inhibitors of NFκB may show beneficial effects for multiple pro-metastatic pathways aside from those described in the previous literature. It is also important to note that while many nutritional compounds have been reported to be inhibitors of NFκB, in some cases it is not known if the compound directly inhibits NFκB or if the compound inhibits a different factor that is upstream of NFκB. In some cases, such as with DHA, the compound may interfere with the binding of NFκB to the target sites on the DNA (Schley et al., 2005). In other cases, the compound may interfere with expression or activation of NFκB. Because NFκB is not a classical pro-metastatic molecule but an important molecule in signaling in many pathways including those of pro-metastatic proteins, compounds that inhibit NFκB may have non-specific effects on other important pathways unrelated to the cancer. This is an important factor that must be considered when using some treatment options.

2.4 Angiogenic factors

Vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) are two examples of proteins that contribute to the process of angiogenesis where tumor cells generate new blood vessels in order to become self sustaining (de Jong et al., 1998; Goldman et al., 1993; Petit et al., 1997). Angiogenesis is an important process for metastasis because it allows migrated cells to form distal tumors in their new tissue locations. Therefore, while some nutritional compounds may directly effect migration and invasion, there are also examples of nutritional compounds that have inhibited angiogenesis, resulting in an indirect effect on metastasis. The compounds isolated from flaxseed oil are an example of anti-angiogenesis factors (Chen, J. et al., 2002; Dabrosin et al., 2002).

2.5 Tumor suppressors

Another example of indirect activity of factors against metastasis would be the tumor suppressors. There are extensive examples of proteins expressed within the cells as in the case of p53 or maspin that function to inhibit the uncontrolled proliferation of cancer cells (Crawford et al., 1981; Mercer et al., 1984; Zou et al., 2000). Many breast cancer phenotypes express very little p53 or the p53 is mutated into an inactive form (Neve et al., 2006). Additionally, there are also extracellular factors, such as secreted proteins or immunomodulatory lymphocytes like CD8+ cytotoxic T lymphocytes or natural killer cells that induce apoptosis in tumor cells (Schild et al., 1987; Talmadge et al., 1980). While this activity is separate from the process of metastasis, it is still a function that contributes to the inhibition of metastasis. When cancer cells are controlled by tumor suppressors, it becomes more difficult to progress towards metastasis. In this sense, it could be said that any treatment that is shown to reduce proliferation or induce apoptosis in tumor cells will also indirectly inhibit metastasis. Additionally, it has been reported that the suppressor maspin

also exhibits anti-metastatic properties (Sheng et al., 1996). High maspin expression has been associated with high CXCR4 expression in patients with advanced breast cancer. (Tsoli et al., 2007). These findings suggest that compounds that promote the expression of maspin will not only suppress tumor formation but will also inhibit metastasis as an added level of protection. Abalone visceral extract and apple peel extract are two examples of nutritional compounds that have been reported to enhance tumor suppression and inhibit metastasis through up-regulation of maspin (Reagan-Shaw et al., 2010; Trapani & Smyth, 2002).

3. Nutritional factors inhibiting metastasis

There are many obvious benefits to the use of nutritional compounds as therapeutic treatments for cancer metastasis. First, the potential for adverse side effects is greatly reduced. Second, the cost and accessibility of nutritional compounds is significantly preferable to those of synthetic drugs. However, there are also disadvantages. One major concern is bioavailability. The amount of the active molecules in nutritional compounds may not be practical for individuals who are seeking the beneficial effects. Therefore, it is necessary to continue to develop new treatments based on the discoveries of active anti-cancer molecules found in various foods to improve the bioavailability. Additionally, while it may not be practical to assume that a patient can be treated by a nutritional compound alone, the compound may also be used in combination with the established cancer therapies in order to enhance their potential.

There is also an issue of practicality in terms of actual consumption. In some cases, it is not difficult to acquire the beneficial effects of a nutritional compound. For example fish and apple peels are readily available to people in most cultures. They are also desirable parts of the human diet. However, certain plants may not be desirable to eat or readily available, but may contain bioactive factors. Therefore, while it is important to continue investigating novel nutritional treatment options, another important step is the isolation and synthesis of the bioactive compounds for easy consumption.

3.1 Fish oil

There have been numerous studies that report that mice fed diets rich in fish oil showed significant reduction of metastasis of transplanted breast cancer cell lines (Ghosh-Choudhury et al., 2009; Rose et al., 1995). The omega-3 polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the main active components of fish oil. DHA and EPA have specifically been shown to have down-modulating effects on migration and invasion of aggressive breast cancer cell lines (Blanckaert et al., 2010). DHA and EPA both decrease signaling through the pro-metastatic molecule CXCR4 *in vitro* (Altenburg & Siddiqui, 2009). Incorporation of DHA or EPA into the cellular plasma membrane results in alterations of signaling patterns (Shaikh et al., 2004). The conformational changes induced in the plasma membrane also render certain surface receptors inaccessible to the ligand (Li et al., 2005; Schley et al., 2007).

Additionally, DHA and EPA inhibit metastasis of MDA-MB-231 xenographs in mice to bone through targeting of CD44, the another prometastatic surface molecule (Mandal et al., 2010). Treatment of aggressive metastatic breast cancer cell lines with DHA and EPA decreased transcription of polycomb group (PcG) protein, enhancer of zeste homologue 2 (EZH2)(Dimri et al.,), a protein that is over expressed in metastatic cells. Conversely, treatment with linoleic acid and arachidonic acid, two n-6 PUFAs, had no effect on EZH2

expression, confirming previous reports that suggest the beneficial effects of fatty acids on metastasis are specific for n-3 PUFAs, while n-6 PUFAs are associated with increased risk of metastasis (Bartsch et al., 1999; Chen et al., 2007; Hubbard & Erickson, 1987). DHA and EPA were also shown to inhibit expression of MMP-2 and MMP-9 (Suzuki et al., 1997). Fish oil as well as the individual DHA and EPA components have also been reported to have inhibitory effects on NFκB signalling in both cancer-related and non-cancer-related pathways, suggesting that decreases in the pro-metastatic proteins may all be related to NFκB (Fickl et al., 2005; Ghosh-Choudhury et al., 2009; Schley et al., 2005; Weise et al.,). However, we observed that treatment of MDA-MB-231 cells with DHA and EPA resulted in decreases of surface expressed CXCR4 with no effects on total expression of CXCR4 (Altenburg & Siddiqui, 2009). This suggests that the NFκB signaling pathway may play a role in the inhibition of some, but not all, pro-metastatic factors.

In addition to using DHA or EPA in order to treat metastatic cancer, the fatty acids may be modified as a mechanism to improve their anti-cancer potency. Limited bioavailability is an issue that affects many of the nutritional compounds reviewed in this chapter. It was reported that patients given a daily supplement of DHA over the course of a month exhibited levels of approximately 200 μM in their plasma (Rusca et al., 2009). By modifying the molecule, this number may be improved. Additionally, the molecule also may show higher potency through mechanisms that are not utilized by the unmodified molecule. For example, DHA and other fatty acids have been conjugated to paclitaxel (Bradley et al., 2001) and methotrexate (Zerouga et al., 2002). In all three of these cases, the conjugated fatty acids have shown increased potency compared to either counterpart alone.

Our lab has developed conjugates of DHA with the commonly used anesthetic 2,6-diisopropylphenol (propofol) (Harvey et al., 2010; Siddiqui et al., 2005). The conjugates showed significantly increased inhibition of proliferation of breast cancer cell lines through increased apoptosis. Conversely, the treatment of the cells with unconjugated DHA combined with propofol did not have a significantly different effect from cells treated with DHA alone. This suggests that the conjugation act resulted in a new molecule with increased antiproliferative potency. The conjugates also have shown a significantly higher potency in decreasing surface expression of CXCR4 in the T acute lymphoblastic leukemia cell lines CEM and Jurkat (Altenburg et al., 2011). Taken together, the results of these studies suggest that the conjugation of DHA with propofol may be a valuable treatment option for patients with metastatic breast cancer.

A phase II clinical trial concluded that patients supplemented with DHA showed significant enhancement of the anti-metastatic potential of an anthracycline-based chemotherapy regimen with no adverse side effects (Bougnoux et al., 2009). The effect was only observed in patients who incorporated high plasma levels of DHA, suggesting that the beneficial effects of DHA supplementation are dependent on the individual profile of the patient and not universally applicable. In addition, DHA has been reported to enhance the effects of other anti-cancer treatments, including celecoxib on prostate cancer cells (Narayanan et al., 2006), and doxorubicin for breast cancer (Bougnoux et al., 2009). This is just one example of how a nutritional compound may be used in combination with other treatments in order to amplify the desired effect.

3.2 Flaxseed oil

In addition to EPA and DHA, beneficial effects of another omega-3 fatty acid, α-linolenic acid (ALA), have been reported for various stages of breast cancer progression (Rose, 1997;

Thompson, 1998). Secoicolariciresinol diglycoside (SDG) is a precursor to the lignans enterolactone and enterodiol that also inhibits breast cancer (Thompson, 1998). It was reported that mice given diets rich in flaxseed oil were protected from metastasis of xenograph transplants of MDA-MB-435 cells (Dabrosin et al., 2002). The authors concluded that the protection was associated with decreased levels of VEGF expression. The same investigators later reported that the anti-metastatic effect was also partially due to decreases in expression of insulin-like growth factor 1 (IGF-1) and epidermal growth factor receptor (EGFR) (Chen, J. et al., 2002). It was also suggested that the components of flaxseed oil exert a synergistic anti-metastatic effect when combined with the drug tamoxifen (Chen & Thompson, 2003).

Flaxseed oil is known to contain very high levels of SDG as well as ALA (Cunnane et al., 1993; Thompson et al., 1991). Wang *et al* investigated the importance of SDG, compared to ALA in terms of effects on breast cancer metastasis (Wang et al., 2005). They reported that both compounds contributed to the effects; however, metastasis in mice given SDG only was not significantly different than the control mice. This suggests a potential synergistic interaction between the SDG and ALA compounds found in flaxseed oil. As a result, it is important to emphasize that in many of the examples of nutritional compounds, such as resveratrol (Castillo-Pichardo et al., 2009) and flaxseed oil that show beneficial effects on various diseases, the entire compound may be a better option than isolating a single component for treatment.

3.3 Curcumin

The phenolic compound curcumin [1,7-bis(4-hydroxy-3-methoxy phenyl) -1,6-heptadiene-3,5-dione] is the major ingredient in the rhizome of the herb *Curcuma longa*. Curcumin has been used in Asian medicine since the second millennium BC (Srimal & Dhawan, 1973). Curcumin displays a wide range of pharmacological activities, including anti-inflammatory, anticancer, antioxidant, wound healing, and antimicrobial effects (Maheshwari et al., 2006). Curcumin has also been shown to decrease expression of CXCR4 in highly metastatic lymphoma cells (Skommer et al., 2007). Another report has shown that curcumin inhibits lung metastasis of paclitaxel-resistant breast cancer cells transplanted into mice through suppression of pro-metastatic protein expression, including COX-2 and MMP-9 (Aggarwal et al., 2005). Curcumin also downregulates the inflammatory chemokines CXCL1 and CXCL2 (Bachmeier et al., 2008). In many cases, the mechanism for which curcumin down-regulates the pro-metastatic factors has been reported to be inhibition of the NFκB pathway. This has been the case for CXCL1 and CXCL2 as well as the angiogenic factors VEGF and EGFR (Bachmeier et al., 2007, 2008).

Demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC) are two derivatives of curcumin that have been reported to have inhibitory effects on the expression and secretion of matrix metalloproteinase 3 (MMP-3), a key molecule for invasion and metastasis (Boonrao et al., 2010). The matrix metalloproteinase family of enzymes is responsible for degrading the extracellular matrix and allowing tumor cells to invade and metastasize (Stetler-Stevenson et al., 1996). Tumor cells expressing increased levels of MMPs are more aggressive. In the study above, the derivatives of curcumin had inhibitory effects on both expression and secretion of MMP-3, but curcumin itself had no effect. This suggests that with some nutritional compounds, the actual compound may not be beneficial to the individual; however, metabolites of the compounds may have strong effects.

Curcumin has also been reported to enhance expression of mammary serine protease inhibitor (maspin) (Prasad et al., 2010). Maspin is a tumor suppressor protein that was reported by Zhang *et al* to inhibit cell motility and angiogenesis (Zhang et al., 2000). Maspin expression is controlled by P53 and is abundant in normal mammary epithelial cells (Zou et al., 2000). Cancer cells with mutated or lost p53 express little if any maspin. In breast cancer cells, maspin is silenced epigenetically as a result of hypermethylated CpG islands (Domann et al., 2000). The mechanism utilized by curcumin to up-regulate maspin expression as reported by Prasad *et al* is unknown at this time. However, they observed that the up-regulation only occurred in MCF7 cells that have wildtype p53 (Prasad et al., 2010). MDA-MB-231 cells that contain mutated p53 showed no increase of maspin expression after curcumin treatment. This suggests that the activity of curcumin toward maspin expression may be modulated through the p53 pathway.

Curcumin is known to have very poor bioavailability, especially compared to DHA or EPA. In a clinical phase I trial with curcumin, patients were given oral doses of 8 grams per day. The measured serum and urine concentrations of curcumin were approximately 2 μM (Cheng et al., 2001). This suggests that while curcumin may show encouraging results in many of the *in vitro* and *in vivo* mouse experiments outlined in these studies, it will be important to conduct human experiments to confirm the validity of the results. Studies are also underway to improve the bioavailability of curcumin or to combine other therapeutic molecules with curcumin in order to enhance the potency (Saw et al., 2010; Swamy et al., 2008). We have very recently reported that curcumin in combination with docosahexaenoic acid (DHA) synergistically inhibits proliferation of the SK-BR-3 breast cancer cell line (Altenburg et al., 2011). We observed that the combination of DHA and curcumin decreased transcription of the pro-metastatic genes for CXCL1, CXCR4, maspin, and goosecoid (GSC) while the two individual compounds had little or no effect on any of these genes.

In a phase I trial it was reported that the advanced metastatic breast cancer patients supplemented with 6,000 mg/day for seven consecutive d every 3 w in addition to a standard dose of the chemotherapeutic drug docetaxel displayed encouraging results (Bayet-Robert et al., 2010). As described with the omega-3 fatty acids, this suggests that while curcumin may have potential as a treatment alone, it may also be used to further enhance the currently used chemotherapies for metastatic breast cancer. This further suggests that while bioavailability of curcumin is of important concern, it is still proving useful in terms of cancer therapy.

3.4 Resveratrol

Resveratrol (*trans*-3, 4′, 5-trihydroxystilbene, $C_{14}H_{12}O_3$) is a polyphenolic compound similar to curcumin that is derived from the skin of grapes as well as other fruits, including blueberries and raspberries. In 1997 it was reported that resveratrol blocks initiation and progression of tumorigenesis in mice treated with the carcinogen dimethylbenz(a)anthracene (DMBA) (Jang et al., 1997). Resveratol blocks expression of MMP-9 induced through hereugulin-beta1 (HRG-β1) (Tang et al., 2008a). HRG-β1 is a growth factor expressed by approximately 30% of breast cancer tumors (Lupu et al., 1996). HRG-β1 signaling through the HER2/neu receptor results in induction of MMP-9 (O-charoenrat et al., 1999; Tsai et al., 2003). Additionally, resveratrol has been shown to inhibit the expression of MMP-2 induced by insulin-like growth factor 1 (IGF-1) (Tang et al., 2008b).

One limitation of these studies is that the authors used concentrations of isolated resveratrol that were higher than those achievable by *in vivo* dietary intake. It was later reported by Castillo-Pachardo *et al* that combinations of total grape polyphenols, including resveratrol, quercetin, and catechin, were more potent at metastasis inhibition at physiologically relevant concentrations than the purified resveratrol (Castillo-Pichardo et al., 2009).

3.5 *Ganoderma Lucidum*

Numerous mushrooms have been shown to possess therapeutic properties covering a wide range of physical ailments. *Ganoderma Lucidum* is the scientific nomenclature for the oyster mushroom. *Ganoderma lucidum* has been used in asian medicine for well over 2000 years. The predominant active component in *Ganoderma lucidum* in regards to anti-cancer effects is the triterpene. The triterpenes in ganoderma lucidum have been reported to inhibit invasion and metastasis of breast cancer cell lines through inhibition of oxidative stress induced interleukin-8 secretion (Thyagarajan et al., 2006). The authors concluded that the mechanism responsible for this effect was inhibition of the AP-1 and NFκB pathways. The same investigators later reported that extract from ganoderma lucidum exerted a synergistic anti-metastatic effect when combined with green tea extract (Thyagarajan et al., 2007). This occurred through a synergistic down-regulation of urokinase plasminogen (uPA) activator secretion.

3.6 Protocatechuic acid

Protocatechuic acid (PCA) is a polyphenol that is found in numerous fruits, vegetables, nuts (Ma et al., 2008), and brown rice (Hudson et al., 2000). PCA was reported in a study in 1993 to exhibit chemopreventative properties on tumorigenesis and progression of colon cancer (Tanaka et al., 1993). Since then, PCA was further reported to have beneficial effects on a wide range of cancers, including breast and liver (Kampa et al., 2004; Yip et al., 2006). Yin *et al* reported that treatments of the T47D aggressive breast cancer cell line with escalating doses of PCA down-regulated production of interleukin-6 (IL-6), interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF) (Yin et al., 2009). Interleukin-8, also known as CXCL1, is known to promote the expression of the prometastatic proteins MMP-2 (Luca et al., 1997) and MMP-9 (Inoue et al., 2000) in prostate cancer cells. VEGF is a factor that promotes angiogenesis and allows tumors access to their own blood supply (Brown et al., 1995; Kolch et al., 1995; Toi et al., 1995). Therefore, metastasized tumors are able to sustain themselves. This study only consisted of *in vitro* cell line experiments. It remains to be seen if the results will be confirmed *in vivo*.

3.7 Abalone visceral extract

Abalones are edible sea snails that can vary in size and are harvested in east Asia as a primary food source. A recent report demonstrated that extract generated from the viscera of the abalone has potent anti-cancer and anti-metastasis properties in mice transplanted with the 4T1 cell line (Lee, C.G. et al., 2010). The authors concluded that abalone visceral extract inhibits metastasis through stimulation of CD8+ cytotoxic T lymphocyte activity, which is well known to have anti-tumor properties (Trapani & Smyth, 2002). Additionally, the abalone visceral extract down-regulated the expression of cyclooxygenase-2 (COX-2) (Lee, C.G. et al., 2010). COX-2 is a key target for treatments against metastatic cancer because it acts in concord with hypoxia inducible factor 1-α (HIF1α) as a transcription factor for

numerous prometastatic proteins, including CXCR4 and various MMPs (Maroni et al., In Press). The bioactive compounds contained in abalone visceral extract are not known at this time.

3.8 Alpha-lipoic acid

α-Lipoic acid (ALA) is a compound that is commonly found in animal and plant cells that has known antioxidant properties (Moini et al., 2002; Packer et al., 1995). A case study was reported of a patient with advanced metastatic pancreatic cancer who was administered treatments of ALA along with low doses of Naltrexone (Berkson et al., 2006). The treatments for this patient began in October of 2002. In January of 2006, the individual showed no symptoms and no visible progression of the malignancy, suggesting that ALA may be a valuable treatment for advanced metastatic cancer. Lee, H.S. et al reported that ALA significantly decreases proliferation, migration, and invasion of the MDA-MB-231 aggressive cell line (Lee, H.S. et al., 2010). The authors in this study concluded that treatment of the cells with ALA resulted in significant decreases in transcription and translation of MMP-2 and MMP-9, suggesting a mechanism for the anti-metastatic effects.

3.9 Butein

Butein is a tetrahydroxychalcone that is derived from numerous plants with the most common being the stembark of cashews (Pandey et al., 2007). Butein has been reported to have anti-proliferative effects on various cancer cell types through inhibition of NFκB and suppression of signal transducer and activator of transcription (STAT)-3 (Pandey et al., 2007). Chua *et al* found that butein also has an inhibitory effect on CXCL12 signaling through CXCR4 (Chua et al., 2010), suggesting a beneficial anti-metastatic effect. These investigators demonstrated through reporter assays and chromatin immunoprecipitation assays that the decrease in CXCR4 expression was at the transcriptional level and not due to receptor degradation. The effect of butein on CXCR4 expression was observed in multiple cancer cell lines, including representative cell lines for breast, liver and prostate cancer. This suggests that while many of the compounds reviewed in this chapter have only been studied for breast cancer metastasis, the findings from these studies may also be applicable to other cancers or other diseases that share similar mechanisms with cancer metastasis. For example, CXCR4 has been shown to be a critical cofactor for the cellular entry of certains strains of HIV-1 (Bleul et al., 1996; Oberlin et al., 1996). It is therefore possible that some nutritional compounds, including butein, curcumin, and DHA, that have decreasing effects on CXCR4 expression would be useful in treatment or prevention of HIV infection. This is an intriguing possibility for some nutritional compounds that remains to be investigated.

3.10 Zerumbone

Zerumbone is a sexquiterpene that is derived from the rhizome of the ginger plant *Zingiber zerumbet.* Zerumbone was reported to induce apoptosis and inhibit the activity of NFκB, resulting in down-modulation of numerous cancer promoting genes, including prometastatic genes like COX-2, MMP-9, and ICAM-1 (Takada et al., 2005). This study also reported effects of zerumbone on different cell lines including those of breast cancer, lung adenocarcinoma, and human squamous cell carcinoma. The same group later published data showing that zerumbone down-regulates the transcription and expression of CXCR4 in

Her2+ MCF7 breast cancer cells (Sung et al., 2008). The authors suggested that the previously reported inhibition of NFκB activity may have been the reason for the decrease in CXCR4 expression due to blocking of NFκB interaction in the CXCR4 promoter region. The authors chose the estrogen receptor$^+$/Her2$^+$ MCF7 cell line because Her2 has been linked along with NFκB to metastasis to increased CXCR4 expression (Li et al., 2004). It will be important to confirm the results of this study with *in vivo* mouse models as well as more aggressive cell lines.

Common name	Bioactive components	Targets
Fish oil	DHA, EPA	CXCR4, CD44, EZH2, MMP-2, MMP-9, NFκB
Flaxseed oil	ALA, SDG	VEGF, IGF-1, EGFR
Turmeric	Curcumin	CXCR4, COX-2, MMP-9, CXCL1, CXCL2, VEGF, EGFR, MMP-3, Maspin, NFκB
Grapes and other fruit	Resveratrol	MMP-2, MMP9
Ganoderma lucidum (oyster mushrooms)	Triterpenes	IL-8, uPA
Olives	Protocatechuic acid	IL-6, IL-8, VEGF
Abalone visceral extract	unknown	COX-2, CXCR3, MMPs
Plant and animal cells	α-Lipoic acid	MMP-2, MMP-9
Cashews	Butein	NFκB, STAT3, CXCR4
Ginger	Zerumbone (sexquiterpene)	NFκB, COX-2, CXCR4, ICAM-1
Apple peel extract	Polyphenolic antioxidants	Maspin
Green tea	polyphenols	uPA, NFκB, AP-1, MMP-2, MMP-9
Loquat methanol extract	triterpenoids	MMP-2, MMP-9

Table 1. Summary of reviewed nutritional compound effects on breast cancer metastasis.

3.11 Apple peel extract

The common apple contains numerous polyphenolic antioxidants, including catechins, epicatechins and procyanidins (Boyer & Liu, 2004). Various case-controlled epidemiologic studies from multiple locations between 1991 and 2002 have shown that diets high in apple consumption are associated with reduced cancer risk (Gallus et al., 2005). Additionally, it was reported that rats given whole apple extract doses equivalent to those of a human eating 1, 3, or 6 apples per day showed significant signs of breast cancer prevention when given the carcinogen DMBA (Liu et al., 2005). Reagen-Shaw *et al* later reported that extract from the apple peel also prevented breast cancer progression (Reagan-Shaw et al., 2010). As in the case of curcumin, the cells treated with apple peel extract displayed significant increases in the expression of maspin (Reagan-Shaw et al., 2010).

3.12 Green tea

Green tea is a widely consumed beverage that is popular for its flavor as well as numerous health benefits (Katiyar & Mukhtar, 1996). Green tea contains an abundance of polyphenols, including epicatechin derivatives, including epicatechingallate and epigallocatechin. The polyphenols of green tea possess antioxidant and anti-inflammatory properties as well as beneficial effects for many cancers, including breast cancer (Katiyar & Mukhtar, 1996; Zheng et al., 1996). Baliga *et al* reported that oral treatment of mice with green tea polyphenols inhibited tumor growth and metastasis of the highly aggressive 4T1 mouse breast cancer cell line (Baliga et al., 2005). This occurred through an upregulation of the pro-apoptotic protein Bax and a down-regulation of Bcl-2 in addition to activation of apoptotic pathways involving cleaved caspase-3 and PARP. This suggests that the anti-metastatic effects of the green tea polyphenols in this particular study were indirect effects brought about by the decreased viability of the cancer cells rather than direct effects on metastasis-specific mechanisms.

It was later reported that green tea polyphenols suppress migration and invasion of MDA-MB-231 cells *in vitro* by down-regulating the expression of urokinase-type plasminogen activator (uPA) through inhibition of the AP-1 and NFκB pathways (Slivova et al., 2005). uPA is a serine protease, which is in part responsible for degradation of the extracellular matrix, which allows cells to migrate into surrounding tissues (Blasi & Carmeliet, 2002). Isolated preparations of (-)-epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea, significantly reduced expression of MMP-2 and MMP-9, two other proteases that degrade the extracellular matrix, in MCF7 cells (Sen et al., 2009).

3.13 Loquat methanol extract

The leaves of the loquat plant have long been used in traditional Japanese and Chinese medicine to treat chronic bronchitis, coughs, phlegm, high fever and gastroenteric disorders. Previous studies have demonstrated that the triterpenoids isolated from the loquat plant have anti-tumor, antiviral and anti-inflammatory activities (Banno et al., 2005; De Tommasi et al., 1992; Liang et al., 1990). Recently, a report showed that the extract from loquat inhibits MDA-MB-231 proliferation, migration, and invasion through down-modulation of MMP-2 and MMP-9 expression (Kim et al., 2009). This observation suggests that the loquat methanol extract has beneficial effects against tissue invasion; however, the results of this study will need to be confirmed with *in vivo* experiments.

4. Conclusions

Metastasis is the leading cause of death in patients with most cancers, including breast cancer. Numerous studies have outlined a wide array of signaling molecules and secreted proteases that contribute to the process of metastasis as well as the conversion of a non-metastatic tumor to a highly aggressive tumor. The purpose of this review is to highlight the wide, diverse range of nutritional compounds that all have been reported to have beneficial effects for breast cancer metastasis. Table 1 shows a brief summary of the nutritional compounds covered in this review, including the bioactive components, the target or activity, and the references associated with those compounds. The nutritional compounds may be exotic to the western population in the case of potential medicines such as abalone visceral extract or zerumbone. The nutritional compounds may also be very common to people who consume the traditional western diets. This is the case for apple peel, grapes, or cashews.

Many of the nutritional medicines contain similar bioactive components, and as a result inhibit metastasis through similar mechanisms. However, it has been shown in some cases that isolating these individual compounds may not provide a superior treatment to the whole extract (Castillo-Pichardo et al., 2009). This suggests that there may either be unknown components or components previously thought to be inactive in some of these extracts. This further suggests that there may be interactions between multiple components within the extract that result in synergistic beneficial effects. This adds an extra layer of complexity to the idea of nutritional compounds being used to prevent or treat breast cancer. For example, it is very possible that two or more nutritional compounds from the same or different sources may be used in combination to exert a synergistic effect. Combinations of DHA and curcumin have been reported to synergistically inhibit the progression of pancreatic cancer as well as inflammation (Saw et al., 2010; Swamy et al., 2008). We have also recently observed that combinations of DHA and curcumin inhibit proliferation of the SK-BR-3 cell line in a synergistic manner (Altenburg et al., 2011).

It is important to note that while some nutritional compounds have been analyzed in clinical human trials, the limited efficacy and bioavailability raise concerns for using only these compounds for treatment. Therefore, it is essential that potential metastatic cancer therapies be used with the assumption that proven medical treatment is the preferable option. Nutritional compounds should be used as potential adjuvants to existing cancer therapies. However, because most of the compounds have little or no side effects and are non-toxic at normal levels, there is no reason that a patient should not be able to supplement their diet with the available nutrition.

5. References

Aggarwal B.B.; Shishodia. S.; Takada, Y.; Banerjee, S.; Newman, R.A.; Bueso-Ramos, C.E. & Price, J.E. (2005) Curcumin suppresses the paclitaxel-induced nuclear factor-kappaB pathway in breast cancer cells and inhibits lung metastasis of human breast cancer in nude mice. *Clin Cancer Res,* Vol. 11, No. 20, pp. 7490-7498, ISSN 1078-0432

Altenburg, J. D.; Harvey, K. A.; McCray, S.; Xu, Z. & Siddiqui, R.A. (2011). A novel 2,6-diisopropylphenyl-docosaheaxaenoamide conjugate induces apoptosis in T cell acute lymphoblastic leukemia cell lines. *Biochem Biophys Res Commun*, Vol. 411, No. 2. (July, 2011), pp427-432, ISSN 0006-291X.

Altenburg, J.D. & Siddiqui, R.A. (2010). Docosahexaenoic acid downregulates interferon gamma-induced expression of CXCL16 in human aortic smooth muscle cells. *Biochem Biophys Res Commun,* Vol. 391, No. 1, (January, 2010), pp. 609-614, ISSN 0006-291X

Altenburg, J.D. & Siddiqui, R.A. (2009). Omega-3 polyunsaturated fatty acids down-modulate CXCR4 expression and function in MDA-MB-231 breast cancer cells. *Mol Cancer Res,* Vol. 7, No. 7, (July, 2009), pp. 1013-1020, 2009, ISSN 1541-7786

Altenburg, J.D.; Bieberich, A.A.; Terry, C.; Harvey, K.A.; VanHorn, J.F.; Davisson, V.J. & Siddiqui, R.A. (2011) A synergistic antiproliferation effect of curcumin and docosahexaenoic acid in SK-BR-3 breast cancer cells: unique signaling not explained by the effects of either compound alone. *BMC Cancer*, Vol. 11, (April, 2011), pp. 149, ISSN 1471-2407

Bachmeier, B.; Nerlich, A.G.; Iancu, C.M.; Cilli, M.; Schleicher, E.; Vene, R.; Dell'Eva, R.; Jochum, M.; Albini, A. & Pfeffer, U. (2007). The chemopreventive polyphenol

Curcumin prevents hematogenous breast cancer metastases in immunodeficient mice. *Physiol Biochem,* Vol. 19 No. 1-4, pp. 137-15, ISSN 1015-8987

Bachmeier, B.E.; Mohrenz, I.V.; Mirisola, V.; Schleicher, E.; Romeo, F.; Hohneke, C.; Jochum, M.; Nerlich, A.G. & Pfeffer, U. (2008) Curcumin downregulates the inflammatory cytokines CXCL1 and -2 in breast cancer cells via NFkappaB. *Carcinogenesis,* Vol. 29, No. 4, (April, 2008), pp. 779-789, ISSN 0143-3334

Baliga, M.S.; Meleth, S. & Katiyar, S.K. (2005) Growth inhibitory and antimetastatic effect of green tea polyphenols on metastasis-specific mouse mammary carcinoma 4T1 cells in vitro and in vivo systems. *Clin Cancer Res,* Vol. 11, No. 5, (March, 2005), pp. 1918-1927, ISSN 1048-0432

Bang, H.O. & Dyerberg, J. (1972). Plasma lipids and lipoproteins in Greenlandic west coast Eskimos. *Acta Med Scand,* Vol. 192, No. 1-2, (July-August, 1972), pp. 85-94, ISSN 1520-765X

Banno, N.; Akihisa, T.; Tokuda, H.; Yasukawa, K.; Taguchi, Y.; Akazawa, H.; Ukiya, M.; Kimura, Y.; Suzuki, T. & Nishino, H. (2005). Anti-inflammatory and antitumor-promoting effects of the triterpene acids from the leaves of Eriobotrya japonica. *Biol Pharm Bull,* Vol. 28, No. 10, (October, 2005), pp. 1995-1999, ISSN 0198-6158

Bartsch, H.; Nair, J. & Owen, R.W. (1999). Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. *Carcinogenesis,* Vol. 20, No. 12, (December, 1999), pp. 2209-2218, ISSN 0143-3334

Bayet-Robert, M.; Kwiatkowski, F.; Leheurteur, M.; Gachon, F.; Planchat, E.; Abrial, C.; Mouret-Reynier, M.A.; Durando, X.; Barthomeuf, C. & Chollet, P. (2010) Phase I dose escalation trial of docetaxel plus curcumin in patients with advanced and metastatic breast cancer. *Cancer Biol Ther,* Vol. 9, No. 1, (January, 2010), pp. 8-14, ISSN 1555-8576

Berkson, B.M.; Rubin, D.M. & Berkson, A.J. (2006) The long-term survival of a patient with pancreatic cancer with metastases to the liver after treatment with the intravenous alpha-lipoic acid/low-dose naltrexone protocol. *Integr Cancer Ther,* Vol. 5, No. 1, (March, 2006), pp. 83-89, ISSN 1534-7354

Blanckaert, V.; Ulmann, L.; Mimouni, V.; Antol, J.; Brancquart, L. & Chenais, B. (2010) Docosahexaenoic acid intake decreases proliferation, increases apoptosis and decreases the invasive potential of the human breast carcinoma cell line MDA-MB-231. *Int J Oncol,* Vol. 36, No. 3, (March, 2010), pp. 737-742, ISSN 1791-2423

Blasi, F. & Carmeliet, P. (2002). uPAR: a versatile signalling orchestrator. *Nat Rev Mol Cell Biol,* Vol. 3, No. 12, (December, 2002), pp. 932-943, ISSN 1471-0072

Bleul, C.C.; Farzan, M.; Choe, H.; Parolin, C.; Clark-Lewis, I.; Sodroski, J. & Springer, T.A. (1996) The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature,* Vol. 382, No. 6594, (August, 2006), pp. 829-833, ISSN 0028-0836

Boonrao, M.; Yodkeeree, S.; Ampasavate, C.; Anuchapreeda, S. & Limtrakul, P. (2010) The inhibitory effect of turmeric curcuminoids on matrix metalloproteinase-3 secretion in human invasive breast carcinoma cells. *Arch Pharm Res,* Vol. 33, No. 7, (July, 2010), pp. 989-998, ISSN 0253-6269

Bougnoux, P.; Hajjaji, N.; Ferrasson, M.N.; Giraudeau, B.; Couet, C. & Le Floch, O. (2009) Improving outcome of chemotherapy of metastatic breast cancer by

docosahexaenoic acid: a phase II trial. *Br J Cancer,* Vol. 101, No.12, (December, 2009), pp. 1978-1985, ISSN 0007-0920

Boyer, J. & Liu, R.H. (2004). Apple phytochemicals and their health benefits. *Nutr J,* Vol. 3, (May, 2004), p. 5, ISSN 1475-2891

Bradley, M.O.; Swindell, C.S.; Anthony, F.H.; Witman, P.A.; Devanesan, P.; Webb, N.L.; Baker, S.D.; Wolff, A.C. & Donehower, R.C. (2001) Tumor targeting by conjugation of DHA to paclitaxel. *J Control Release,* Vol. 74, No. 10, (October, 2001), pp. 233-236, ISSN 0168-3659

Brown, L.F.; Berse, B.; Jackman, R.W.; Tognazzi, K.; Guidi, A.J.; Dvorak, H.F.; Senger, D.R.; Connolly, J.L. & Schnitt, S.J. (1995). Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Hum Pathol,* Vol. 26, No. 1, (January, 1995), pp. 86-91, ISSN 0046-8177

Castillo-Pichardo, L.; Martinez-Montemayor, M.M.; Martinez, J.E.; Wall, K.M.; Cubano, L.A. & Dharmawardhane, S. (2009). Inhibition of mammary tumor growth and metastases to bone and liver by dietary grape polyphenols. *Clin Exp Metastasis,* Vol. 26, No. 6, pp. 505-516, ISSN 0262-0898

Chen, J.; Stavro, P.M. & Thompson, L.U. (2002). Dietary flaxseed inhibits human breast cancer growth and metastasis and downregulates expression of insulin-like growth factor and epidermal growth factor receptor. *Nutr Cancer,* Vol. 43, No.2, pp. 187-192, ISSN 0163-5581

Chen, J. & Thompson, L.U. (2003) Lignans and tamoxifen, alone or in combination, reduce human breast cancer cell adhesion, invasion and migration in vitro. *Breast Cancer Res Treat,* Vol. 80, No. 2, (July, 2003), pp. 163-170, ISSN 0167-6806

Chen, Y.; Stamatoyannopoulos, G. & Song, C.Z. (2003) Down-regulation of CXCR4 by inducible small interfering RNA inhibits breast cancer cell invasion in vitro. *Cancer Res,* Vol. 63, No 16, (August, 2003), pp. 4801-4804, ISSN 0008-5472

Chen, Y.Q.; Edwards, I.J.; Kridel, S.J.; Thornburg, T. & Berquin, I.M. (2007). Dietary fat-gene interactions in cancer. *Cancer Metastasis Rev,* Vol. 26, No. 3-4, (December, 2007), pp. 535-551, ISSN 0167-7659

Cheng, A.L.; Hsu, C.H.; Lin, J.K.; Hsu, M.M.; Ho, Y.F.; Shen, T.S.; Ko, J.Y.; Lin, J.T.; Lin, B.R.; Ming-Shiang, W.; Yu, H.S.; Jee, S.H.; Chen, G.S.; Chen, T.M.; Chen, C.A.; Lai, M.K.; Pu, Y.S.; Pan, M.H.; Wang, Y.J.; Tsai, C.C. & Hsieh, C.Y. (2001). Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res,* Vol. 21, No. 4B, (July-August, 2001), pp. 2895-2900, ISSN 0250-7005

Chua, A.W.; Hay, H.S.; Rajendran, P.; Shanmugam, M.K.; Li, F.; Bist, P.; Koay, E.S.; Lim, L.H.; Kumar, A.P. & Sethi, G. (2010). Butein downregulates chemokine receptor CXCR4 expression and function through suppression of NF-kappaB activation in breast and pancreatic tumor cells. *Biochem Pharmacol,* Vol. 80, No. 10, (November, 2010), pp. 1553-1562, ISSN 0006-2952

Connolly, J.M.; Gilhooly, E.M. & Rose, D.P. (1999) Effects of reduced dietary linoleic acid intake, alone or combined with an algal source of docosahexaenoic acid, on MDA-MB-231 breast cancer cell growth and apoptosis in nude mice. *Nutr Cancer,* Vol. 35, No. 1, pp. 44-49, ISSN 0163-5581

Crawford, L.V.; Pim, D.C.; Gurney, E.G.; Goodfellow, P. & Taylor-Papadimitriou, J. (1981) Detection of a common feature in several human tumor cell lines--a 53,000-dalton

protein. *Proc Natl Acad Sci U S A,* Vol. 78, No.1, (January, 1981), pp. 41-45, ISSN 0027-8424

Cunnane, S.C.; Ganguli, S.; Menard, C.; Liede, A.C.; Hamadeh, M.J.; Chen, Z.Y.; Wolever, T.M. & Jenkins, D.J. (1993) High alpha-linolenic acid flaxseed (Linum usitatissimum): some nutritional properties in humans. *Br J Nutr,* Vol. 69, No. 2, (March, 1993), pp. 443-453, ISSN 0007-1145

Dabrosin, C.; Chen, J.; Wang, L. & Thompson, L.U. (2002). Flaxseed inhibits metastasis and decreases extracellular vascular endothelial growth factor in human breast cancer xenografts. *Cancer Lett,* Vol. 185, No. 1, (November, 2002), pp. 31-3, ISSN 0304-3835

de Jong, J.S.; van Diest, P.J.; van der Valk, P. & Baak, J.P. (1998) Expression of growth factors, growth-inhibiting factors, and their receptos in invasive breast cancer. II: Correlations with proliferation and angiogenesis. *J Pathol,* Vol. 184, No. 1, (January, 1998), pp. 53-57, ISSN 0022-3417

De Tommasi, N.; De Simone, F.; Pizza, C.; Mahmood, N.; Moore, P.S.; Conti, C.; Orsi, N. & Stein, M.L. (1992) Constituents of Eriobotrya japonica. A study of their antiviral properties. *J Nat Prod,* Vol. 55, No. 8, (August, 1992), pp. 1067-1073, ISSN 0970-129X

Dimri, M.; Bommi, P.V.; Sahasrabuddhe, A.A.; Khandekar, J.D. & Dimri, G.P. (2010). Dietary omega-3 polyunsaturated fatty acids suppress expression of EZH2 in breast cancer cells. *Carcinogenesis,* Vol. 31, No. 3, (March, 2010), pp. 489-495, ISSN 0143-3334

Domann, F.E.; Rice, J.C.; Hendrix, M.J. & Futscher, B.W. (2000) Epigenetic silencing of maspin gene expression in human breast cancers. *Int J Cancer,* Vol. 85, No. 6, (March, 2000), pp. 805-810, ISSN 0020-7136

Egeblad, M. & Werb, Z. (2002) New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer,* Vol. 2, No. 3, (March, 2002), pp. 161-174, ISSN 1474-175X

Fickl, H.; Cockeran, R.; Steel, H.C.; Feldman, C.; Cowan, G.; Mitchell, T.J. & Anderson, R. (2005). Pneumolysin-mediated activation of NFkappaB in human neutrophils is antagonized by docosahexaenoic acid. *Clin Exp Immunol,* Vol. 140, No. 2, (May, 2005), pp. 274-281, ISSN 0009-9104

Forget, M.A.; Desrosiers, R.R. & Beliveau, R. (1999) Physiological roles of matrix metalloproteinases: implications for tumor growth and metastasis. *Can J Physiol Pharmacol,* Vol. 77, No. 7, (July, 1999), pp. 465-480, ISSN 0008-4212

Gallus, S.; Talamini, R.; Giacosa, A.; Montella, M.; Ramazzotti, V.; Franceschi, S.; Negri, E. & La Vecchia, C. (2005) Does an apple a day keep the oncologist away? *Ann Oncol,* Vol. 16, No. 11, (November, 2005), pp. 1841-1844, ISSN 0923-7534

Ghosh-Choudhury, T.; Mandal, C.C.; Woodruff, K.; St Clair, P.; Fernandes, G.; Choudhury, G.G. & Ghosh-Choudhury, N. (2009) Fish oil targets PTEN to regulate NFkappaB for downregulation of anti-apoptotic genes in breast tumor growth. *Breast Cancer Res Treat,* Vol. 118, No. 1, (November, 2009), pp. 213-228, ISSN 0167-6806

Goldman, C.K.; Kim, J.; Wong, W.L.; King, V.; Brock, T. & Gillespie, G.Y. (1993). Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. *Mol Biol Cell,* Vol. 4, No. 1, (January, 1993), pp. 121-133, ISSN 1059-1524

Harvey, K.A.; Xu, Z.; Whitley, P.; Davisson, V.J. & Siddiqui, R.A. (2010) Characterization of anticancer properties of 2,6-diisopropylphenol-docosahexaenoate and analogues in

breast cancer cells. *Bioorg Med Chem,* Vol. 18, No. 5, (March, 2010), pp. 1866-1874, ISSN 0968-0896

Helbig, G.; Christopherson, K.W., 2nd; Bhat-Nakshatri, P.; Kumar, S.; Kishimoto, H.; Miller, K.D.; Broxmeyer, H.E. & Nakshatri, H. (2003). NF-kappaB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. *J Biol Chem,* Vol. 278, No. 24, (June, 2003), pp. 21631-21638, ISSN 0021-9528

Hubbard, N.E. & Erickson, K.L. (1987) Enhancement of metastasis from a transplantable mouse mammary tumor by dietary linoleic acid. *Cancer Res,* Vol. 47, No. 23, (December, 1987), pp. 6171-6175, ISSN 0008-5472

Hudson, E.A.; Dinh, P.A.; Kokubun, T.; Simmonds, M.S. & Gescher, A. (2000) Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. *Cancer Epidemiol Biomarkers Prev,* Vol. 9, No. 11, (November, 2000), pp. 1163-1170, ISSN 1055-9965

Inoue, K.; Slaton, J.W.; Eve, B.Y.; Kim, S.J.; Perrotte, P.; Balbay, M.D.; Yano, S.; Bar-Eli, M.; Radinsky, R.; Pettaway, C.A. & Dinney, C.P. (2000) Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. *Clin Cancer Res,* Vol. 6, No. 5, (May, 2000), pp. 2104-2119, ISSN 1078-0432

Jang, M.; Cai, L.; Udeani, G.O.; Slowing, K.V.; Thomas, C.F.; Beecher, C.W.; Fong, H.H.; Farnsworth, N.R.; Kinghorn, A.D.; Mehta, R.G.; Moon, R.C. & Pezzuto, J.M. (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science,* Vol. 275, No. 5297, (January, 1997), pp. 218-220, ISSN 0036-8075

Kampa, M.; Alexaki, V.I.; Notas, G.; Nifli, A.P.; Nistikaki, A.; Hatzoglou, A.; Bakogeorgou, E.; Kouimtzoglou, E.; Blekas, G.; Boskou, D.; Gravanis, A. & Castanas, E. (2004) Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells: potential mechanisms of action. *Breast Cancer Res,* Vol. 6, No. 2, pp. R63-74, ISSN 1465-5411

Kang, H.; Watkins, G.; Parr, C.; Douglas-Jones, A.; Mansel, R.E. & Jiang, W.G. (2005) Stromal cell derived factor-1: its influence on invasiveness and migration of breast cancer cells in vitro, and its association with prognosis and survival in human breast cancer. *Breast Cancer Res,* Vol. 7, No. 4, pp. R402-410, ISSN 1465-5411

Kang, Y.; Siegel, P.M.; Shu, W.; Drobnjak, M.; Kakonen, S.M.; Cordon-Cardo, C.; Guise, T.A. & Massague, J. (2003) A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell,* Vol. 3, No. 6, (June, 2003), pp. 537-549, ISSN 1535-6108

Katiyar, S.K. & Mukhtar, H. (1996) Tea consumption and cancer. *World Rev Nutr Diet,* Vol. 79, pp. 154-184, ISSN 0084-2230

Kim, M.S.; You, M.K.; Rhuy, D.Y.; Kim, Y.J.; Baek, H.Y. & Kim, H.A. (2009) Loquat (Eriobotrya japonica) extracts suppress the adhesion, migration and invasion of human breast cancer cell line. *Nutr Res Pract,* Vol. 3, No. 4, (Winter, 2009), pp. 259-264, ISSN 1976-1457

Kluger, H.M.; Chelouche Lev, D.; Kluger, Y.; McCarthy, M.M.; Kiriakova, G.; Camp, R.L.; Rimm, D.L. & Price, J.E. (2005) Using a xenograft model of human breast cancer metastasis to find genes associated with clinically aggressive disease. *Cancer Res,* Vol. 65, No. 13, (July, 2005), pp. 5578-5587, ISSN 0008-5472

Kolch, W.; Martiny-Baron, G.; Kieser, A. & Marme, D. (1995) Regulation of the expression of the VEGF/VPS and its receptors: role in tumor angiogenesis. *Breast Cancer Res Treat,* Vol. 36, No. 2, pp. 139-155, ISSN 0167-6806

Lee, C.G.; Kwon, H.K.; Ryu, J.H.; Kang, S.J.; Im, C.R.; Ii Kim, J. & Im, S.H. (2010) Abalone visceral extract inhibit tumor growth and metastasis by modulating Cox-2 levels and CD8+ T cell activity. *BMC Complement Altern Med,* Vol. 10, p. 60, ISSN 1472-6882

Lee, H.S.; Na, M.H. & Kim, W.K. (2010) alpha-Lipoic acid reduces matrix metalloproteinase activity in MDA-MB-231 human breast cancer cells. *Nutr Res,* Vol. 30, No. 6, (June, 2010), pp. 403-409, ISSN 0271-5317

Lee, S.O.; Jeong, Y.J.; Kim, M.; Kim, C.H. & Lee, I.S. (2008) Suppression of PMA-induced tumor cell invasion by capillarisin via the inhibition of NF-kappaB-dependent MMP-9 expression. *Biochem Biophys Res Commun,* Vol. 366, No. 4, (February, 2008), pp. 1019-1024, ISSN 0006-291X

Li, Q.; Wang, M.; Tan, L.; Wang, C.; Ma, J.; Li, N.; Li, Y.; Xu, G. & Li, J. (2005) Docosahexaenoic acid changes lipid composition and interleukin-2 receptor signaling in membrane rafts. *J Lipid Res,* Vol. 46, No. 9, (September, 2005), pp. 1904-1913, ISSN 0022-2275

Li, Y.M.; Pan, Y.; Wei, Y.; Cheng, X.; Zhou, B.P.; Tan, M.; Zhou, X.; Xia, W.; Hortobagyi, G.N.; Yu, D. & Hung, M.C. (2004) Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. *Cancer Cell* Vol. 6, No. 5, (November, 2004), pp. 459-469, ISSN 1535-6108

Liang, Z.Z.; Aquino, R.; Feo, V.D.; Simone, F.D. & Pizza C. (1990) Polyhydroxylated Triterpenes from Eriobotrya japonica. *Planta Med,* Vol. 56, No. 3, (June, 1990), pp. 330-332, ISSN 0032-0943

Liu, R.H.; Liu, J. & Chen, B. (2005) Apples prevent mammary tumors in rats. *J Agric Food Chem* Vol. 53, No. 6, (March, 2005), pp. 2341-2343, ISSN 0021-8561

Luca, M.; Huang, S.; Gershenwald, J.E.; Singh, R.K.; Reich, R. & Bar-Eli, M. (1997) Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. *Am J Pathol,* Vol. 151, No. 4, (October, 1997), pp. 1105-1113, ISSN 0887-8005

Lupu, R.; Cardillo, M.; Cho, C.; Harris, L.; Hijazi, M.; Perez, C.; Rosenberg, K.; Yang, D. & Tang, C. (1996) The significance of heregulin in breast cancer tumor progression and drug resistance. *Breast Cancer Res Treat,* Vol. 38, No. 1, pp. 57-66, ISSN 0167-6806

Ma, Y.Q.; Ye, X.Q.; Fang, Z.X.; Chen, J.C.; Xu, G.H. & Liu, D.H. (2008) Phenolic compounds and antioxidant activity of extracts from ultrasonic treatment of Satsuma Mandarin (Citrus unshiu Marc.) peels. *J Agric Food Chem,* Vol. 56, No. 14, (July, 2008), pp. 5682-5690, ISSN 0021-8561

Maheshwari, R.K.; Singh, A.K.; Gaddipati, J. & Srimal, R.C. (2006) Multiple biological activities of curcumin: a short review. *Life Sci,* Vol. 78, No. 18, (March, 2006), pp. 2081-2087,ISSN 0024-3205

Mandal, C.C.; Ghosh-Choudhury, T.; Yoneda, T.; Choudhury, G.G. & Ghosh-Choudhury, N. (2010) Fish oil prevents breast cancer cell metastasis to bone. *Biochem Biophys Res Commun,* Vol. 402, No. 4, (November, 2010), pp. 602-607, ISSN 0006-291X

Maroni, P.; Matteucci, E.; Luzzati, A.; Perrucchini, G.; Bendinelli, P. & Desiderio, M.A. (2010) Nuclear co-localization and functional interaction of COX-2 and HIF-1alpha characterize bone metastasis of human breast carcinoma. *Breast Cancer Res Treat*. November 10, Epub ahead of press, ISSN 0167-6806

Mercer, W.E.; Avignolo, C. & Baserga, R. (1984) Role of the p53 protein in cell proliferation as studied by microinjection of monoclonal antibodies. *Mol Cell Biol,* Vol. 4, No. 2, (February, 1984), pp. 276-281, ISSN 0270-7306

Minn, A.J.; Gupta, G.P.; Siegel, P.M.; Bos, P.D.; Shu, W.; Giri, D.D.; Viale, A.; Olshen, A.B.; Gerald, W.L. & Massague, J. (2005) Genes that mediate breast cancer metastasis to lung. *Nature,* Vol. 436, No. 7249, (June, 2005), pp. 518-524, ISSN 0028-0836

Moini, H.; Packer, L. & Saris, N.E. (2002) Antioxidant and prooxidant activities of alpha-lipoic acid and dihydrolipoic acid. *Toxicol Appl Pharmacol,* Vol. 182, No. 1, (July, 2002), pp. 84-90, ISSN 0041-008X

Muller, A.; Homey, B.; Soto, H.; Ge, N.; Catron, D.; Buchanan, M.E.; McClanahan, T.; Murphy, E.; Yuan, W.; Wagner, S.N.; Barrera, J.L.; Mohar, A.; Verastegui, E. & Zlotnik, A. Involvement of chemokine receptors in breast cancer metastasis. *Nature,* Vol. 410, No. 6824, (March, 2001), pp. 50-56, ISSN 0028-0836

Mundy, G.R. (2002) Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer,* Vol. 2, No. 8, (August, 2002), pp. 584-593, ISSN 1474-175X

Narayanan, N.K.; Narayanan, B.A.; Bosland, M.; Condon, M.S. & Nargi, D. (2006) Docosahexaenoic acid in combination with celecoxib modulates HSP70 and p53 proteins in prostate cancer cells. *Int J Cancer,* Vol. 119, No. 7, (October, 2006), pp. 1586-1598, ISSN 0020-7136

Neve, R.M.; Chin, K.; Fridlyand, J.; Yeh, J.; Baehner, F.L.; Fevr, T.; Clark, L.; Bayani, N.; Coppe, J.P.; Tong, F.; Speed, T.; Spellman, P.T.; DeVries, S.; Lapuk, A.; Wang, N.J.; Kuo, W.L.; Stilwell, J.L.; Pinkel, D.; Albertson, D.G.; Waldman, F.M.; McCormick, F.; Dickson, R.B.; Johnson, M.D.; Lippman, M.; Ethier, S.; Gazdar, A. & Gray, J.W. (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell,* Vol. 10, No. 6, (December, 2006), pp. 515-527, ISSN 1535-6108

Nguyen, D.X.; Bos, P.D. & Massague, J. (2009) Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer,* Vol. 9, No. 4, (April, 2009), pp. 274-284, ISSN 1474-175X

Noe, V.; Fingleton, B.; Jacobs, K.; Crawford, H.C.; Vermeulen, S.; Steelant, W.; Bruyneel, E.; Matrisian, L.M. & Mareel, M. (2001) Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. *J Cell Sci,* Vol. 114, Pt. 1, (January, 2001), pp. 111-118, ISSN 0021-9533

Oberlin, E.; Amara, A.; Bachelerie, F.; Bessia, C.; Virelizier, J.L.; Arenzana-Seisdedos, F.; Schwartz, O.; Heard, J.M.; Clark-Lewis, I.; Legler, D.F.; Loetscher, M.; Baggiolini, M. & Moser B. (1996) The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature,* Vol. 382, No.6594, (August, 1996), pp. 833-835, ISSN 0028-0836

O-charoenrat, P.; Rhys-Evans, P.; Court, W.J.; Box, G.M. & Eccles, S.A. (1999) Differential modulation of proliferation, matrix metalloproteinase expression and invasion of human head and neck squamous carcinoma cells by c-erbB ligands. *Clin Exp Metastasis,* Vol. 17, No. 7, pp. 631-639, ISSN 0262-0898

Packer, L.; Witt, E.H. & Tritschler, H.J. (1995) alpha-Lipoic acid as a biological antioxidant. *Free Radic Biol Med,* Vol. 19, No. 2, (August, 1995), pp. 227-250, ISSN 0891-5849

Pandey, M.K.; Sandur, S.K.; Sung, B.; Sethi, G.; Kunnumakkara, A.B. & Aggarwal, B.B. (2007) Butein, a tetrahydroxychalcone, inhibits nuclear factor (NF)-kappaB and NF-

kappaB-regulated gene expression through direct inhibition of IkappaBalpha kinase beta on cysteine 179 residue. *J Biol Chem,* Vol. 282, No. 24, (June, 2007), pp. 17340-17350, ISSN 0021-9528

Perkins, N.D. (2007) Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol,* Vol. 8, No. 1, (January, 2007), pp. 49-62, ISSN 1471-0072

Perrotte, P.; Matsumoto, T.; Inoue, K.; Kuniyasu, H.; Eve, B.Y.; Hicklin, D.J.; Radinsky, R. & Dinney, C.P. (1999) Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res,* Vol. 5, No. 2, (February, 1999), pp. 257-265, ISSN 1078-0432

Petit, A.M.; Rak, J.; Hung, M.C.; Rockwell, P.; Goldstein, N.; Fendly, B. & Kerbel, R.S. (1997) Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol,* Vol. 151, No. 6, (December, 1997), pp. 1523-1530, ISSN 0887-8005

Prasad, C.P.; Rath, G.; Mathur, S.; Bhatnagar, D. & Ralhan, R. (2010). Expression analysis of maspin in invasive ductal carcinoma of breast and modulation of its expression by curcumin in breast cancer cell lines. *Chem Biol Interact,* Vol. 183, No. 3, (February, 2010), pp. 455-461, ISSN 0009-2797

Quesada, V.; Ordonez, G.R.; Sanchez, L.M.; Puente, X.S. & Lopez-Otin, C. (2009). The Degradome database: mammalian proteases and diseases of proteolysis. *Nucleic Acids Res,* Vol. 37, (January, 2009), pp. D239-243, ISSN 0305-1048

Reagan-Shaw, S.; Eggert, D.; Mukhtar, H. & Ahmad, N. (2010). Antiproliferative effects of apple peel extract against cancer cells. *Nutr Cancer,* Vol. 62, No. 4, pp. 517-524, ISSN 0163-5581

Rehman, A.O. & Wang, C.Y. (2008) SDF-1alpha promotes invasion of head and neck squamous cell carcinoma by activating NF-kappaB. *J Biol Chem,* Vol. 283, No. 29, (July, 2008), pp. 19888-19894, ISSN 0021-9528

Rose, D.P.; Connolly, J.M.; Rayburn, J. & Coleman, M. (1995). Influence of diets containing eicosapentaenoic or docosahexaenoic acid on growth and metastasis of breast cancer cells in nude mice. *J Natl Cancer Inst,* Vol. 87, No. 8, (April, 1995), pp. 587-592, ISSN 0027-8874

Rose, D.P. (1997). Effects of dietary fatty acids on breast and prostate cancers: evidence from in vitro experiments and animal studies. *Am J Clin Nutr,* Vol. 66, No. 6-suppl, (December, 1997), pp. 1513S-1522S, ISSN 0002-9165

Rusca, A.; Di Stefano, A.F.; Doig, M.V.; Scarsi, C. & Perucca, E. (2009). Relative bioavailability and pharmacokinetics of two oral formulations of docosahexaenoic acid/eicosapentaenoic acid after multiple-dose administration in healthy volunteers. *Eur J Clin Pharmacol,* Vol. 65, No. 5, (May, 2009), pp. 503-510, ISSN 0031-6970

Sato, H. & Seiki, M. (1993). Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. *Oncogene,* Vol. 8, No. 2, (February, 1993), pp. 395-405, ISSN 0950-9232

Saw, C.L.; Huang, Y. & Kong, A.N. (2010). Synergistic anti-inflammatory effects of low doses of curcumin in combination with polyunsaturated fatty acids:

docosahexaenoic acid or eicosapentaenoic acid. *Biochem Pharmacol,* Vol. 79, No. 3, (February, 2010), pp. 421-430, ISSN 0006-2952

Schild, H.J.; Kyewski, B.; Von Hoegen, P. & Schirrmacher, V. (1987). CD4+ helper T cells are required for resistance to a highly metastatic murine tumor. *Eur J Immunol,* Vol. 17, No. 12, (December, 1987), pp. 1863-1866, ISSN 0014-2980

Schley, P.D.; Jijon, H.B.; Robinson, L.E. & Field, C.J. (2005). Mechanisms of omega-3 fatty acid-induced growth inhibition in MDA-MB-231 human breast cancer cells. *Breast Cancer Res Treat,* Vol. 92, No. 2, (July, 2005), pp. 187-195, ISSN 0167-6806

Schley, P.D.; Brindley, D.N. & Field, C.J. (2007). (n-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. *J Nutr,* Vol. 137, No. 3, (March, 2007), pp. 548-553, ISSN 0022-3166

Sen, T.; Moulik, S.; Dutta, A.; Choudhury, P.R.; Banerji, A.; Das, S.; Roy M, & Chatterjee, A. (2009). Multifunctional effect of epigallocatechin-3-gallate (EGCG) in downregulation of gelatinase-A (MMP-2) in human breast cancer cell line MCF-7. *Life Sci,* Vol. 84, No. 7-8, (February, 2009), pp. 194-204, ISSN 0024-3205

Sen, T.; Dutta, A. & Chatterjee, A. (2010), Epigallocatechin-3-gallate (EGCG) downregulates gelatinase-B (MMP-9) by involvement of FAK/ERK/NFkappaB and AP-1 in the human breast cancer cell line MDA-MB-231. *Anticancer Drugs,* Vol. 21, No. 6, (July, 2010), pp. 632-644, ISSN 0959-4973

Shaikh, S.R.; Dumaual, A.C.; Castillo, A.; LoCascio, D.; Siddiqui, R.A.; Stillwell, W. & Wassall, S.R. (2004). Oleic and docosahexaenoic acid differentially phase separate from lipid raft molecules: a comparative NMR, DSC, AFM, and detergent extraction study. *Biophys J,* Vol. 87, No. 3, (September, 2004), pp. 1752-1766, ISSN 1542-0086

Sheng, S.; Carey, J.; Seftor, E.A.; Dias, L.; Hendrix, M.J. & Sager, R. (1996). Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. *Proc Natl Acad Sci U S A,* Vol. 93, No.21, (October, 1996), pp. 11669-11674, ISSN 0027-8424

Siddiqui, R.A.; Zerouga, M.; Wu, M.; Castillo, A.; Harvey, K.; Zaloga, G.P. & Stillwell, W. (2005). Anticancer properties of propofol-docosahexaenoate and propofol-eicosapentaenoate on breast cancer cells. *Breast Cancer Res,* Vol. 7, No. 5, pp. R645-654, ISSN 1465-5411

Skommer, J.; Wlodkowic, D. & Pelkonen, J. (2007). Gene-expression profiling during curcumin-induced apoptosis reveals downregulation of CXCR4. *Exp Hematol,* Vol. 35, No. 1, (January, 2007), pp. 84-95, ISSN 0301-472X

Slivova, V.; Zaloga, G.; DeMichele, S.J.; Mukerji, P.; Huang, Y.S.; Siddiqui, R.; Harvey, K.; Valachovicova, T. & Sliva, D. (2005). Green tea polyphenols modulate secretion of urokinase plasminogen activator (uPA) and inhibit invasive behavior of breast cancer cells. *Nutr Cancer,* Vol. 52, No. 1, pp. 66-73, ISSN 0163-5581

Srimal, R.C. & Dhawan, B.N. (1973). Pharmacology of diferuloyl methane (curcumin), a non-steroidal anti-inflammatory agent. *J Pharm Pharmacol,* Vol. 25, No. 6, (June, 1973), pp. 447-452, ISSN 0022-3573

Stetler-Stevenson, W.G.; Hewitt, R. & Corcoran, M. (1996). Matrix metalloproteinases and tumor invasion: from correlation and causality to the clinic. *Semin Cancer Biol,* Vol. 7, No. 3, (June, 1996), pp. 147-154, ISSN 1044-579X

Sung, B.; Jhurani, S.; Ahn, K.S.; Mastuo, Y.; Yi, T.; Guha, S.; Liu, M. & Aggarwal, B.B. (2008). Zerumbone down-regulates chemokine receptor CXCR4 expression leading to inhibition of CXCL12-induced invasion of breast and pancreatic tumor cells. *Cancer Res,* Vol. 68, No. 21, (November, 2008), pp. 8938-8944, ISSN 0008-5472

Suzuki, I.; Iigo, M.; Ishikawa, C.; Kuhara, T.; Asamoto, M.; Kunimoto, T.; Moore, M.A.; Yazawa, K.; Araki, E. & Tsuda, H. (1997). Inhibitory effects of oleic and docosahexaenoic acids on lung metastasis by colon-carcinoma-26 cells are associated with reduced matrix metalloproteinase-2 and -9 activities. *Int J Cancer,* Vol. 73, No. 4, (November, 1997), pp. 607-612, ISSN 0020-7136

Swamy, M.V.; Citineni, B.; Patlolla, J.M.; Mohammed, A.; Zhang, Y. & Rao, C.V. (2008). Prevention and treatment of pancreatic cancer by curcumin in combination with omega-3 fatty acids. *Nutr Cancer,* Vol. 60, No. Suppl 1, pp. 81-89, ISSN 0163-5581

Takada, Y.; Murakami, A. & Aggarwal, B.B. (2005). Zerumbone abolishes NF-kappaB and IkappaBalpha kinase activation leading to suppression of antiapoptotic and metastatic gene expression, upregulation of apoptosis, and downregulation of invasion. *Oncogene,* Vol. 24, No. 46, (October, 2005), pp. 6957-6969, ISSN 0950-9232

Talmadge, J.E.; Meyers, K.M.; Prieur, D.J. & Starkey, J.R. (1980). Role of NK cells in tumour growth and metastasis in beige mice. *Nature,* Vol. 284, No. 5757, (April, 1980), pp. 622-624, ISSN 0028-0836

Tanaka, T.; Kojima, T.; Suzui, M. & Mori, H. (1993). Chemoprevention of colon carcinogenesis by the natural product of a simple phenolic compound protocatechuic acid: suppressing effects on tumor development and biomarkers expression of colon tumorigenesis. *Cancer Res,* Vol. 53, No. 17, (September, 1993), pp. 3908-3913, ISSN 0008-5472

Tang, F.Y.; Chiang, E.P. & Sun, Y.C. (2008a). Resveratrol inhibits heregulin-beta1-mediated matrix metalloproteinase-9 expression and cell invasion in human breast cancer cells. *J Nutr Biochem,* Vol. 19 No. 5, (May, 2008), pp. 287-294, ISSN 0955-2863

Tang, F.Y.; Su, Y.C.; Chen, N.C.; Hsieh, H.S. & Chen, K.S. (2008b). Resveratrol inhibits migration and invasion of human breast-cancer cells. *Mol Nutr Food Res,* Vol. 52, No. 6, (June, 2008), pp. 683-691, ISSN 1613-4125

Thompson, L.U.; Robb, P.; Serraino, M. & Cheung, F. (1991). Mammalian lignan production from various foods. *Nutr Cancer,* Vol. 16, No.1, pp. 43-52, ISSN 0163-5581

Thompson, L.U. (1998). Experimental studies on lignans and cancer. *Baillieres Clin Endocrinol Metab,* Vol. 12, No. 4, (December, 1998), pp. 691-705, ISSN 0950-351X

Thyagarajan, A.; Jiang, J.; Hopf, A.; Adamec, J. & Sliva, D. (2006). Inhibition of oxidative stress-induced invasiveness of cancer cells by Ganoderma lucidum is mediated through the suppression of interleukin-8 secretion. *Int J Mol Med,* Vol. 18, No. 4, (October, 2006), pp. 657-664, ISSN 1107-3756

Thyagarajan, A.; Zhu, J. & Sliva, D. (2007). Combined effect of green tea and Ganoderma lucidum on invasive behavior of breast cancer cells. *Int J Oncol,* Vol. 30, No. 4, (April, 2007), pp. 963-969, ISSN 1019-6439

Toi, M.; Inada, K.; Hoshina, S.; Suzuki, H.; Kondo, S. & Tominaga, T. (1995). Vascular endothelial growth factor and platelet-derived endothelial cell growth factor are frequently coexpressed in highly vascularized human breast cancer. *Clin Cancer Res,* Vol. 1, No. 9, (September, 1995), pp. 961-964, ISSN 1078-0432

Trapani, J.A. & Smyth, M.J. (2002). Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol,* Vol. 2, No. 10, (October, 2002), pp. 735-747, ISSN 1474-1733

Tsai, M.S.; Shamon-Taylor, L.A.; Mehmi, I.; Tang, C.K. & Lupu, R. (2003). Blockage of heregulin expression inhibits tumorigenicity and metastasis of breast cancer. *Oncogene,* Vol. 22, No. 5, (February, 2003), pp. 761-768, ISSN 0950-9232

Tsoli, E.; Tsantoulis, P.K.; Papalambros, A.; Perunovic, B.; England, D.; Rawlands, D.A.; Reynolds, G.M.; Vlachodimitropoulos, D.; Morgan, S.L.; Spiliopoulou, C.A.; Athanasiou, T. & Gorgoulis, V.G. (2007). Simultaneous evaluation of maspin and CXCR4 in patients with breast cancer. *J Clin Pathol,* Vol. 60, No. 3, (March, 2007), pp. 261-266, ISSN 0021-9746

Vila-Coro, A.J.; Rodriguez-Frade, J.M.; Martin De Ana, A.; Moreno-Ortiz, M.C.; Martinez, A.C. & Mellado, M. (1999). The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *Faseb J,* Vol. 13, No. 13, (October, 1999), pp. 1699-1710, ISSN 0892-6638

Wang, J.; He, L.; Combs, C.A.; Roderiquez, G. & Norcross, M.A. (2006). Dimerization of CXCR4 in living malignant cells: control of cell migration by a synthetic peptide that reduces homologous CXCR4 interactions. *Mol Cancer Ther,* Vol. 5, No. 10, (October, 2006), pp. 2474-2483, ISSN 1535-7163

Wang, L.; Chen, J. & Thompson, L.U. (2005). The inhibitory effect of flaxseed on the growth and metastasis of estrogen receptor negative human breast cancer xenograftsis attributed to both its lignan and oil components. *Int J Cancer,* Vol. 116, No. 5, (September, 2005), pp. 793-798, ISSN 0020-7136

Weise, C.; Hilt, K.; Milovanovic, M.; Ernst, D.; Ruhl, R. & Worm, M. (2011). Inhibition of IgE production by docosahexaenoic acid is mediated by direct interference with STAT6 and NFkappaB pathway in human B cells. *J Nutr Biochem,* Vol. 22, No. 3, (March, 2011), pp. 269-275, ISSN 0955-2863

Wu, M.; Harvey, K.A.; Ruzmetov, N.; Welch, Z.R.; Sech, L.; Jackson, K.; Stillwell, W.; Zaloga, G.P. & Siddiqui, R.A. (2005). Omega-3 polyunsaturated fatty acids attenuate breast cancer growth through activation of a neutral sphingomyelinase-mediated pathway. *Int J Cancer,* Vol. 117, No. 3, (November, 2005), pp. 340-348, ISSN 0020-7136

Yin, M.C.; Lin, C.C.; Wu, H.C.; Tsao, S.M. & Hsu, C.K. (2009). Apoptotic effects of protocatechuic acid in human breast, lung, liver, cervix, and prostate cancer cells: potential mechanisms of action. *J Agric Food Chem,* Vol. 57, No. 14, (July, 2009), pp. 6468-6473, ISSN 0021-8561

Yip, E.C.; Chan, A.S.; Pang, H.; Tam, Y.K. & Wong, Y.H. (2006). Protocatechuic acid induces cell death in HepG2 hepatocellular carcinoma cells through a c-Jun N-terminal kinase-dependent mechanism. *Cell Biol Toxicol,* Vol. 22, No. 4, (July, 2006), pp. 293-302, ISSN 0742-2091

Yu, L.; Cecil, J.; Peng, S.B.; Schrementi, J.; Kovacevic, S.; Paul, D.; Su, E.W. & Wang, J. (2006). Identification and expression of novel isoforms of human stromal cell-derived factor 1. *Gene,* Vol. 374, (June, 2006), pp. 174-179, ISSN 0378-1119

Yu, Q. & Stamenkovic, I. (2000). Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev,* Vol. 14, No. 2, (January, 2000), pp. 163-176, ISSN 0890-9369

Zerouga, M.; Stillwell, W. & Jenski, L.J. (2002). Synthesis of a novel phosphatidylcholine conjugated to docosahexaenoic acid and methotrexate that inhibits cell proliferation. *Anticancer Drugs,* Vol. 13, No. 3, (March, 2002), pp. 301-311, ISSN 0959-4973

Zhang, M.; Volpert, O.; Shi, Y.H. & Bouck, N. (2000). Maspin is an angiogenesis inhibitor. *Nat Med,* Vol. 6, No. 2, (February, 2000), pp. 196-199, ISSN 1078-8956

Zheng, H.; Dai, T.; Zhou, B.; Zhu, J.; Huang, H.; Wang, M. & Fu, G. (2008). SDF-1alpha/CXCR4 decreases endothelial progenitor cells apoptosis under serum deprivation by PI3K/Akt/eNOS pathway. *Atherosclerosis,* Vol. 201, No. 1, (November, 2008), pp. 36-42, ISSN 0021-9150

Zheng, W.; Doyle, T.J.; Kushi, L.H.; Sellers, T.A.; Hong, C.P. & Folsom, A.R. (1996). Tea consumption and cancer incidence in a prospective cohort study of postmenopausal women. *Am J Epidemiol,* Vol. 144, No. 2, (July, 1996), pp. 175-182, ISSN 0002-9262

Zou, Z.; Gao, C.; Nagaich, A.K.; Connell, T.; Saito, S.; Moul, J.W.; Seth, P.; Appella, E. & Srivastava, S. (2000). P53 regulates the expression of the tumor suppressor gene maspin. *J Biol Chem,* Vol. 275, No. 9, (March, 2000), pp. 6051-6054, ISSN 0021-9528

16

Legume-Derived Bioactive Compounds for the Prevention and Treatment of Breast Cancer

Graziella Joanitti, Sonia Freitas and Ricardo Azevedo
University of Brasilia,
Brazil

1. Introduction

Breast cancer is one of the most prevalent cancer types among women worldwide (Jemal et al., 2011); however, its incidence rates among populations are heterogeneous. Epidemiologic studies have shown that breast cancer incidence in Asian women is 40% lower than in Caucasian women (Goldin et al., 1986). A reasonable explanation for the difference in the cancer incidence rates could be related to intrinsic biological characteristics present in each population. For example, in general, breast cancer growth requires the presence of estrogen and it is known that Asian women have lower estrogen serum levels than Caucasian women (Shimizu et al., 1990). Nevertheless, epidemiologic studies have reported that when Asian women moved to western countries the breast cancer incidence of their subsequent generations were similar to the Caucasian women (Wu et al., 1996). Therefore, it seems that other factors have been influencing breast cancer incidence rates in each population.

In fact, it is known that only 10-15% of all breast cancers cases are caused by genetic predisposition such as BRCA and Li-Fraumeni mutations; whereas the remaining 85-90% of cases are attributed to environmental, reproductive, lifestyle factors including radiation, chemicals, late pregnancy, early menarche, nulliparity, diet and reduced physical activity (Colditz et al., 1995). The diet is an important factor affecting breast cancer incidence rates and is estimated to be correlated with 50% of new diagnosed cases (Willett, 1995). It has been described that diets based on the consumption of garlic, onion, tomato, vegetables, fruits and legumes are associated with reduced breast cancer risk. One of the major differences between western and Asian populations is their diet. The consumption of legumes (soy, beans, peas) in Asian populations is expressively higher than in western populations. These disparities on breast cancer risk and on legume intake have attracted the attention of scientists and since then this topic has been the goal of innumerable researches (Messina et al., 2006).

In addition to their importance as a nutritive food source, legumes and their bioactive compounds have also been described to show protective and therapeutic effects not only in breast cancer, but also in symptoms of menopause, heart disease and osteoporosis. On the other hand, findings suggesting no effects or possible risks in legume intake and breast cancer have also been published (Messina et al., 2008). Therefore, the evaluation of the effects of legume consumption on women at high risk for breast cancer and breast cancer patients is an important public health goal (Messina et al., 2006). In this chapter, we provide a comprehensive review of the biological, nutritional and economic background on legumes

and gather the current knowledge regarding the benefits and risks of their bioactive molecules in breast cancer prevention and treatment.

2. Legumes - biological, nutritional and economic aspects

The legumes are classified in the family Fabacea (or Leguminosae) – including around 700 legume genera and 20,000 species – and are the third largest flowering plant family (Doyle et al., 2003; Gepts et al., 2005). They present a large range of variation and are also well adapted to several temperatures and climates (Doyle et al., 2003). Despite the large number of species, only a few legumes are generally known due to their use as feeds and foods. Clovers (*Trifolium* sp.), vetches (*Vicia faba*) and alfafa (*Medicago sativa*) are mainly grown for animal feeding; while beans (*Phaseolus vulgaris*), soybeans (*Glycine max*), lentils (*Lens esculenta*), peas (*Pisum sativum*), and peanuts (*Arachis hypogaea*) are the main species grown for food (Doyle et al., 2003; Gepts et al., 2005).

Legumes nutritional profile includes dietary fibers, low glycemic indexes, no cholesterol, low levels of fat (2- 5%), and high amounts of carbohydrates (55- 60%). In addition, essential minerals and vitamins for human health are also present (Rochfort et al., 2007). High protein content (20-40%) is another notable feature of legumes which is known to be 2-3 times higher than cereals. Along with their high protein content, they also produce a good balance of all essential aminoacids, with the exception of methionine (Rochfort et al., 2007).

Among legumes, soybeans have not only the highest protein content but also the highest protein digestibility, which is typically 90%. Soybeans and peanuts are considered an exception among legumes in terms of nutritional profile. Despite producing high protein contents, they are low in carbohydrates but high in fat (40% total energy) (Messina et al., 2010). In addition, soy is a component widely used to fortify school breakfast and lunch programs and is also present in upwards 60% of processed foods (Patisaul et al., 2010).

Some legumes produce antinutritional factors which have shown to induce allergy and intestinal disturbance when eaten raw. However, the majority of these toxins can be eliminated through heating or other industrially processing (Gepts et al., 2005).

The intake rates of legumes vary dramatically among populations worldwide. In Asian countries, legumes have been consumed for centuries representing ~50% of their diet which achieve and even surpass the minimum intake recommended. On the other hand, the rates of legume consumption by North American and European countries are low (Messina, 2010).

A few decades ago researchers have reported that some compounds produced by legumes could promote protective and therapeutic effects on human health. This new vision of legumes as functional foods has induced a profound impact in sales and consumption of legumes in countries which had low consumption rates as the EUA and European countries (Messina et al., 2001; Messina, 2010; Patisaul et al., 2010).

3. Legume bioactive compounds and breast cancer

Hypothesis regarding protective and therapeutic effects of legumes in breast cancer have been formulated mainly based on epidemiological studies suggesting a negative correlation between legume intake and breast cancer incidence among populations worldwide. Asian women, who traditionally consume high amounts of legumes daily, are 4 to 10 times less likely to be diagnosed with and die from breast cancer than are people in the United States

(Fournier et al., 1998). Interestingly, genetic variations do not seem to be the main factors involved in this disparity since Asian women who immigrate to the United States and adopted a "Western" lifestyle, particularly a diet poor in legumes, develop breast cancer risk comparable to Caucasian women within two generations (Wu et al., 1996).
These evidences have triggered several researches seeking for the identification of potential legume molecules involved in breast cancer prevention and treatment. Isoflavones, protease inhibitors and peptides are the main legume bioactive compounds evaluated in this field. In the next sections, their anticancer properties as well as synergistic effects are discussed.

4. Isoflavones

Legumes produce large amounts and several isoflavones isoforms which are assumed to have antimicrobial activity and to play an important role in plant protection (Rochfort et al., 2007). In particular, soybeans produce 12 isoforms of isoflavones and contain the highest dietary-relevant amounts of these compounds among legumes (Franke et al., 1998). For example, each gram of soy protein in soybeans contains approximately 3.5 mg of isoflavones; while no significant amounts are present in lentils (Murphy et al., 1999). For this reason, the majority of published data regarding their activities in breast cancer involve isoflavones found in soy.

4.1 Structural and bioavailability

The isoflavones are a subclass of flavonoids and belong to the group of naturally occurring heterocyclic phenols. Their basic structure is composed of 2 benzene rings linked through a heterocyclic pyrane ring. Isoflavones are named glycoside (inactive form) when conjugated to glucose or carbohydrate moieties and glycone (active form) when unconjugated (Franke et al., 1998) (Fig.1).

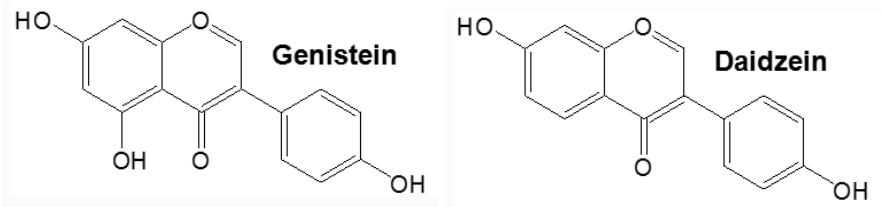


Fig. 1. Chemical structures of the genistein (4', 5, 7-trihydroxyisoflavone) and daidzein (4', 7-dihydroxyisoflavone), the most abundant isoflavones found in soy. The figure was drawn by ChemDraw (Cambridge Soft, version 9.0).

The primary isoflavones found in soybeans are the glycones genistein (4',5,7-trihydroxyisoflavone), daidzein (4',7-dihydroxyisoflavone) and glycitein (7,49- dihydroxy-6-methoxyisoflavone), and their respective glycosides genistin, daidzin and glycitin (Messina et al., 2001). Genistein and daidzein are the most abundant isoflavones in soybeans representing 50% and 40% of the total isoflavone content respectively (Rochfort et al., 2007).
The majority of isoflavones found in raw soybeans are almost entirely as glycosides (genistin, daidzin, and glycitin) while only 1 – 3% account for their active form glycone (Murphy et al., 1999). After ingestion, they are rapidly absorbed entering systemic circulation predominantly as conjugated forms (95%) with limited bioavailability (Messina

et al., 2006). Isoflavones then are further deconjugated by the action of glucosidases produced in intestinal bacteria. Interestingly, there is considerable inter-individual variation in intestinal bacteria metabolism of genistein and daidzein. The bioconvertion of daidzein to one of its metabolites (equol) is performed by a very specific type of intestinal bacteria which have been found only in 30–50% of individuals (Patisaul et al., 2010).
The resulting isoflavone metabolites are widely biodistributed and their serum levels can reach the low micromolar range according to the amount ingested (Messina et al., 2001). Soy isoflavones have half-lives of approximately 8 hours and are nearly all excreted within 24 hours after consumption (Messina et al., 2006).

4.2 Exploring isoflavones biological effects on breast cancer

4.2.1 Estrogen-receptor dependent properties

Breast cancer development is significantly influenced by the exposure to estrogens. These hormones have been described to induce proliferation of malignant breast cells contributing to breast cancer promotion and progression. Therefore, the control of estrogen exposure is a key factor in breast cancer chemoprevention (Bouker et al., 2000).
The structures of soy isoflavones are similar to mammalian estrogens (Messina et al., 2001). By the early 1960s, isoflavones were characterized as ligands of estrogen receptors and thus labelled as phytoestrogens (Cheng et al., 1953). These findings led to an initial enthusiasm mainly based on the possibility that isoflavones might exert antiestrogenic effects on breast tissue as other known estrogen antagonists such as tamoxifen (Messina et al., 2001).
Folman & Pope were the first to conduct assays establishing the relative binding affinity isoflavones for the estrogen receptor (ER) (Folman et al., 1966). There are two major ER subtypes in mammals, ER-α and ER-β presenting different tissue distributions. Soy isoflavones have singular affinities for each ER and for this reason are classified as selective estrogen receptor modulators (SERMs) (Messina et al., 2006). Genistein is 7- to 48-fold more selective for ER-β than Er-α and is 1,000-fold more potent at triggering transcriptional activity with ER-β than Er-α (Kuiper et al., 1998). However, isoflavones are considered to be weak estrogens, showing binding affinity to ER-α and ER-β of nearly 20- and 5-fold less than estradiol, respectively (Kuiper et al., 1998).
There are some evidences suggesting that activation of ER-β inhibits proliferation in breast cells (Patisaul et al., 2010). Because genistein preferentially binds to ER-β, it may induce antiestrogenic effects through this receptor (Bouker et al., 2000). Moreover, soy isoflavones can act as an antiestrogen through other mechanisms. Genistein has the ability to inhibit the enzyme 17β-hydroxysteroid oxidoreductase type 1 (HSOR-1), which is necessary for estradiol secretion from the ovaries in premenopausal women and is essential for the reduction of estrone to estradiol in the adipose tissues. Isoflavones are also involved in the inhibition of the aromatase enzyme, which is responsible for the conversion of androgens to estrone in peripheral (adipose) tissues (Bouker et al., 2000). Thus, the inhibition of estrogen–metabolizing enzymes can lead to a decreased total estradiol level and intensify isoflavones antiestrogen effects. It is important to highlight that complex feedback mechanisms associated with the hypothalamic/ pituitary/gonadal axis are involved in controlling the levels of estrogen and that the effects of isoflavones in this network are unclear and demand further studies (Bouker et al., 2000).
The main biological effects of soy isoflavones in breast cancer cells involve cell growth arrest and induction of apoptosis (Lamartiniere et al., 1998). Genistein have been shown to inhibit

growth factor- and cytokine -stimulated growth of breast cancer cells (Peterson et al., 1996). Indeed, at the molecular level, this isoflavone can influence the regulation of cell cycle molecules by inducing significant up-regulation of p21/WAF1 expression (cell cycle inhibitor) in the treatment of breast cancer cells (Banerjee et al., 2008). In addition, the treatment of breast cancer cells with genistein influences the regulation of apoptotic molecules such as down-regulating anti-apoptotic molecules (Bcl-2, Bcl-xL, and HER-2/neu) and up-regulating pro-apoptotic ones (Bax and caspases). It has been suggested that genistein could also induce the regulation of those genes through the inhibition of proteasome. Overall, these findings suggest that ER stress, cell cycle arrest and apoptosis induction may represent part of the molecular mechanism by which isoflavones exert their anticarcinogenic effects (Banerjee et al., 2008).

4.2.2 Estrogen-receptor independent properties

Interestingly, the effects of soy isoflavones on cell cycle arrest and apoptosis has been detected not only in ER-positive but also in ER-negative breast cancer cells (Theil et al., 2010) suggesting that isoflavones anticancer activity might also occur independently of ER modulation. Indeed, several non-estrogenic targets for isoflavones have been described.

Isoflavones have been described as specific inhibitors of protein-tyrosine kinase (PTK), which is an enzyme frequently overexpressed in cancer cells. PTKs are crucial molecules for tumor development and thus soy isoflavones can potentially slow tumorigenesis by inhibiting their mediated signalling mechanisms (Banerjee et al., 2008; Patisaul et al., 2010).

Isoflavones can modulate and block the activity of several molecules involved in breast cancer cell growth and survival pathways such as topoisomerase I and II, mitogen activated protein kinases (MAPK), urokinase- type plasminogen activator (uPA), and nuclear factor-κB (NF-κB). Isoflavones are also implicated in the growth inhibition of various cancer cells through the regulation of gene activity by modulating epigenetic events that are intimately related to the regulation of cell cycle and apoptosis such as DNA methylation and/or histone acetylation (Messina et al., 2001; Banerjee et al., 2008).

Furthermore, soy isoflavones are able to inhibit invasion, metastasis, and angiogenesis *in vitro* and *in vivo* in a number of cancers including breast cancer. Genistein was described to inhibit the secretion of matrix metalloproteinases (crucial enzymes for invasion and metastasis) in MDA-MB-435 breast cancer cells and blocked invasion of a highly meta-static subline of BALB/c mammary carcinoma cells (Bouker et al., 2000; Messina et al., 2001).

Antioxidant activity is also included among the described effects of soy isoflavones. They are able to protect cells against reactive oxygen species by scavenging free radicals and inhibiting the expression of stress–response related genes which is an interesting approach for cancer prevention (Ruiz-Larrea et al., 1997).

Overall, it is clear that when evaluating biological effects of isoflavones it is necessary to look beyond the estrogen receptor and consider their non-hormone-related activities (Messina et al., 2001).

4.2.3 Dose-dependent effects

Soy isoflavones can induce different effects on breast cancer cells according to the dose used. For example, the effects of soy isoflavones in MCF-7 and BT20 breast cancer cells were only observed in the highest dose tested (50 μg/mL) (Theil et al., 2010). Similar effects were reported showing that isoflavone doses higher than 10 μM could inhibit the growth of breast

cancer cells (Wang et al., 1996). In particular, the IC50 values of genistein able to induce growth arrest in both hormone-dependent and hormone-independent breast cancer cells have been described to range from 10 to 50 μM (Messina et al., 2001).

Some researchers question the relevance of these results by claiming that the high isoflavone concentrations used *in vitro* would not be achieved after ingestion in an *in vivo* system. Most Asians or Caucasians that consume a diet rich in soy have serum genistein levels smaller than 1 μM (Bouker et al., 2000). In order to address these claims, several assays evaluated the effects of isoflavones in low concentrations and, surprisingly, showed a different outcome. Low doses (0.01–1 μM) of genistein were shown to stimulate proliferation in human breast cancer cell lines (Bouker et al., 2000; Messina et al., 2001).

In animal studies, soy isoflavones also induce different activities according not only to the dose but to the animal model and the route of administration used (Barnes et al., 1997). Injections of 0.8 mg genistein in rats significantly reduced MNU-induced tumor multiplicity and marginally reduced tumor incidence. Similarly, a high dose of daidzein (0.8 mg) decreased tumor multiplicity without affecting incidence, whereas a low dose (0.4 mg) was ineffective. Other study showed that rats fed with a low dose of biochanin A (10 mg/kg), which is an isoflavone that is converted to genistein *in vivo*, significantly reduced tumor multiplicity and that a higher dose (50 mg/kg) also reduced tumor incidence (Barnes et al., 1997).

Conversely, instead of decreasing tumor growth as previously commented, soy isoflavones have also been described to stimulate breast tumor growth *in vivo* (Helferich et al., 2008; Bouker et al., 2000; Allred et al., 2004). The effects of dietary level of genistein were studied in an athymic BALB/c ovariectomized mice model subcutaneously injected with human estrogen-dependent cells (MCF-7). They found that in mice fed with a standard (control) AIN-93G diet, tumors reduced completely; however, mice fed with diets containing either isoflavone-rich isolated soy protein or isoflavone extracts had tumor growth stimulated (Hsieh et al., 1998). The use of a non-ovariectomized athymic mice model injected with ER-negative breast cancer cells to evaluate the effects of high dietary levels of soy isoflavones intake showed that daidzein increased while genistein decreased mammary tumor growth by 38 and 33% respectively. In addition, daidzein increased lung and heart metastases while genistein decreased bone and liver metastases. Combined soy isoflavones did not affect primary tumor growth but increased metastasis to all organs tested, which include lung, liver, heart, kidney, and bones (Martinez-Montemayor et al., 2010).

In general, the evidences indicate that at lower concentrations isoflavones exert estrogen-like effects while at higher concentrations other non-estrogen receptor–mediated effects are induced (Messina et al., 2001). Data from *in vivo* studies suggest that in a low-estrogen environment (as exists in postmenopausal women), genistein is estrogenic and has a proliferative effect on breast tissue. However, in a high estrogen environment (similar to that in premenopausal women), it has an antiproliferative and possibly antiestrogenic effect (Hsieh et al., 1998; Allred et al., 2004). Therefore, isoflavones can both inhibit or stimulate proliferation of breast cancer cells showing a biphasic effect according to the dose (Bouker et al., 2000).

Several hypothesis and discussion about experimental models limitations/weaknesses have been elaborated to address these conflicting results (Messina et al., 2008). First, considering that both estrogen and soy isoflavones bind to ER, differences in endogenous estrogen levels may interfere in the results. Premenopausal women have high levels of estrogen while basal levels of estrogen are found in postmenopausal women. Thus, *in vitro* estrogen-depleted

conditions and ovariectomized animals (with no basal estrogen levels) would not be suitable models because there are no sufficient estrogen levels to promote or even to maintain estrogen-dependent tumors. Even weak estrogenic compounds, such as isoflavones, could stimulate the growth of estrogen-sensitive mammary tumors in such environment (Messina et al., 2006). Thus, these models would not accurately reflect conditions in either premenopausal or postmenopausal women (Messina et al., 2008). Researchers have supported this hypothesis showing that although genistein stimulated proliferation of MCF-7 cells and enhanced expression of the estrogen-responsive pS2 gene in an estrogen-depleted *in vitro* environment, it inhibited estrogen-induced proliferation and reduced pS2 expression of MCF-7 breast cancer cells when in the presence of a maintained level of estrogen (Wang et al., 1996; So et al., 1997). Nevertheless, breast tumor growth stimulation by both dietary and subcutaneously injected genistein has still been noted in animal models in which estrogen levels were more similar to the amounts of pre and postmenopausal women (Messina et al., 2006).

The second critique addresses the use of mice lacking the immune properties (athymic or nude). This animal feature is a necessary element of these models in order to allow the growth of human tumor cells in a murine environment. However, the lack of immune function may eliminate a potential mechanism by which soy isoflavones reduce tumor development (Messina et al., 2008). A recent research in B6C3F1 mice showed that enhanced immune function resulting from pre-treatment with genistein (20 ppm) is correlated with protection against chemically-induced mammary tumors (Guo et al., 2007).

The third critique relates to isoflavone dose. In many studies, breast cancer cells and animals are exposed to high amounts of genistein (750 ppm) which exceeds typical dietary intake. In Japan, adults consume about 15–20 mg genistein daily (total average isoflavone intake is approximately 40 mg), which equates to a dietary concentration of about 30–40 ppm (Messina et al., 2008). However, it is important to highlight that isoflavone biodistribution is not homogeneous. Isoflavone concentrations in breast tissue are two- to threefold higher than paired serum concentrations. It suggests that breast tissue may be exposed to higher levels of biologically active isoflavones than was previously thought (Pasqualini et al., 2005) and supports investigations of high concentrations of isoflavones.

Another aspect of oral doses of isoflavones relies in the amount of free (unconjugated) isoflavones processed by intestinal bacteria. The rodent gut bacteria are able to convert daidzein to the metabolite equol more effectively than humans. Furthermore, even in humans who are classified as equol producers, genistein is the predominant serum isoflavone in response to the ingestion of soy or mixed isoflavones, whereas equol predominates in most other species, including both rodents and monkeys (Gu et al., 2006).

The fourth consideration is based on the fact that it is not clear to what extent the existing MCF-7 xenoplants in nude mice resemble tumors in human breast cancer. These tumors are fully transformed and composed of cells that are extremely sensitive to the growth-stimulating effects of estrogen (Messina et al., 2008). Thus, a better comprehension of the current existing animal models and the development of new ones would contribute to the interpretation and translation of isoflavone effects in humans.

Given the conflicting data and limited *in vitro* and *in vivo* models, the controversy about the effects of isoflavones either from soy foods or supplements would be unlikely solved by additional animal research (Messina et al., 2009). Then, epidemiologic data should be another alternative to study and conclude about isoflavone intake and breast cancer. Current data discussing this topic is provided in the next section.

4.2.4 Aspects of isoflavone intake in humans

Women with high risk of breast cancer, breast cancer patients and survivors are among the group of consumers who have embraced soy products, isoflavone supplements and isoflavone-enriched foods, seeking for their health-promoting properties. Nevertheless, the estrogenic/antiestrogenic effects of these molecules and the disparities of *in vitro* and *in vivo* data have led to considerable controversy and misinterpretation among health professionals and consumers over the use of soy by this group of women. Due to the phytoestrogenic nature of isoflavone, several oncologists often discourage and even prohibit its intake by breast cancer patients, particularly those with ER-positive tumors (Messina et al., 2001).

As previously discussed, early epidemiologic studies have reported that high isoflavone intake was related to low cancer rates regardless of intrinsic genetic and biological differences among populations worldwide (Wu et al., 1996). Since then, more researchers have attempted to refine the knowledge of this matter and further investigate the correlations of isoflavone intake among breast cancer biomarkers, time of exposition, and age.

Soy intake was found to be significantly associated with a decreased risk of death from breast cancer and/or recurrence when evaluated in 5,042 Chinese women aged from 25 to 75 followed for 5 years (Shu et al., 2009). These benefits remained significant even after adjusting the results for 17 factors including tumor, node, metastasis stage, ER, progesterone receptor status and the type of treatment received. It was also observed that women who had the highest level of soyfood intake and did not take tamoxifen had a lower risk of mortality and a lower recurrence rate than women who had the lowest level of soyfood intake and used tamoxifen (Shu et al., 2009; Messina et al., 2010). The association of decreased breast cancer risk and soy isoflavone intake has been observed even in Asian-American women, both pre- and postmenopausal, living in the West (Wu et al., 1996). Overall, evidences show that for Asian women the risk of developing breast cancer reduces as soy intake rises. Even a soy intake of as little as 10 mg per day was sufficient to decrease breast cancer risk by 12% (Patisaul et al., 2010).

Effects of isoflavone intake have also been investigated in non-Asian populations. In a US study, it was observed that breast cancer survivors (n= 1,954; followed for 6.3 years) had reduced risk of cancer recurrence with increasing amounts of isoflavone among postmenopausal women and tamoxifen users. Interestingly, more pronounced effects were observed in women with ER-positive breast cancer (Guha et al., 2009). Beneficial effects of isoflavone intake were also observed in a Dutch study, which compared serum isoflavone concentrations in women with and without breast cancer. It was observed that high plasma concentrations of genistein were associated with a 32% reduction in breast cancer risk (Verheus et al., 2007). Furthermore, it was reported that isoflavone intake was associated with a reduced risk of all-cause mortality during the 5-y follow-up period among postmenopausal U.S. breast cancer patients (Fink et al., 2007).

Conversely, other investigations have failed in detecting benefits in soy isoflavone intake. One of them did not find significant differences in soy daidzein or genistein intake between breast cancer cases and their controls in Shanghai (Zheng et al., 1999). Other investigation showed that soy food intake was unrelated to survival of Chinese breast cancer patients during the 5.2-y follow-up period (Boyapati et al., 2005). Several other researchers have also suggested that soy consumption is not associated with a reduced risk of breast cancer; however, no harmful effects were found in these studies (Bouker et al., 2000).

Breast tissue density has been used as a non-invasive breast cancer biomarker to evaluate isoflavone intake. It was observed that both intervention and epidemiologic studies have not

shown evidence of neither harm or benefit of isoflavone on breast cancer density (Messina et al., 2006). Analysis of breast cell proliferation has also been used as a biomarker of potential tumor promotion. Comparison of biopsies taken before and after exposure to soy products did not show increased cell proliferation in any of the four different trials involving breast cancer patients, healthy subjects, and women undergoing breast biopsy or definitive surgery for breast cancer. Daily isoflavone intake in these trials ranged from 36 to more than 100 mg, with study periods ranging from 2 weeks to one year (Messina et al., 2009). Another study examining more than one breast cancer biomarker found no statistically significant differences in cell proliferation (Ki67 index), histology (hyperplasia with or without atypia), or ER expression in 6 and 12 months of soy intake (Messina et al., 2006). Conversely, a study evaluating the effects of soy consumption (38 g soy protein isolate, 80 mg isoflavones) over 5 months showed an association of isoflavone intake and a two- to sixfold increase in breast nipple aspirate fluid (NAF) secretion of premenopausal but not postmenopausal women (Petrakis et al., 1996).

The effects of isoflavone intake in serum estrogen and androgen levels have been widely investigated. The consumption of textured vegetable (i.e., soy) protein for 2 weeks elicited an ER-mediated response detected by increased pS2 levels (protein expressed in response to estrogen) in breast biopsies taken from premenopausal women (Hargreaves et al., 1999). However, high levels of soy products have been described to induce no changes or even decrease plasma estradiol concentrations in premenopausal women. None of these effects were observed in postmenopausal women (Bouker et al., 2000). These contrasting findings could be partially explained by the observation that, despite binding to ERs, isoflavones are also able to inhibit enzymes related to estrogen synthesis and metabolism. Therefore, it has been hypothesized that the presence of other additional simultaneous stimuli may result in either reduced or increased circulating estradiol concentrations (Bouker et al., 2000).

Isoflavone intake has been implicated to interfere in serum levels of other menstrual cycle hormones, such as progesterone. Studies have reported that isoflavone intake was associated with a significant reduction in serum progesterone levels and in luteal phase lengths (Lu et al., 2000). These findings suggest that isoflavones may reduce the probability of neoplastic transformation and breast cancer development since breast cells are more proliferative during the luteal phase of the menstrual cycle, when progesterone concentrations are the highest (Lu et al., 2000).

Overall, inconsistent results about isoflavone intake and breast cancer are also present in epidemiological studies. Indeed, researchers have highlighted the difficulties of comparing clinical studies since several variables are not properly considered. In a meta-analysis by Trock et al., 18 studies (12 case-control and 6 cohort) published between 1978 and 2004 were evaluated. After making several assumptions, the authors showed that there is a small inverse correlation between soy intake and breast cancer for both pre- and postmenopausal women; however data limitations cannot exclude the possibility that this result could be an artifact of the analysis (Trock et al., 2006).

An important variable to be considered when evaluating risks and benefits of soy isoflavone intake comprises the extent and the period of life in which women are exposed to soy food. Studies attempting to address this topic are discussed in the next section.

4.2.5 Lifetime exposition of isoflavones

Throughout the life span, estrogens can induce mammary cell proliferation or cell differentiation depending on the overall hormonal environment. Since isoflavones present

estrogenic or antiestrogenic activities, they may have a different impact on the breast if the exposure occurs in utero; during childhood, puberty, or pregnancy; premenopausally; or during postmenopause (Bouker et al., 2000). Studies have been carried out to determine if the putative preventive effects of soy isoflavones are related to the lifetime period of exposition.

Different outcomes have been shown from perinatal/neonatal exposure of isoflavones in animals. The offspring of pregnant rats receiving subcutaneously administration of high doses of genistein exhibited abnormal mammary gland development and higher susceptibility to develop 7,12-dimethylbenz[a]anthracene (DMBA)- induced mammary tumorigenesis (Padilla-Banks et al., 2006; Patisaul et al., 2010). Conversely, it was reported that rat pups born to mothers consuming high levels of genistein during gestation and lactation developed fewer breast tumors (Fritz et al., 1998). Protective effects of isoflavones were also reported when soy exposure occurred perinatally. Rats receiving genistein through diet or subcutaneously during the first days postpartum showed lower tumor incidence after DMBA mammary tumor induction (Lamartiniere, 2000).

The period between puberty and a first full-term pregnancy is when the breast is particularly vulnerable to the effects of carcinogens. During this time, there are a high percentage of indiferentiated breast cells, named terminal end buds (TEBs), actively proliferating. Several investigations in animal models have shown protective effects of prepubertal isoflavone exposure on mammary tumorigenesis induced by DMBA (Murrill et al., 1996; Lamartiniere et al., 1998).

Epidemiological studies support *in vivo* findings showing that isoflavone intake during adolescence and adulthood is correlated with low risk of breast cancer. Shu et al. reported that women consuming tofu (11 g soy protein/day) during their teenage years (13–15 years) were less likely to develop premenopausal and postmenopausal breast cancer as adults (Shu et al., 2001). Other epidemiologic studies have supported these results when protective effects with reductions in risk of breast cancer ranging from 28 to 60% were observed (Messina, 2010).

Investigations on mammary gland morphology and cell differentiation were carried out in animal models to understand how isoflavone exerts the described protective effects. The results indicated that isoflavones might have been exerting its chemoprotective effect by stimulating early cell differentiation leading to a reduction in the number of least differentiated structures in the breast tissue (TEBs) which are susceptible to chemical carcinogens (Bouker et al., 2000; Lamartiniere, 2000).

Therefore, animal and epidemiological studies are consistent with the hypothesis that childhood and/or adolescence is the critical period for isoflavone of exposure (Messina et al., 2006) and also corroborates with speculations that Asian low breast cancer rates is derived from early exposure to soy products, including during pregnancy (Lamartiniere, 2000).

4.2.6 Variables influencing inconsistent outcomes

To better interpret and understand data about isoflavone intake and breast cancer it is important to consider the strengths and weaknesses of a wide variety of experimental models and designs (Messina et al., 2006). As commented previously, the major sources of limitation in those studies are the inappropriate experimental designs and incomplete or unclear information provided about food isoflavone content, patient description, serum

isoflavone levels, time of exposition, and isoflavone metabolism. There are studies in which some variables are not even considered. Differences in these factors can considerably difficult comparisons among studies generating misinterpretations of data and are probably related to the majority of inconsistent results in the literature.

Epidemiologic studies should take into consideration that the amounts of isoflavones are not equal in all soy foods. Indeed, there are notably differences in soy food preparations and isoflavone content. Raw soybeans contain nearly 1.0 mg/g (range of 0.4 –2.4 mg/g of total isoflavones while traditional soy foods (i.e., tofu, miso, natto) typically contain 0.2– 0.4 mg/g (Messina et al., 2001). In addition, the content and structure of isoflavones are altered when soy food undergo processing, which was shown to potentially affect the effects on breast cancer (Murphy et al., 1999; Allred et al., 2004).

Moreover, soy isoflavone content can vary according to local, weather, seed maturation, and breeding conditions. It was observed that during the process of seed maturation the contents of isoflavones decrease, whereas sprouting led to a continuing increase of isoflavone content. Interestingly, the protein extracts from the developing seeds showed clearly opposite effects on cell viability and inhibition of foci formation compared with those from sprouting seeds (Park et al., 2005).

Individual differences in the absorption and metabolism of ingested isoflavones are another variable not usually addressed on breast cancer studies. To evaluate the potential risks and benefits of phytochemicals to human health, it is important to know the physiological behavior of these compounds after ingestion (Hsieh et al., 2010). As previously commented, isoflavones are metabolized by intestinal microorganisms, which may be heterogeneous among individuals. This variability may have large contributing effects on the serum levels of free isoflavones and correspondent metabolites and thereby on the resulting physiologic effects. Furthermore, differences in isoflavone metabolism and bioavaiability should also be considered when analyzing data from rodent animal models since a higher percentage of both genistein and daidzein appear in the free or glycone form in rats (Gu et al., 2006).

Individual variabilities should also be considered when analyzing the effects of isoflavone intake in estrogen and progesterone serum levels. Menstrual cycle length varies significantly among women and analysis of reproductive hormone levels in single periods may not provide accurate data on isoflavone effects. In this case, it would be more appropriate to measure the hormone levels during the whole menstrual cycle (Lu et al., 2000). Different times of isoflavone exposition are yet another type of limitation that influences data interpretation. For example, short periods of exposition (such as 2 weeks) may provide data regarding only the acute effects of soy isoflavone intake on breast cancer, thus, limiting comparisons with long-term studies.

Other potential source of variability in clinical studies includes incomplete patient description. Different and more specific correlations would be obtained whether patients were also addressed in subgroups by ER status, serum estrogen levels, and type of treatment being received (e.g. tamoxifen) for example. Those detailed patient information should also be used as valuable adjustment parameters for raw data in order to improve interpretation accurateness (Shu et al., 2009; Messina et al., 2010).

Clearly, there is a need to encourage further detailed studies to reduce the heterogeneity of soy exposure data (Rochfort et al., 2007). Several recommendations have been made to improve study conditions and data interpretation such as: provide clear information about the isoflavone content (including glycone amount) on test products; include detailed description of products, concentrations, and amounts used; relate study conditions to usual

soy and isoflavone intakes and/or tissue levels of isoflavones; consider the risks and benefits of research findings for human health; and outline the benefits and limitations of the model system used when conducting cell culture or animal studies (Erdman et al., 2004).

5. Protease Inhibitors

5.1 Structure and bioavalability

Protease inhibitors (PIs) have been isolated from black-eyed peas (*Vigna unguiculata*), soy (*Glycine max*), brazilian pink bean (*Phaseolus vulgaris*), pea (*Pisum sativum*) and lentil (*Lens culinaris*) (Losso, 2008). In seeds, these molecules are involved in the regulation of endogenous proteases and in defense-related strategies against seed-eating insects and microorganisms (Ryan, 1990). The concentration of PIs is affected by the stage of seed development and sprouting. For example, soy-derived BBI content increases during the process of seed maturation while it decreases with soaking time during sprouting (Park et al., 2005).

PIs are classified in more than 20 families according to their inhibitory activity and structural features (Laskowski et al., 2000; Joanitti et al., 2006). The primary families found in legumes are the Kunitz and the Bowman-Birk which are involved in the inhibition of serine proteases (Joanitti et al., 2006; Losso, 2008). Kunitz PIs consist of proteins with molecular mass ranging from 6-20 kDa. These inhibitors are cross-linked by 2-3 disulfide bonds and have one reactive site that generally binds to trypsin. Bowman-Birk PIs are small proteins (6-15 kDa) presenting 5-7 disulfide bonds and 2 different and independent reactive sites located at opposite regions of the molecule (Fig. 2). Due to this double-headed configuration, these inhibitors can interact with 2 enzymes simultaneously (e.g. trypsin and chymotrypsin or trypsin and trypsin (Freitas et al., 1999; Ventura et al., 1966)). The disulfide bonds content of Kunitz and Bowman-Birk PIs are responsible for their remarkable structural stability (Joanitti et al., 2006). It has been reported that the inhibitory activity of these molecules is preserved after being exposed to a wide range of temperatures (up to 100°C) and pHs (2-13) (Silva et al., 2001; Ho et al., 2008; Ye et al., 2009).

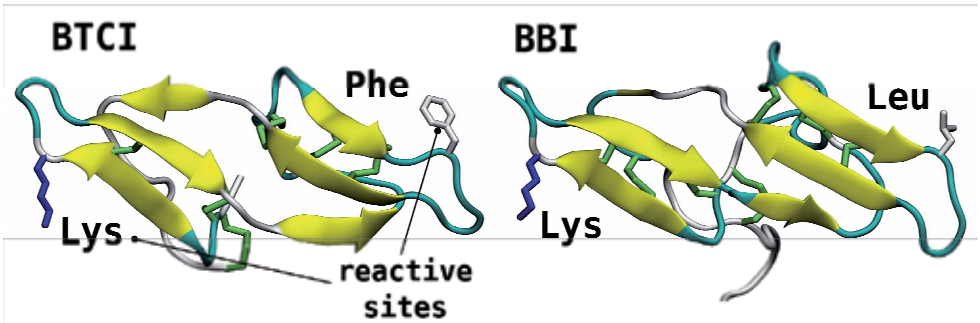


Fig. 2. Tridimensional structure of the Black-eyed pea trypsin and chymotrypsin inhibitor (BTCI) (Barbosa et al., 2007); PDB access number 2G81) and Bowman-Birk inhibitor (BBI) (PDB access number 1BBI) in ribbon representation. The disulfide bonds (in green) and reactive sites for trypsin (Lys) and chymotrypsin (Phe or Leu) are indicated. The image was made with VMD (Theoretical and Computational Biophysics Group, NIH Resource for Macromolecular Modeling and Bioinformatics, Beckman Institute, University of Illinois, Urbana-Champaign).

Because Kunitz and Bowman-Birk PIs are involved in the inhibition of serine proteases, these molecules have been considered as antinutritional factors able to impair digestion. Proper thermal seed processing have been described to eliminate the majority of legume antinutritional factors (Lajolo et al., 2002); nevertheless, some Kunitz and Bowman-Birk PIs are able to resist both acidic conditions and the action of proteolytic enzymes and pass through the stomach and small intestine without major degradation (Clemente et al., 2010). The metabolism and absorption of a soy Bowman-Birk PI (termed BBI) have been well characterized following oral administrations. After ingestion, BBI are rapidly metabolized and absorbed. BBI can resist stomach and small intestine conditions permitting the reach of significant amounts to the large intestine in the active form to exert their reported anticancer and anti-inflammatory properties (Kennedy, 1998). This PI is widely distributed throughout the body including breast tissue (Kennedy, 1998). In mice, BBI metabolites can be found in the liver, serum, and kidneys of mice 1.5 hours after ingestion (Wang et al., 2000). In humans, BBI excretion rates reach the peak within 6 hours and decrease to baseline levels within 12-24 hours (Wan et al., 2000).

PIs have been viewed as toxic agents inhibiting the growth of young animals and, perhaps, contributing to the development of pancreatic cancer. However, the effect on the promotion of atypical growth in rat pancreata, is not expected to occur in humans (Kennedy, 1998). Despite preserving their biological activity after passing through the gastrointestinal tract, it seems that these inhibitors do not act as antinutritional factors since they are not implicated in significant side effects even when ingested in concentrations far higher than the therapeutic dose (Kennedy, 1998).

5.2 Effects on breast cancer

PIs have been considered promising compounds in several economic and clinical areas. These inhibitors are multiple functional molecules with properties varying from insecticide to therapeutical activity in fields as immune systems, microorganism and viral infections, hemostasis, and cancer (Joanitti et al., 2006). PIs have shown promising anticancer effects on the prevention and suppression of cancer in several organ systems and tissue types (e.g. colon, liver, lung, esophagus, oral epithelium, ovarian, prostate, hematopoietic cells, and connective tissue), *in vitro* and *in vivo* (Kennedy, 1998). Particularly, BBI (Bowman-Birk Inhibitor from soy) was recognized as an investigational new drug by the Food and Drug Administration (FDA) and is currently being evaluated in clinical trials against pre-malignant oral cancer lesions, showing successful results on the reduction of cancer lesions with low or no side effects (Armstrong et al., 2000). Despite having their anticancer activity being widely investigated on different tumor types, few studies have addressed the effects of Kunitz and Bowman-Birk PIs specifically on breast cancer.

The effects of soy BBI on DMBA-induced transformation were investigated using an *in vitro* whole organ culture system of mouse mammary glands. It was observed that soy BBI and its palmitic acid conjugate (Pal-BBI) were effective in preventing DMBA-induced transformation, especially when added to the medium during the developing period after the exposure of mammary glands to DMBA, showing 35.9 and 53.4% prevention, respectively. Pal-BBI was also effective in decreasing the transformation incidence (32.2%) while BBI was not (10.3%) when only present in the medium before the promotion period. Possibly, the high lipophilicity of Pal-BBI increased its tissue retention and resulted in higher chemopreventive effects (Du et al., 2001).

An interesting *in vivo* study reported the effects of one year-long feeding of field bean meal (a rich source of PIs with 24% protein content), on mouse mammary tumor virus (MMTV)-induced mammary tumorigenesis in mice. The animals were fed chow with 2%, 4%, and 8% field bean protein (FBP) for 49 weeks and the incidence of mammary tumors was recorded in 58 weeks. A suppressive effect on mammary tumorigenesis was observed with increased FBP intake as mice fed with 2%, 4%, and 8% FBP showed significant tumor incidence reduction of 68%, 75% and 81% respectively. Adverse growth effects were observed only in mice receiving the 8% FBP-fed group (Fernandes et al., 1997).

In addition to chemopreventive properties, PI effects on the viability of breast cancer cells have also been reported. A Bowman-Birk PI isolated from Hokkaido large black soybeans seeds and a Kunitz PI isolated from Chinese black soybean seeds have been described to suppress the proliferation of breast cancer cells (MCF-7) *in vitro* (Ho et al., 2008; Ye et al., 2009). Similar effects were observed in MCF-7 cells treated with Bowman-Birk PIs derived from soy or black-eyed peas (Zhang et al., 1999; Joanitti et al., 2010). Conversely, it was described that soy BBI was not able to induce anticancer effects on ER-negative breast cancer cell MDA-MB 231 (Hsieh et al., 2010). It has been suggested that additional investigations should be made to determine whether tumor cell types and their specific carcinogenesis pathways may be determinants of the cancer chemopreventive properties of BBI (Hsieh et al., 2010).

PIs have been described to act on different cancer stages and activities impairing malignant cell transformation, altered gene expression and proteolytic activity, tumor growth, angiogenesis, and metastasis. These effects have been suggested to be linked to their protease inhibition activity (Kennedy, 1998; Joanitti et al., 2006). Nevertheless, the precise mechanisms by which PIs exerts their preventive and suppressive anticancer effects are not completely elucidated on breast cancer.

One of the main effects observed in tumor cells treated with PIs is a reduction on cell proliferation rates (Wan et al., 1998; Zhang et al., 1999; Chen et al., 2005). In 2005, Chen et al. reported a landmark research study revealing some clues about the mechanisms involved in this growth inhibition. They observed that the soy-derived BBI was able to inhibit proteasome activity, specifically the chymotrypsin-like domain, *in vitro* and *in vivo* in MCF-7 breast cancer cells. Proteasomes are large protein complexes acting in the degradation of misfolded proteins and regulation of particular proteins levels involved in intracellular pathways. Proteasome inhibition by BBI resulted in an accumulation of ubiquitinated proteins and proteasome substrates, such as cell cycle inhibitors p21Cip1/WAF1 and p27Kip1, inducing cell cycle arrest at G1/S phase. Furthermore, an up-regulation of MAP kinase phosphatase-1 (MKP-1) accompanied by a decrease of phosphorylated extracellular signal-related kinases (ERK1/2), which is a pathway involved in cell division, was also observed (Chen et al., 2005).

In addition to tumor growth inhibition, the BBI found in black-eyed peas (termed BTCI) was also shown to induce death on MCF-7 cells (Joanitti et al., 2010). The treatment of cells with BTCI induced significant reduction of the cell proliferation (arrest in S and G2/M phase) accompanied by significative DNA fragmentation, mitochondrial swelling, morphologic alterations, annexin-V+ cell number increase, and mitochondrial membrane potential reduction. These cytotoxic effects at first suggested that BTCI induced apoptosis cell death on MCF-7 cell. However, other features observed such as large lysosomes presenting weak acidification pattern followed by an increase in cytoplasmic acidification indicated another cell death pathway related to lysosomes: the lysosomal membrane permeabilization (LMP).

LMP is characterized by a perturbation of lysosomal membrane function leading to the translocation of lysosomal hydrolases from the lysosomal lumen to the cell cytoplasm. Therefore, the authors suggested that BTCI was able to induce both LMP and apoptosis processes on breast cancer cells (Joanitti et al., 2010).
The ability to induce tumor growth inhibiton and or cell death might be determined by PIs structural features, especially on the reactive sites, leading to different affinities to the targets. Moreover, variations in the dose and time of exposure are also important factors to be considered. For example, low Bowman–Birk PIs concentrations (10–40 μM) and long incubation periods (6–14 days) are frequently associated with proliferation inhibition (Wan et al., 1998; Zhang et al., 1999); while high concentration (200 μM) and short incubation periods (3 days) has been described to induce both tumor growth inhibition and cell death (Joanitti et al., 2010). Overall, these findings indicate that PIs are promising anticancer molecules and encourage more studies of these compounds on breast cancer.

6. Peptides

6.1 Structure and bioavailability

Among bioactive peptides found in legumes, a peptide isolated from soybean cotyledon has stand out as a potential anticancer agent. Lunasin is a 43-amino acid peptide with structurally conserved helix region containing Arg- Gly-Asp (RGD) cell adhesion motif followed by 9 aspartic acid residues at the carboxyl end. This peptide exhibits the primary sequence, SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD (Hernandez-Ledesma et al., 2009).
Lunasin has been identified in several soybean varieties with concentrations ranging from 4.4 to 70.5 mg lunasin/g protein (Gonzalez de Mejia et al., 2004; Hernandez-Ledesma et al., 2008). These concentrations are affected by the stage of seed development and sprouting. A notable increase of lunasin content is observed during seed maturation while the opposite occurs during sprouting. Breeding conditions (light and dark cycles) do not seem to affect the content of this peptide (Park et al., 2005). Large-scale processing of soy also influences lunasin concentration which was observed to vary from 12 to 44 mg lunasin/g of flour among different U.S. commercially available soy proteins (Gonzalez de Mejia et al., 2004).
Besides presenting heat stability, *in vitro* digestibility studies have shown that lunasin is digested by pancreatin (Galvez et al. 2001). However, animal studies indicate that when lunasin is ingested in combination with soy protein extract, it survives digestion and about 35% of the total oral dose is absorbed within 3 hours (de Lumen, 2005). These findings suggest that other components of soy are protecting lunasin from degradation (see section 7). Lunasin is biodistributed in various tissues including lung, liver, mammary gland, prostate and even the brain within 6 hours after administration. In addition, analysis of the liver and blood showed that this peptide was present in an intact and bioactive form (Jeong et al., 2007; Hsieh et al., 2010).

6.2 Effects on breast cancer

Lunasin was first discovered during studies regarding soy seed development. Early soybean seed development is characterized by orchestrated events of rapid cell division and differentiation. It was observed that the stage of seed cell expansion (massive synthesis of storage molecules) began after cell division had ceased and that a temporal production of lunasin coincided with this mitotic arrest. This data led to the hypothesis that lunasin could

also be involved in the disruption of mammalian cell division such as cancer cells. Indeed, lunasin was shown to block mitosis in mammalian cells by binding to chromatin and impairing the formation of the kinetochore complex in the centromere. These effects lead to the failure of microtubules to attach the centromeres and thereby to mitotic arrest and cell death (Galvez et al., 1999).

The mechanism of action for lunasin in the prevention of cell malignant transformation is related to chromatin binding. The dynamics of histone acetylation and deacetylation in non-cancerous cells is involved in chromatin remodelling which is implicated in cell cycle control (Hernandez-Ledesma et al., 2009). These processes are tightly regulated by tumor suppressor molecules which have among other activities the function to keep the histone core in a deacetylated (repressed) state. Nevertheless, during cell malignant transformation, many tumor suppressor molecules are inactivated by chemical and viral carcinogens which lead to the exposure of histones core. At this stage, lunasin is able to inhibit histone acetylation by binding deacetylated histones which prevents transcription and represses cell cycle progression (Galvez et al., 1999; Hernandez-Ledesma et al., 2009). In this context, lunasin can act as a surrogate tumor suppressor. Therefore, it has been suggested that lunasin selectively kills cells that are being transformed by disrupting the dynamics of histone acetylation-deacetylation when a transforming event occurs (Hernandez-Ledesma et al., 2008).

The RGD motif also contributes to the anticancer effects of lunasin. Since RGD motif is implicated in the attachment of tumor cells to the extracellular matrix, peptides containing this motif have been described to prevent metastasis of tumor cells by competitive adhesion to the extracellular matrix. Furthermore, it has been suggested that the internalization of lunasin in MCF-7 cells would be mediated by a functional RGD motif (Galvez et al., 1999).

In *in vitro* studies, lunasin was shown to suppress colony formation induced by the ras-oncogene in MCF-7 cells (Jeong et al., 2003). The *in vivo* effects of lunasin were investigated on an ER-negative MDA-MB-231 breast cancer model in which athymic mice received intraperitoneal injections of lunasin (4 or 20 mg/kg body weight) for 2 months prior to tumor implantation. After 7 weeks, mice treated with lunasin showed a significant reduction in breast tumor incidence and a delay in the appearance of tumors. In addition, histologic analysis revealed low proliferation and high apoptosis indexes in tumors of lunasin-treated mice (Hsieh et al., 2010).

7. Combined effects of bioactive compounds

The combination of therapies has emerged as an interesting approach for cancer prevention and treatment. This alternative strategy is based on synergistic effects of 2 or more anticancer compounds able to act in multiple targets resulting in a more efficacious treatment. Moreover, the combination of agents can result in significant activity at concentrations where the single agent is inactive. Thus, there is possibility to regulate an optimal dose and reduce unwanted side-effects (Lane, 2006).

As extensively commented here, legumes are rich sources of anticancer compounds including not only isoflavones, protease inhibitors and peptides but also saponins, phytic acid, and inositol phosphates. These molecules have different mechanisms of action on cancer cells, which suggests that their combination would result in synergistic effects. For example, a soy extract containing isoflavones and saponins significantly reduced the incidence of mammary tumour induced by DMBA (Gallo et al., 2001; Jin et al., 2002).

Inhibition of both breast tumor growth and metastasis was observed in animals treated with isolated soy proteins and isoflavones (Yan et al., 2002). Supplementation of isoflavone-containing crude soy protein to a transgenic mouse model for mmammary tumor significantly prolonged the latency period of tumour development (Jin et al., 2002).

The effects of combining legume bioactive compounds with other dietary molecules have also been studied. The combination of soy phytochemicals at a low dietary level with tea showed synergistic effects on inhibiting the growth of MCF-7 tumours (Zhou et al., 2004). Velie et al. undertook a large diet-based cohort study (40,559 postmenopausal women) and found that the only diet with significant negative correlation with invasive breast cancer was the traditional southern diet, which comprises high legume intake, low mayonnaise intake, and potentially cabbage intake (Velie et al., 2005).

The potential synergisms among legume bioactive compounds provide a clue to explain the different effects observed between studying the bioactive compound alone and evaluating a specific bioactive compound often associated with other anticancer molecules present in the legume. Therefore, researchers should consider the characterization of each legume extract or food not only in terms of quantity of the studied molecule but also in terms of identifying the presence of other potential bioactive compounds. This approach should improve data quality and allow more reliable comparisons and conclusions regarding the benefits and risks of legume intake.

In addition, the elucidation of these synergistic mechanisms may be useful to clarify the real effect of each bioactive compound and, based on this knowledge, aid in the design of novel preventive/therapeutic approaches and dietary guidelines. Chiesa et al. compared the breast tumor development of mice receiving a diet with different concentrations of isoflavones and soy protein and concluded that only animals receiving an isoflavone-poor soy protein concentrate diet showed reduction in tumor progression rates and metastasis development (Chiesa et al., 2008). Another study aimed to elucidate the synergistic effects between the major bioactive components present in a soy extract termed "Bowman-Birk Inhibitor Concentrate" (BBIC). BBIC was developed for use in large-scale human cancer-prevention trials and has been extensively studied for its bioactivity against several cancer types (Kennedy, 1998). Its anticancer effects have been mainly attributed to the soy-derived BBI, which is present in high concentrations in this extract (Kennedy, 1998). In addition to BBI, lunasin peptide is also present and both represent 44% of total protein in BBIC. BBI and lunasin peptide were administered intraperitoneally and separately in mice for 2 months prior to the implantation of MDA-MB-231 breast cancer cells. Surprisingly, it was observed that only lunasin was effective in inducing significant chemopreventive and therapeutic effects. In this context, BBI and other PIs present in BBIC complement lunasin activity in other manner. Since lunasin is easily degraded by gastrointestinal enzymes when ingested in a pure form, it has been reported that those PIs protect lunasin from digestion and make this peptide bioavailable (Hsieh et al., 2010).

Approaches of combining legumes bioactives compounds with conventional chemotherapeutic drugs have been shown promising results. Ito et al. reported that the combination of 10% miso diet (soy food) with 2.5 mg/kg tamoxifen resulted in a significant reduction in the incidence and multiplicity of MNU-induced mammary carcinomas mice (Ito et al., 1996). The combination of tamoxifen and soy protein isolate provided a better protection than using the components alone. The tumor incidence in DMBA-induced mammary carcinogenesis mice was reduced 29% by tamoxifen, 37% by soy protein isolate and 62% by the combination (Constantinou et al., 2001).

The high intake of soy isoflavones was associated with reduced risk of ER-positive breast cancer recurrence in patients receiving anastrozole treatment (aromatase inhibitor). In addition, it was suggested that the observed effect might be related to a synergistic inhibitory effect of isoflavones and anastrozole on the synthesis of estrogen (Kang et al., 2010). Another interesting combination with centchroman (selective estrogen receptor modulator) and soy intake was investigated on DMBA-induced mammary carcinogenesis. The doses and periods of treatment were optimized and a maximum tumor regression of 98.6% were achieved with centchroman 5 mg/kg per day, alone/in combination with soy 3×10^4 mg/kg per day at 5 weeks treatment period (Mishra et al., 2010).

8. Conclusion

Undoubtedly, plant species are notable sources of compounds essential to human health not only in terms of nutrition but also in a therapeutic aspect. In particular, the variety of legume-derived bioactive compounds has attracted the attention of researchers for their health-promoting properties on breast cancer. Encouraging results have identified the preventive and treatment effects of these compounds on breast cancer in *in vitro*, *in vivo* and epidemiological studies. However, other data sets indicate harmful or neutral outcomes. Therefore, the discussed data do not allow conclusive statements regarding the effects of legumes and their bioactive compounds on humans, especially on women at high risk for breast cancer and breast cancer patients.

Re-evaluation of current data and further studies are crucial to elucidate such doubts. Nevertheless, in order to provide expressive contributions and to make studies comparable, the design and interpretation of new experiments should be considered. In this context, the influences of variables, such as dose and time of exposition, and potential synergisms or antagonisms among compounds need to be investigated. Indeed, further studies on the effects and mechanisms of action of these molecules on breast cancer will provide a better comprehension regarding the safety of legume intake and the elaboration of suitable dietary guidelines.

The conclusive characterization of legumes as preventive and therapeutic anticancer compounds would lead to interesting future perspectives. Among other options, it would be possible to develop therapies based on legumes compounds; to improve their bioavailability by the use of drug delivery systems; to use them as templates in the process of rational design of new anticancer drugs; and even to control the amounts of specific bioactive compounds produced by genetically engineered legume crops.

9. Acknowledgment

We would like to thank Alexandre Ferreira de Souza Dias for his important comments during the preparation of this chapter. We also would like to thank the financial support provided by the Pós-Graduação em Biologia Molecular/CEL - University of Brasilia, Brazil.

10. References

Allred, C. D.; Allred, K. F.; Ju, Y. H.; Goeppinger, T. S.; Doerge, D. R. & Helferich, W. G. (2004). Soy processing influences growth of estrogen-dependent breast cancer

tumors. *Carcinogenesis,* Vol. 25, No. 9, (September 2004), pp. 1649-1657. ISSN 0143-3334.

Armstrong, W. B.; Kennedy, A. R.; Wan, X. S.; Taylor, T. H.; Nguyen, Q. A.; Jensen, J.; Thompson, W.; Lagerberg, W. & Meyskens, F. L., Jr. (2000). Clinical modulation of oral leukoplakia and protease activity by Bowman-Birk inhibitor concentrate in a phase IIa chemoprevention trial. *Clinical Cancer Research,* Vol. 6, No. 12, (December 2000), pp. 4684-4691. ISSN 1078-0432.

Banerjee, S.; Li, Y.; Wang, Z. & Sarkar, F. H. (2008). Multi-targeted therapy of cancer by genistein. *Cancer Letters,* Vol. 269, No. 2, (October 2008), pp. 226-242. ISSN 1872-7980.

Barbosa, J. A.; Silva, L. P.; Teles, R. C.; Esteves, G. F.; Azevedo, R. B.; Ventura, M. M. & de Freitas, S. M. (2007). Crystal structure of the Bowman-Birk Inhibitor from Vigna unguiculata seeds in complex with beta-trypsin at 1.55 A resolution and its structural properties in association with proteinases. *Biophysical Journal*, Vol. 92, No. 5, (March 2007), pp. 1638-1650. ASSN 1542-0086.

Barnes, S. (1997). The chemopreventive properties of soy isoflavonoids in animal models of breast cancer. *Breast cancer research and treatment*, Vol. 46, No. 2-3, (November 1997), pp. 169-179. ASSN 0167-6806.

Bouker, K. B. & Hilakivi-Clarke, L. (2000). Genistein: does it prevent or promote breast cancer? *Environmental health perspectives,* Vol. 108, No. 8, (August 2000), pp. 701-708. ISSN 0091-6765.

Boyapati, S. M.; Shu, X. O.; Ruan, Z. X.; Dai, Q.; Cai, Q.; Gao, Y. T. & Zheng, W. (2005). Soyfood intake and breast cancer survival: a followup of the Shanghai Breast Cancer Study. *Breast cancer research and treatment,* Vol. 92, No. 1, (July 2005), pp. 11-17. ISSN 0167-6806.

Chen, Y. W.; Huang, S. C.; Lin-Shiau, S. Y. & Lin, J. K. (2005). Bowman-Birk inhibitor abates proteasome function and suppresses the proliferation of MCF7 breast cancer cells through accumulation of MAP kinase phosphatase-1. *Carcinogenesis,* Vol. 26, No. 7, (July 2005), pp. 1296-1306. ISSN 0143-3334.

Cheng, E.; Story, C. D.; Yoder, L.; Hale, W. H. & Burroughs, W. (1953). Estrogenic activity of isoflavone derivatives extracted and prepared from soybean oil meal. *Science,* Vol. 118, No. 3058, (August 1953), pp. 164-165. ISSN 0036-8075.

Chiesa, G.; Rigamonti, E.; Lovati, M. R.; Disconzi, E.; Soldati, S.; Sacco, M. G.; Cato, E. M.; Patton, V.; Scanziani, E.; Vezzoni, P.; Arnoldi, A.; Locati, D. & Sirtori, C. R. (2008). Reduced mammary tumor progression in a transgenic mouse model fed an isoflavone-poor soy protein concentrate. *Molecular nutrition & food research,* Vol. 52, No. 10, (October 2008), pp. 1121-1129. ISSN 1613-4133.

Colditz, G. A. & Frazier, A. L. (1995). Models of breast cancer show that risk is set by events of early life: prevention efforts must shift focus. *Cancer Epidemiology, Biomarkers & Prevention,* Vol. 4, No. 5, (July 1995), pp. 567-571. ISSN 1055-9965.

Constantinou, A.; Lantvit, D.; Lim, E.; Xu, H. & Pezzuto, J. M. (2001). Consumption of soy products may enhance tamoxifen's breast cancer preventive effects. *Proceedings of the American Association for Cancer Research,* Vol. 42, No. 2001), pp. 826.

de Lumen, B. O. (2005). Lunasin: a cancer-preventive soy peptide. *Nutrition reviews,* Vol. 63, No. 1, (January 2005), pp. 16-21. ISSN 0029-6643.

Doyle, J. J. & Luckow, M. A. (2003). The rest of the iceberg. Legume diversity and evolution in a phylogenetic context. *Plant Physiology,* Vol. 131, No. 3, (March 2003), pp. 900-910. ISSN 0032-0889.

Du, X.; Beloussow, K. & Shen, W. C. (2001). Bowman-Birk protease inhibitor and its palmitic acid conjugate prevent 7,12-dimethylbenz[a]anthracene-induced transformation in cultured mouse mammary glands. *Cancer Letters,* Vol. 164, No. 2, (March 2001), pp. 135-141. ISSN 0304-3835.

Erdman, J. W.; Badger, T. M.; Lampe, J. W.; Setchell, K. D. R. & Messina, M. (2004). Not All Soy Products Are Created Equal: Caution Needed in Interpretation of Research Results. *Journal of Nutrition,* Vol. 134, No. (May 2004), pp. 1229S-1233S. ISSN

Fernandes, A. O. & Banerji, A. P. (1997). Long-term feeding of field bean protein containing protease inhibitors suppresses virus-induced mammary tumors in mice. *Cancer Letters,* Vol. 116, No. 1, (June 1997), pp. 1-7. ISSN 0304-3835.

Fink, B. N.; Steck, S. E.; Wolff, M. S.; Britton, J. A.; Kabat, G. C.; Gaudet, M. M.; Abrahamson, P. E.; Bell, P.; Schroeder, J. C.; Teitelbaum, S. L.; Neugut, A. I. & Gammon, M. D. (2007). Dietary flavonoid intake and breast cancer survival among women on Long Island. *Cancer Epidemiology, Biomarkers & Prevention,* Vol. 16, No. 11, (November 2007), pp. 2285-2292. ISSN 1055-9965.

Folman, Y. & Pope, G. S. (1966). The interaction in the immature mouse of potent oestrogens with coumestrol, genistein and other utero-vaginotrophic compounds of low potency. *The Journal of endocrinology,* Vol. 34, No. 2, (February 1966), pp. 215-225. ISSN 0022-0795.

Fournier, D. B.; Erdman, J. W., Jr. & Gordon, G. B. (1998). Soy, its components, and cancer prevention: a review of the in vitro, animal, and human data. *Cancer Epidemiology, Biomarkers & Prevention,* Vol. 7, No. 11, (November 1998), pp. 1055-1065. ISSN 1055-9965.

Franke, A. A.; Custer, L. J.; Wang, W. & Shi, C. Y. (1998). HPLC analysis of isoflavonoids and other phenolic agents from foods and from human fluids. *Proceedings of the Society for Experimental Biology and Medicine,* Vol. 217, No. 3, (March 1998), pp. 263-273. ISSN 0037-9727.

Freitas, S.M.; Ikemoto, H. & Ventura, M.M. (1999). Thermodynamics of the binding of chymotrypsin with the black-eyed pea trypsin and chimotrypsin inhibitor (BTCI). *Journal of Protein Chemistry*, Vol. 18, No. 3. (April 1999), pp 307–313. ISSN 0277-8033.

Fritz, W. A.; Coward, L.; Wang, J. & Lamartiniere, C. A. (1998). Dietary genistein: perinatal mammary cancer prevention, bioavailability and toxicity testing in the rat. *Carcinogenesis,* Vol. 19, No. 12, (December 1998), pp. 2151-2158. ISSN 0143-3334.

Gallo, D.; Giacomelli, S.; Cantelmo, F.; Zannoni, G. F.; Ferrandina, G.; Fruscella, E.; Riva, A.; Morazzoni, P.; Bombardelli, E.; Mancuso, S. & Scambia, G. (2001). Chemoprevention of DMBA-induced mammary cancer in rats by dietary soy. *Breast cancer research and treatment,* Vol. 69, No. 2, (September 2001), pp. 153-164. ISSN 0167-6806.

Galvez, A. F. & de Lumen, B. O. (1999). A soybean cDNA encoding a chromatin-binding peptide inhibits mitosis of mammalian cells. *Nature Biotechnology,* Vol. 17, No. 5, (May 1999), pp. 495-500. ISSN 1087-0156.

Gepts, P.; Beavis, W. D.; Brummer, E. C.; Shoemaker, R. C.; Stalker, H. T.; Weeden, N. F. & Young, N. D. (2005). Legumes as a model plant family. Genomics for food and feed

report of the Cross-Legume Advances Through Genomics Conference. *Plant Physiology,* Vol. 137, No. 4, (April 2005), pp. 1228-1235. ISSN 0032-0889.

Goldin, B. R.; Adlercreutz, H.; Gorbach, S. L.; Woods, M. N.; Dwyer, J. T.; Conlon, T.; Bohn, E. & Gershoff, S. N. (1986). The relationship between estrogen levels and diets of Caucasian American and Oriental immigrant women. *The American journal of clinical nutrition,* Vol. 44, No. 6, (December 1986), pp. 945-953. ISSN 0002-9165.

Gonzalez de Mejia, E.; Vasconez, M.; de Lumen, B. O. & Nelson, R. (2004). Lunasin concentration in different soybean genotypes, commercial soy protein, and isoflavone products. *Journal of agricultural and food chemistry,* Vol. 52, No. 19, (September 2004), pp. 5882-5887. ISSN 0021-8561.

Gu, L.; House, S. E.; Prior, R. L.; Fang, N.; Ronis, M. J.; Clarkson, T. B.; Wilson, M. E. & Badger, T. M. (2006). Metabolic phenotype of isoflavones differ among female rats, pigs, monkeys, and women. *The Journal of nutrition,* Vol. 136, No. 5, (May 2006), pp. 1215-1221. ISSN 0022-3166.

Guha, N.; Kwan, M. L.; Quesenberry, C. P., Jr.; Weltzien, E. K.; Castillo, A. L. & Caan, B. J. (2009). Soy isoflavones and risk of cancer recurrence in a cohort of breast cancer survivors: the Life After Cancer Epidemiology study. *Breast cancer research and treatment,* Vol. 118, No. 2, (November 2009), pp. 395-405. ISSN 1573-7217.

Guo, T. L.; Chi, R. P.; Hernandez, D. M.; Auttachoat, W. & Zheng, J. F. (2007). Decreased 7,12-dimethylbenz[a]anthracene-induced carcinogenesis coincides with the induction of antitumor immunities in adult female B6C3F1 mice pretreated with genistein. *Carcinogenesis,* Vol. 28, No. 12, (December 2007), pp. 2560-2566. ISSN 1460-2180.

Hargreaves, D. F.; Potten, C. S.; Harding, C.; Shaw, L. E.; Morton, M. S.; Roberts, S. A.; Howell, A. & Bundred, N. J. (1999). Two-week dietary soy supplementation has an estrogenic effect on normal premenopausal breast. *The Journal of clinical endocrinology and metabolism,* Vol. 84, No. 11, (November 1999), pp. 4017-4024. ISSN 0021-972X.

Helferich, W. G.; Andrade, J. E. & Hoagland, M. S. (2008). Phytoestrogens and breast cancer: a complex story. *Inflammopharmacology,* Vol. 16, No. 5, (October 2008), pp. 219-226. ISSN 0925-4692.

Hernandez-Ledesma, B. & de Lumen, B. O. (2008). Lunasin: a novel cancer preventive seed Peptide. *Perspectives in medicinal chemistry,* Vol. 2, pp. 75-80. ISSN 1177-391X.

Hernandez-Ledesma, B.; Hsieh, C. C. & de Lumen, B. O. (2009). Lunasin, a novel seed peptide for cancer prevention. *Peptides,* Vol. 30, No. 2, (February 2009), pp. 426-430. ISSN 0196-9781.

Ho, V. S. & Ng, T. B. (2008). A Bowman-Birk trypsin inhibitor with antiproliferative activity from Hokkaido large black soybeans. *Journal of Peptide Science,* Vol. 14, No. 3, (March 2008), pp. 278-282. ISSN 1075-2617.

Hsieh, C. C.; Hernandez-Ledesma, B.; Jeong, H. J.; Park, J. H. & de Lumen, B. O. (2010). Complementary roles in cancer prevention: protease inhibitor makes the cancer preventive peptide lunasin bioavailable. *PLoS One,* Vol. 5, No. 1, pp. e8890. ISSN 1932-6203.

Hsieh, C. Y.; Santell, R. C.; Haslam, S. Z. & Helferich, W. G. (1998). Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7)

cells in vitro and in vivo. *Cancer Research,* Vol. 58, No. 17, (September 1998), pp. 3833-3838. ISSN 0008-5472.

Ito, A.; Goto, T.; Okamoto, T.; Yamada, K. & Roy, G. (1996). A combined effect of tamoxifen (Tam) and miso for the development of mammary tumors induced with MNU in SD rats. *Proceedings of the American Association for Cancer Research,* Vol. 37, pp. 271.

Jemal, A.; Bray, F.; Center, M. M.; Ferlay, J.; Ward, E. & Forman, D. (2011). Global cancer statistics. *CA: a cancer journal for clinicians,* Vol. 61, No. 2, (March 2011), pp. 69-90. ISSN 1542-4863.

Jeong, H. J.; Jeong, J. B.; Kim, D. S. & de Lumen, B. O. (2007). Inhibition of core histone acetylation by the cancer preventive peptide lunasin. *Journal of agricultural and food chemistry,* Vol. 55, No. 3, (February 2007), pp. 632-637. ISSN 0021-8561.

Jeong, H. J.; Park, J. H.; Lam, Y. & de Lumen, B. O. (2003). Characterization of lunasin isolated from soybean. *Journal of agricultural and food chemistry,* Vol. 51, No. 27, (December 2003), pp. 7901-7906. ISSN 0021-8561.

Jin, Z. & MacDonald, R. S. (2002). Soy isoflavones increase latency of spontaneous mammary tumors in mice. *The Journal of nutrition,* Vol. 132, No. 10, (October 2002), pp. 3186-3190. ISSN 0022-3166.

Joanitti, G. A.; Azevedo, R. B. & Freitas, S. M. (2010). Apoptosis and lysosome membrane permeabilization induction on breast cancer cells by an anticarcinogenic Bowman-Birk protease inhibitor from Vigna unguiculata seeds. *Cancer Letters,* Vol. 293, No. 1, (July 2010), pp. 73-81. ISSN 1872-7980.

Joanitti, G. A.; Freitas, S. M. & Silva, L. P. (2006). Proteinaceous Protease Inhibitors: Structural Features and Multiple Functional Faces. *Current Enzyme Inhibition,* Vol. 2, No. 3, (August 2006), pp. 199-217. ISSN

Kang, X.; Zhang, Q.; Wang, S.; Huang, X. & Jin, S. (2010). Effect of soy isoflavones on breast cancer recurrence and death for patients receiving adjuvant endocrine therapy. *Canadian Medical Association Journal,* Vol. 182, No. 17, (November 2010), pp. 1857-1862. ISSN 1488-2329.

Kennedy, A. R. (1998). The Bowman-Birk inhibitor from soybeans as an anticarcinogenic agent. *The American journal of clinical nutrition,* Vol. 68, No. 6 Suppl, (December 1998), pp. 1406S-1412S. ISSN 0002-9165.

Kuiper, G. G.; Lemmen, J. G.; Carlsson, B.; Corton, J. C.; Safe, S. H.; van der Saag, P. T.; van der Burg, B. & Gustafsson, J. A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology,* Vol. 139, No. 10, (October 1998), pp. 4252-4263. ISSN 0013-7227.

Lajolo, F. M. & Genovese, M. I. (2002). Nutritional significance of lectins and enzyme inhibitors from legumes. *Journal of agricultural and food chemistry,* Vol. 50, No. 22, (October 2002), pp. 6592-6598. ISSN 0021-8561.

Lamartiniere, C. A. (2000). Protection against breast cancer with genistein: a component of soy. *The American journal of clinical nutrition,* Vol. 71, No. 6 Suppl, (June 2000), pp. 1705S-1707S. ISSN 0002-9165.

Lamartiniere, C. A.; Murrill, W. B.; Manzolillo, P. A.; Zhang, J. X.; Barnes, S.; Zhang, X.; Wei, H. & Brown, N. M. (1998). Genistein alters the ontogeny of mammary gland development and protects against chemically-induced mammary cancer in rats. *Proceedings of the Society for Experimental Biology and Medicine,* Vol. 217, No. 3, (March 1998), pp. 358-364. ISSN 0037-9727.

Lane, D. (2006). Designer combination therapy for cancer. *Nature Biotechnology,* Vol. 24, No. 2, (February 2006), pp. 163-164. ISSN 1087-0156.

Laskowski, M., & M. A. Qasim (2000). What can the structures of enzyme-inhibitor complexes tell us about the structures of enzyme substrate complexes? *Biochimica et Biophysica Acta*, Vol. 1477, (March 2000), pp. 324–337. ISSN 0006-3002.

Losso, J. N. (2008). The biochemical and functional food properties of the bowman-birk inhibitor. *Critical reviews in food science and nutrition,* Vol. 48, No. 1, (January 2008), pp. 94-118. ISSN 1040-8398.

Lu, L. J.; Anderson, K. E.; Grady, J. J.; Kohen, F. & Nagamani, M. (2000). Decreased ovarian hormones during a soya diet: implications for breast cancer prevention. *Cancer Research,* Vol. 60, No. 15, (August 2000), pp. 4112-4121. ISSN 0008-5472.

Martinez-Montemayor, M. M.; Otero-Franqui, E.; Martinez, J.; De La Mota-Peynado, A.; Cubano, L. A. & Dharmawardhane, S. (2010). Individual and combined soy isoflavones exert differential effects on metastatic cancer progression. *Clinical & experimental metastasis,* Vol. 27, No. 7, (October 2010), pp. 465-480. ISSN 1573-7276.

Messina, M. (2010). Insights gained from 20 years of soy research. *The Journal of nutrition,* Vol. 140, No. 12, (December 2010), pp. 2289S-2295S. ISSN 1541-6100.

Messina, M.; Abrams, D. I. & Hardy, M. (2010). Can clinicians now assure their breast cancer patients that soyfoods are safe? *Womens Health,* Vol. 6, No. 3, (May 2010), pp. 335-338. ISSN 1745-5065.

Messina, M.; McCaskill-Stevens, W. & Lampe, J. W. (2006). Addressing the soy and breast cancer relationship: review, commentary, and workshop proceedings. *Journal of the National Cancer Institute,* Vol. 98, No. 18, (September 2006), pp. 1275-1284. ISSN 1460-2105.

Messina, M. & Wu, A. H. (2009). Perspectives on the soy-breast cancer relation. *The American journal of clinical nutrition,* Vol. 89, No. 5, (May 2009), pp. 1673S-1679S. ISSN 1938-3207.

Messina, M. J. & Loprinzi, C. L. (2001). Soy for breast cancer survivors: a critical review of the literature. *The Journal of nutrition,* Vol. 131, No. 11, (November 2001), pp. 3095S-3108S. ISSN 0022-3166.

Messina, M. J. & Wood, C. E. (2008). Soy isoflavones, estrogen therapy, and breast cancer risk: analysis and commentary. *Nutrition journal,* Vol. 7, No. 2008), pp. 17. ISSN 1475-2891.

Mishra, R.; Tiwari, A.; Bhadauria, S.; Mishra, J.; Murthy, P. K. & Murthy, P. S. (2010). Therapeutic effect of centchroman alone and in combination with glycine soya on 7,12-dimethylbenz[alpha]anthracene-induced breast tumor in rat. *Food and Chemical Toxicology,* Vol. 48, No. 6, (June 2010), pp. 1587-1591. ISSN 1873-6351.

Murphy, P. A.; Song, T.; Buseman, G.; Barua, K.; Beecher, G. R.; Trainer, D. & Holden, J. (1999). Isoflavones in retail and institutional soy foods. *Journal of agricultural and food chemistry,* Vol. 47, No. 7, (July 1999), pp. 2697-2704. ISSN 0021-8561.

Murrill, W. B.; Brown, N. M.; Zhang, J. X.; Manzolillo, P. A.; Barnes, S. & Lamartiniere, C. A. (1996). Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. *Carcinogenesis,* Vol. 17, No. 7, (July 1996), pp. 1451-1457. ISSN 0143-3334.

Padilla-Banks, E.; Jefferson, W. N. & Newbold, R. R. (2006). Neonatal exposure to the phytoestrogen genistein alters mammary gland growth and developmental

programming of hormone receptor levels. *Endocrinology,* Vol. 147, No. 10, (October 2006), pp. 4871-4882. ISSN 0013-7227.

Park, J. H.; Jeong, H. J. & de Lumen, B. O. (2005). Contents and bioactivities of lunasin, bowman-birk inhibitor, and isoflavones in soybean seed. *Journal of agricultural and food chemistry,* Vol. 53, No. 20, (October 2005), pp. 7686-7690. ISSN 0021-8561.

Pasqualini, J. R. & Chetrite, G. S. (2005). Recent insight on the control of enzymes involved in estrogen formation and transformation in human breast cancer. *The Journal of steroid biochemistry and molecular biology,* Vol. 93, No. 2-5, (February 2005), pp. 221-236. ISSN 0960-0760.

Patisaul, H. B. & Jefferson, W. (2010). The pros and cons of phytoestrogens. *Frontiers in neuroendocrinology,* Vol. 31, No. 4, (October 2010), pp. 400-419. ISSN 1095-6808.

Peterson, G. & Barnes, S. (1996). Genistein inhibits both estrogen and growth factor-stimulated proliferation of human breast cancer cells. *Cell Growth & Differentiation,* Vol. 7, No. 10, (October 1996), pp. 1345-1351. ISSN 1044-9523.

Petrakis, N. L.; Barnes, S.; King, E. B.; Lowenstein, J.; Wiencke, J.; Lee, M. M.; Miike, R.; Kirk, M. & Coward, L. (1996). Stimulatory influence of soy protein isolate on breast secretion in pre- and postmenopausal women. *Cancer Epidemiology, Biomarkers & Prevention,* Vol. 5, No. 10, (October 1996), pp. 785-794. ISSN 1055-9965.

Rochfort, S. & Panozzo, J. (2007). Phytochemicals for health, the role of pulses. *Journal of agricultural and food chemistry,* Vol. 55, No. 20, (October 2007), pp. 7981-7994. ISSN 0021-8561.

Ruiz-Larrea, M. B.; Mohan, A. R.; Paganga, G.; Miller, N. J.; Bolwell, G. P. & Rice-Evans, C. A. (1997). Antioxidant activity of phytoestrogenic isoflavones. *Free Radical Research,* Vol. 26, No. 1, (January 1997), pp. 63-70. ISSN 1071-5762.

Ryan, C. A. (1990). Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. *Annual Review of Phytopathology,* Vol. 28, pp. 425–449.

Sakla, M. S.; Shenouda, N. S.; Ansell, P. J.; Macdonald, R. S. & Lubahn, D. B. (2007). Genistein affects HER2 protein concentration, activation, and promoter regulation in BT-474 human breast cancer cells. *Endocrine,* Vol. 32, No. 1, (August 2007), pp. 69-78. ISSN 1355-008X.

Shimizu, H.; Ross, R. K.; Bernstein, L.; Pike, M. C. & Henderson, B. E. (1990). Serum oestrogen levels in postmenopausal women: comparison of American whites and Japanese in Japan. *British journal of cancer,* Vol. 62, No. 3, (September 1990), pp. 451-453. ISSN 0007-0920.

Shu, X. O.; Jin, F.; Dai, Q.; Wen, W.; Potter, J. D.; Kushi, L. H.; Ruan, Z.; Gao, Y. T. & Zheng, W. (2001). Soyfood intake during adolescence and subsequent risk of breast cancer among Chinese women. *Cancer Epidemiology, Biomarkers & Prevention,* Vol. 10, No. 5, (May 2001), pp. 483-488. ISSN 1055-9965.

Shu, X. O.; Zheng, Y.; Cai, H.; Gu, K.; Chen, Z.; Zheng, W. & Lu, W. (2009). Soy food intake and breast cancer survival. *Jama,* Vol. 302, No. 22, (December 2009), pp. 2437-2443. ISSN 1538-3598.

Silva, L. P.; Leite, J. R. S. A.; Jr., C. B. & Freitas, S. M. (2001). Thermal stability of a black eyed pea trypsin/chymotrypsin inhibitor (BTCI). *Protein and Peptide Letters,* Vol. 7, No. 2001), pp. 397–401. ISSN

So, F. V.; Guthrie, N.; Chambers, A. F. & Carroll, K. K. (1997). Inhibition of proliferation of estrogen receptor-positive MCF-7 human breast cancer cells by flavonoids in the

presence and absence of excess estrogen. *Cancer Letters,* Vol. 112, No. 2, (January 1997), pp. 127-133. ISSN 0304-3835.

Theil, C.; Briese, V.; Gerber, B. & Richter, D. U. (2010). The effects of different lignans and isoflavones, tested as aglycones and glycosides, on hormone receptor-positive and -negative breast carcinoma cells in vitro. *Archives of gynecology and obstetrics,* Vol. No. (September 2010), pp. ISSN 1432-0711.

Trock, B. J.; Hilakivi-Clarke, L. & Clarke, R. (2006). Meta-analysis of soy intake and breast cancer risk. *Journal of the National Cancer Institute,* Vol. 98, No. 7, (April 2006), pp. 459-471. ISSN 1460-2105.

Velie, E. M.; Schairer, C.; Flood, A.; He, J. P.; Khattree, R. & Schatzkin, A. (2005). Empirically derived dietary patterns and risk of postmenopausal breast cancer in a large prospective cohort study. *The American journal of clinical nutrition,* Vol. 82, No. 6, (December 2005), pp. 1308-1319. ISSN 0002-9165.

Ventura, M.M.; Xavier-Filho, J. (1966). A trypsin and chymotrypsin inhibitor from black-eyed pea (*Vigna sinensis*).I. Purification and partial characterization. *Anais da Academia Brasileira de Ciências*, Vol. 38 (1966), pp 553–566.

Verheus, M.; van Gils, C. H.; Keinan-Boker, L.; Grace, P. B.; Bingham, S. A. & Peeters, P. H. (2007). Plasma phytoestrogens and subsequent breast cancer risk. *Journal of Clinical Oncology,* Vol. 25, No. 6, (February 2007), pp. 648-655. ISSN 1527-7755.

Wan, X. S.; Hamilton, T. C.; Ware, J. H.; Donahue, J. J. & Kennedy, A. R. (1998). Growth inhibition and cytotoxicity induced by Bowman-Birk inhibitor concentrate in cisplatin-resistant human ovarian cancer cells. *Nutrition and cancer,* Vol. 31, No. 1, 1998), pp. 8-17. ISSN 0163-5581.

Wan, X. S.; Lu, L. J.; Anderson, K. E.; Ware, J. H. & Kennedy, A. R. (2000). Urinary excretion of Bowman-Birk inhibitor in humans after soy consumption as determined by a monoclonal antibody-based immunoassay. *Cancer Epidemiology, Biomarkers & Prevention,* Vol. 9, No. 7, (July 2000), pp. 741-747. ISSN 1055-9965.

Wang, J. & Shen, W. C. (2000). Gastric retention and stability of lipidized Bowman-Birk protease inhibitor in mice. *International journal of pharmaceutics,* Vol. 204, No. 1-2, (August 2000), pp. 111-116. ISSN 0378-5173.

Wang, T. T.; Sathyamoorthy, N. & Phang, J. M. (1996). Molecular effects of genistein on estrogen receptor mediated pathways. *Carcinogenesis,* Vol. 17, No. 2, (February 1996), pp. 271-275. ISSN 0143-3334.

Willett, W. C. (1995). Diet, nutrition, and avoidable cancer. *Environmental health perspectives,* Vol. 103, No. 8, (November 1995), pp. 165-170. ISSN 0091-6765.

Wu, A. H.; Ziegler, R. G.; Horn-Ross, P. L.; Nomura, A. M.; West, D. W.; Kolonel, L. N.; Rosenthal, J. F.; Hoover, R. N. & Pike, M. C. (1996). Tofu and risk of breast cancer in Asian-Americans. *Cancer Epidemiology, Biomarkers & Prevention,* Vol. 5, No. 11, (November 1996), pp. 901-906. ISSN 1055-9965.

Yan, L.; Li, D. & Yee, J. A. (2002). Dietary supplementation with isolated soy protein reduces metastasis of mammary carcinoma cells in mice. *Clinical & experimental metastasis,* Vol. 19, No. 6, 2002), pp. 535-540. ISSN 0262-0898.

Ye, X. & Ng, T. B. (2009). A trypsin-chymotrypsin inhibitor with antiproliferative activity from small glossy black soybeans. *Planta Medica,* Vol. 75, No. 5, (April 2009), pp. 550-556. ISSN 1439-0221.

Zhang, L.; Wan, X. S.; Donahue, J. J.; Ware, J. H. & Kennedy, A. R. (1999). Effects of the Bowman-Birk inhibitor on clonogenic survival and cisplatin- or radiation-induced cytotoxicity in human breast, cervical, and head and neck cancer cells. *Nutrition and cancer,* Vol. 33, No. 2, 1999), pp. 165-173. ISSN 0163-5581.

Zheng, W.; Dai, Q.; Custer, L. J.; Shu, X. O.; Wen, W. Q.; Jin, F. & Franke, A. A. (1999). Urinary excretion of isoflavonoids and the risk of breast cancer. *Cancer Epidemiology, Biomarkers & Prevention,* Vol. 8, No. 1, (January 1999), pp. 35-40. ISSN 1055-9965.

Zhou, J. R.; Yu, L.; Mai, Z. & Blackburn, G. L. (2004). Combined inhibition of estrogen-dependent human breast carcinoma by soy and tea bioactive components in mice. *International journal of cancer,* Vol. 108, No. 1, (January 2004), pp. 8-14. ISSN 0020-7136.

Part 5

Novel Therapeutics: Gene Therapy, Nanoparticles, Experimental Therapeutics

17

Nanobody, New Agent for Combating Against Breast Cancer Cells

Fatemeh Rahbarizadeh[1], Fatemeh Rahimi Jamnani[2] and Farnoush Jafari Iri-Sofla[1]
[1]*Department of Medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran*
[2]*National Cell Bank of Iran, Pasteur Institute of Iran, Tehran*
Iran

1. Introduction

Breast cancer (BC) is a major public health problem among women throughout the world. More than 1.1 million cases are diagnosed annually and more than 410,000 patients die of it worldwide (Ferlay et al., 2010). BC is a complex and intrinsically heterogeneous disease with different morphologies, molecular profiles and clinical behavior which require different treatments (Bosch et al., 2010). Cancer treatment has come a long way from surgery alone to combined therapies to control tumour growth. By introducing the new therapeutic agents, combinations of existing therapies and targeted therapies, there would be promising future to improve survival and life span of patients who deal with this disease.

2. Breast cancer and therapeutic approaches

Breast cancer remains a threatening health problem in both developed and developing countries. However, its mortality rates have declined in recent years because of the broad advancement in the treatment of BC; including modifications in surgical procedures that reduce the risk of surgical morbidity and improvement in the delivery of radiation using novel imaging techniques that allow enhanced dosing to specified locations with fewer side effects on normal tissues (Moulder and Hortobagyi, 2008). Also, developments in systemic therapies include chemotherapy, hormonal therapy and biological therapy or combinations of these supportive cares, hold great promise for future breast cancer therapy.

2.1 HER2 targeted therapy by clinically approved drugs

Receptor tyrosine kinases (RTKs) play great roles in the transmission of extracellular signals that lead to cancer cell growth, survival and differentiation. These proteins are localized within the cell membrane and contain three parts. An extracellular ligand-binding domain (domains I, II, III, IV), a transmembrane domain that anchors the receptor to the cell membrane and a kinase domain that contains the ATP-binding site that phosphorylated a number of downstream target proteins. Phosphorylation causes the activation of signaling

cascades such as the mitogen-activated protein kinase and phosphoinositol 3'OH kinase (PI3K) pathways (Kruser and Wheeler, 2010). The HER family consists of four RTKs with similar homology: HER1 (EGFR/ErbB1), HER2 (neu, C-erbB2), HER3 (ErbB3) and HER4 (ErbB4) (Moulder and Hortobagyi, 2008). HER2 is the preferred dimerization partner for activation of other HER receptors. Receptor activation via ligand binding leads to downstream signaling including mitogen-activated protein kinases (MAPK), PI3K and signal transducers and activators of transcription (Stats). Ultimately, cell proliferation, angiogenesis, invasion and metastasis can be promoted by these cascades (Kruser and Wheeler, 2010; Nielsen et al., 2009). Aberrant expression or activity of two members of this family HER1 and HER2 have been connected with the occurrence of breast cancer. Two key parts of HER1 and HER2 (ligand binding domain and tyrosine kinase domain) have attracted scientists to inhibit their activity.

After many endeavors, the dream for generating an antibody that could bind to HER2 and block it became true. Trastuzumab (Herceptin®) (Genentech Inc. San Francisco, CA, USA; Hoffmann-La Roche Ltd. Basel, Switzerland) is the only anti-HER2 antibody that approved by FDA. This antibody is a humanized monoclonal antibody that targets the extracellular juxtamembranal domain of HER2 (Park et al., 2010). Trastuzumab by binding to HER2 reveals therapeutic efficacy in HER2-positive early stage and metastatic breast cancer. (Emde et al., 2010; Nielsen et al., 2009).

2.2 Immunotherapy

The clinical purpose of cancer immunotherapy is to enhance immune responses against tumour cells with the lowest side effects on healthy tissue (Guinn et al., 2007; Leen et al., 2007). Two therapeutic strategies have been developed to stimulate anti-tumour immunity in patients with breast cancer; vaccination (active immunization) and antibody/ immune cell therapy (passive immunization) (June, 2007; King et al., 2008).Vaccination of patients with breast cancer has not showed satisfactory results (Stauss et al., 2007). Antibody/T cell therapy possess potential benefits, since two main arms of immune system participate in destroying tumours and prevention of cancer recurrence by developing the immunological memory (Guinn et al., 2007).

The advent of monoclonal antibodies (mAbs) revolutionized the treatment of cancer. Monoclonal antibodies have always been encouraging for scientist as if they have developed novel approaches to produce various antibody formations; recombinant mAbs (include antibody fragments), chimeric antibodies and more recently recombinant polyclonal antibodies (Elbakri et al., 2010). Monoclonal antibodies have emerged as a class of novel oncology therapeutics. The unique pharmacokinetic characteristics, high specificity and the ability to engage and activate the immune system have made them valuable therapeutic agents in breast cancer treatment (Yan et al., 2008). Monoclonal antibodies exert their effects through activating antibody dependent cellular cytotoxicity and complement-dependent cytotoxicity, triggering apoptosis and blockade of growth factor receptors (King et al., 2008). Several monoclonal antibodies against tumour-associated antigen (TAA) have been used as fascinating targeting agents in breast cancer therapy. Clinical success has been observed with passively acquired monoclonal antibodies directed against a number of targets including HER2, HER1, MUC1 and vascular endothelial growth factor (King et al., 2008). However, these molecules have shown some side effects, resistance, immunogenicity and toxicity that have limited their uses. Endeavors to find solutions for these drawbacks led to advent several alternatives (Elbakri et al., 2010).

2.2.1 Nanobody; an old concept and new tool for immunotargeting

The large molecular size and immunogenicity are some complications that explain why treatment of solid tumours by mAbs is so elusive. These problems in therapeutic efficacy of mAbs have been partly solved with applying novel approaches such as phage display in the development of new therapeutically effective antibody domains. Antibody domains which lack the Fc region such as Fabs, diabodies, single chain variable fragments (scFvs), bispecific antibodies and variable domains (VH) offer many therapeutic advantages for therapeutic applications (Elbakri et al., 2010). By serendipity, in 1993 the nicest substitutes, the new generation of magic bullets that called heavy-chain antibodies (HCAbs) was found in *Camelidae* (Hamers-Casterman et al., 1993) and opened new window in breast cancer therapy (Rahbarizadeh et al., 2004b). HCAbs have evolved to be fully functional in the absence of a light chain. The smallest antigen-binding fragment, harbouring the full binding capacity of the naturally occurring HCAbs, is called VHH (variable domain of heavy chain antibodies) or single domain antibody. The crystal structure of an isolated single domain antibody is a particle of 2.5 nm in diameter and about 4 nm height, and is termed nanobody because of its size in nm scale and single domain structure.

2.2.2 Nanobody; structure and characteristics

Sequence analysis and studies on the crystal structure of nanobodies have revealed several structural features of nanobody domains (Hamers-Casterman et al., 1993; Muyldermans et al., 1994). These molecules contain four framework regions (FRs) that form the core structure of the immunoglobulin domain and three CDRs that are involved in antigen binding. When compared to human VH domains, the FRs show sequence homology of more than 80%, and their 3D structures can be superimposed (Desmyter et al., 1996). The genes of gamma 2 and gamma 3 chains of *Camelidae* HCAb show four amino acid changes at positions 42 (F, Y), 49 (E, Q), 50 (C, R) and 52 (F, G, L, W) according to the IMGT unique numbering, that are involved in forming the hydrophobic interface with VL domains. This co-evolution of the variable region (more hydrophilic) and of the constant region (absence of CH1 due to a mutation in the splicing site) is particularly remarkable. Occasionally, antigen-binding single domain antibody fragments that lack these characteristic FR2 substitutions are isolated from camelids. These fall into two categories:

- VHH-like conventional VHs: low-affinity binders isolated from a non-immune library which was originated from conventional antibodies, presumably because of the polymerase chain reaction crossover cloning artifact, as they were linked to the CH1 domain (Tanha et al., 2002)
- Conventional-like VHH domains: single-domain antibody fragments with conventional-like FR2 sequences that bind antigens with high affinity, isolated from immune libraries, which might have a hydrophobic residue at position 103 (mostly arginine)(Conrath et al., 2001; Harmsen and De Haard, 2007; Saerens et al., 2004).

CDRs of nanobodies are somewhat unique. The first amino acids of CDR1 are highly variable (Harmsen et al., 2000; Nguyen et al., 2000; Vu et al., 1997). VHH libraries generated from immunized camelids retain full functional diversity. High-affinity antigen binding domains can be isolated through screening a limited number of clones from immune libraries (Frenken et al., 2000; Harmsen et al., 2005).

This outstanding nature of nanobodies results in several advantages over the classical antibody families. The single domain nature of a nanobody makes molecular manipulation

easy and also facilitates the production of multivalent formats of them compared to conventional recombinant antibodies, in which the linking of specific length VH and VL domains often results in aggregation and reduced affinity due to mispairing of VH and VL domains. Nanobodies can be used readily for the production of such formats because they allow more flexible linker designs. This is important for simultaneous binding to multivalent antigens (Copier et al., 2009; Roovers et al., 2007).

The nanobody amino acid sequence closely resembles the family III of human variable heavy chain, with significant difference in FR2 and the CDRs (Harmsen et al., 2000; Vu et al., 1997). Nanobodies consist of three distinguished hyper-variable regions and CDRs. The CDRs of nanobodies have unique features when compared to mouse or human VH fragments. These are:

- Only three CDRs are involved in the binding surface of the nanobody (six in conventional antibody fragments) (Arbabi Ghahroudi et al., 1997)
- CDR1 and CDR2 loops are not canonical in structure (Decanniere et al., 2000)
- CDR1 loop might begin closer to the N terminus (Harmsen et al., 2000)
- A longer CDR3 loop with 17 residues on average (12 residues in human, 9 in mouse) (Wu et al., 1993).This long CDR3 results in new antigen binding modes, like binding the active site of the enzyme and also covers the hydrophobic interface that would be formed with the VL domain (Desmyter et al., 2002a; Desmyter et al., 1996)
- A second intra-domain disulfide bond connecting CDR3 with the CDR1, or a core residue between CDR1 and CDR2 (Muyldermans et al., 1994)

Although the amino acid sequences are similar between the nanobody and their classical VH counterparts, several hallmark changes occur in the frameworks encoded by the germ line V genes of the former. The most prominent change is seen in position 42 of nanobodies, which is exclusively occupied by either a F or Y adding to its hydrophobic character. In classical VH3 domains a smaller aliphatic residue, such as V or L, is seen in this position. Studies on crystal structures show that this change results in the packing of the residues from CDR3 against F/Y 42, forming a small hydrophobic core (Desmyter et al., 1996; Spinelli et al., 2001). Another change also contributes to this hydrophobic core, a substitution of an R at position 50 in place of the classical L or V. The long aliphatic side-chain of this R packs against F/Y 42, allowing the guanidium group to be present on the outer surface. A substitution of S for Y at position 52 and A for L/R at position 106 have been seen, but no clear consequences have been described (Bond et al., 2003). The presence of the additional intra-domain disulfide bond (connecting CDR3 and CDR1 or CDR1 and CDR2) may have two outcomes:

- Anchoring the CDR3 against the former interface
- Predispose the orientation of CDR3 for appropriate presentation to the antigen

Based on crystallographic studies the above mentioned substitutions of amino acids result in the conversion of a hydrophobic surface, seen in human and murine VH, into a more hydrophilic surface, thus resisting the VH-VL pairing (Chothia et al., 1985). These changes also seem to contribute to the high solubility of nanobodies compared to other single domain antibodies (sdAbs). Several studies have shown that the loss of the light chain binding partner in camelid nanobody is compensated through the interaction of CDR3 and the former light chain interface with CDR3 residues packing against F 42 (Decanniere et al., 1999; Desmyter et al., 2002b; Spinelli et al., 2000), forming a small hydrophobic core. It should be mentioned that these interactions vary considerably in a wide spectrum of

structural solutions. However, all result in the same outcome: sequestration of the hydrophobic framework residues from the solvent.
Nanobodies rapidly pass the renal filter and because of their small size (15 kDa); they are expected to clear rapidly from blood. This small size also is the reason for fast tissue penetration, an advantage for targeting tumours with nanobody coupled toxic substances (Cortez-Retamozo et al., 2004), *in vivo* diagnosis, imaging and treatment of snake bites.

2.2.3 Unique features of nanobodies

The unique intrinsic properties of nanobodies make them better options for medical and biotechnological applications, compared to antigen binding fragments of conventional antibodies. Nanobodies with a molecular weight of ~15 kDa have a size almost half of that for the scFv (30 kDa). Their smaller size also results in lower immunologic response and better pharmacokinetics. Nanobodies are highly soluble, and this is thought to be due to a tetrad of highly conserved hydrophilic substitutions compared to classical antibodies. As mentioned above, another typical feature of nanobodies is their long CDR3, enabling nanobodies to recognize alternative epitopes on an antigen. The lack of post-translational modifications makes the overexpression and production of regular antibodies in bacteria almost impossible. Due to nanobody being active without the modifications mentioned, and as single chains without the Fc domain are easily expressed in bacteria and yeast (Arbabi-Ghahroudi et al., 2005), expression in bacteria should be successful.
Nanobodies are highly stable to extremes of pH and can bind to their target at high concentrations of chaotropic agents (Dumoulin et al., 2002). They also have a remarkable resistance to high temperatures. Studies have shown that nanobodies regain their antigen binding property, even after prolonged incubation at temperatures of 80–92 ^{o}C. It should be mentioned that two other nanobody fragments elicited against a hydrophobic azo dye still exhibited activity in a binding assay at 90 °C. A possible explanation for this is that nanobodies do not aggregate during temperature denaturation, resulting in the reversibility of their folding to the native conformation upon cooling (Dumoulin et al., 2002; Ewert et al., 2003).

2.2.4 Nanobodies in cancer diagnosis and therapy

Based on their unique biophysical and pharmacological properties, nanobodies should be ideally placed to become a new class of cancer therapeutics. In addition to therapy, nanobodies are also expected to have a future as a tool for the diagnosis of cancer.

2.2.4.1 Nanobodies as cancer diagnostic tools

The superior penetration potential of nanobodies, due to their high affinity target binding and fast clearance from the circulation of the excess of non-targeted nanobodies, represenets an ideal basis for imaging purposes. Early detection and staging of prostate cancer is based on the detection of prostate-specific antigen (PSA) in the blood circulation. New nanobodies have been generated that can discriminate between different isoforms of PSA (Saerens et al., 2004). Nanobodies were also used as targeting probes for imaging the *in vivo* biodistribution of specific cell types, using a couple of nanobodies raised against mouse dendritic cells. The observed *in vivo* biodistribution for the two selected nanobodies with different cellular specificities nicely reflects the main *in vivo* locations of the cells that have been determined *in vitro* to be recognized by the nanobodies (De Groeve et al., 2010).
The successful selection and the characterization of antagonistic anti-EGFR nanobodies were shown by Roovers et al. (2007). These researchers isolated nanobodies from immune phage

nanobody repertoires, and showed that they specifically competed for EGF binding to the EGFR and were effective in delaying the outgrowth of A431-derived solid tumours in an *in vivo* murine xenograft model. Recently, llama single-domain antibody fragment was exploited for the *in vivo* radioimmunodetection of EGFR overexpressing tumours by means of a single photon emission computed tomography (SPECT) in mice. The nanobody (8B6) was then labeled with Technetium (99mTc-8B6) through its C-terminal histidine tail. The EGFR-specific nanobody investigated in this study showed high specificity and selectivity towards EGFR over expressing cells. Pinhole SPECT analysis with 99mTc-8B6 nanobody enabled *in vivo* discrimination between tumours with high and moderate EGFR over expression. The favorable biodistribution further corroborates the suitability of nanobodies for *in vivo* tumour imaging (Huang et al., 2008). The high tumour uptake, rapid blood clearance, and low liver uptake of nanobodies make them powerful probes for noninvasive imaging of antigen expression. In other study pinhole SPECT/micro-CT was used in an experiment to evaluate *in vivo* tumour uptake and biodistribution of two specific anti-EGFR nanobodies. Uptake in EGFR expressing tumours was high for both compounds, whereas the EGFR negative tumours showed only minor uptake. This confirms the selective targeting of anti-EGFR nanobodies that have affinities in the nanomolar range (Gainkam et al., 2008).

In another study, for finding better antibody formats for *in vivo* imaging and/or therapy of cancer, three types of sdAb-based molecules directed against EGFR were constructed, characterized and tested. Eleven sdAbs were isolated from a phage display library constructed from the sdAb repertoire of a llama immunized with a variant of EGFR. A pentameric sdAb, or pentabody, V2C-EG2 was constructed by fusing one of the sdAbs, EG2, to a pentamerization protein domain. A chimeric HCAb (cHCAb), EG2-hFc, was constructed by fusing EG2 to the fragment crystallizable (Fc) of human IgG1. Whereas EG2 and V2C-EG2 localized mainly in the kidneys after *i.v.* injection, EG2-hFc exhibited excellent tumour accumulation, and this was largely attributed to its long serum half life, which is comparable to that of IgGs. The moderate size (80 kDa) and intact human Fc make HCAbs a unique antibody format which may outperform whole IgGs as imaging and therapeutic reagents (Bell et al., 2010).

2.2.4.2 Therapeutic applications of nanobody

Although the high specificity of antibodies makes them suitable in many applications and processes, antibodies fragments are mostly applied in human therapy and *in vivo* diagnosis. PCR and the development of powerful panning techniques led to the generation of large libraries of scFv from which several binders could be selected successfully. Gene cloning and expression in bacteria facilitate the mutagenesis of the antigen binding site and improve the immunological and pharmacokinetic qualities (Skerra and Pluckthun, 1988). However, the application of these techniques is quite complex. The expression yield, stability, and functionality of scFv often turn out to be problematic. The immunogenicity and unsatisfactory yield of functional and monomeric products in heterologous expression systems are still some drawbacks in the development of scFvs for therapeutic applications (Whitlow et al., 1993).

The discovery of nanobody raised new hopes to obtain soluble single domain antibodies with a small size. The high affinity and specificity of nanobodies, plus their small size make them suitable for targeting antigens in obstructed locations, such as tumours due to their poorly vascularized tissue. The delivery of toxins or radioisotopes to diseased tissues

(Carter, 2001) is another potential therapeutic use for nanobodies, ensuring targeted delivery of the toxin to specific tissues and minimizing the time of exposure of normal cells. The short half-life of these antibodies is well suited for certain applications where rapid clearance is essential.

Cancer diagnosis in early stages requires an imaging agent able to deliver a sufficient amount of label to the site through prompt tumour penetration and rapid clearance of the unbound conjugate (Rosebrough and Hashmi, 1996; Sundaresan et al., 2003; Zuckier LS, 1997). The small size and high affinity target binding of nanobodies and their fast clearance result in significant penetration of these agents into tumour tissues. There are several reports of successful isolation of nanobodies against tumour markers. MUC1 is a tumour-associated marker with an extensive extracellular domain. In the breast, ovarian, lung, prostate, colon and pancreatic cancer tissues, not only is MUC1 over-expressed, but the core protein is also aberrantly glycosylated, making the tumour-associated mucin antigenically distinct from the normal mucin. Anti-MUC1 antibodies are used for *in vivo* targeting of breast and ovarian tumours, and there is considerable interest in MUC1 as a possible target antigen for immunotherapy of breast cancer (Taylor-Papadimitriou et al., 2002). For the first time, we have reported immunization of one- and two-humped camels by cancerous tissues and tumour markers, preparation of VHH gene libraries from these lymphocytes and isolation of single domain antibodies against MUC1 tumour marker. Furthermore, this was the first report of the production of a nanobody against a tumour-associated peptide (Rahbarizadeh et al., 2004a). Nanobodies against this tumour associated antigen (TAA) showed good specificity toward the synthetic peptides and have been successfully expressed in *E. coli* (Rahbarizadeh et al., 2005), P. pastoris (Rahbarizadeh et al., 2006), Tobacco plants (Ismaili et al., 2007; Rajabi-Memari et al., 2006) and CHO cells (Bazl et al., 2007). Moreover, mouse models with breast cancer tumour originated from MCF-7 (human breast cancer cell line) were used for *in vivo* tumour targeting. We have labeled ER46-28, anti-MUC1 nanobody, with ^{131}I and injected to these mice. The results of this assay confirmed our concept about nanobodies' effectiveness. Although short half life and rapid clearance of nanobodies are favourable in the case of cancer imaging, a longer serum half-life is more suitable for therapy. PEGylation (Chapman, 2002), conjugation and fusion to an Fc fragment of an antibody (Smith et al., 2001) successfully increase the serum half-life of nanobodies. Fusion of nanobodies with proteins such as albumin can provide multifunctional proteins with several binding sites (Bender et al., 1993; Harmsen et al., 2005).

The peculiar nature of nanobodies endows them more ability and they can be shared of immune-constructs. nanobodies act as a recognition site in chimeric T cell receptor, targeting vehicle for imaging and scanning of tumors and as a targeting agent on nanoparticles to drug or gene delivery to tumor associated antigens.

2.3 Combating against cancer with the multifunctional arms of immune system

CD4+ and CD8+ T lymphocytes are valuable components of adaptive immunity, which play pivotal roles in the elimination of tumours. The unique properties of T cells such as the capability of proliferation, homing, extravasation, and target rejection make them attractive candidates for adoptive immunotherapy (Cartellieri et al., 2010). Adoptive immunotherapy encompasses *ex vivo* manipulation and expansion of autologous T cells, followed by their re-infusion into tumour-bearing hosts. The *ex vivo* expansion of lymphokine-activated killer (LAK) cells or tumour-infiltrating lymphocytes (TIL) has attained some noteworthy

response rates in cancer patients. However, despite encouraging responses in patients with melanoma, response rates for several cancers such as breast cancer have remained low. This is partly because of the difficulty in isolating, expanding low frequent endogenous tumour-reactive T cells and their poor persistence after transfer (Berry et al., 2009; Hawkins et al., 2010). Meanwhile, tumours deploy strategies to persist and proliferate even if a large numbers of tumour-specific T cells exist. These strategies include; the low or absent expression of tumour-specific antigens, expression of antigens that are shared with normal cells at certain developmental stages, major histocompatibility complex I (MHCI) loss through structural defects, changes in β2-microglobulin synthesis, defects in transporter-associated antigen processing or actual MHC I gene loss (allelic or locus loss), production of immunosuppressive molecules by the tumour itself or by the tumour microenvironment such as Interleukin-10 (IL-10), transforming growth factor β (TGF-β) and soluble Fas ligand, Indolamine-2,3-Dioxygenase, recruit regulatory T cells (Treg), impaired dendritic cell (DC) function via inactivation (anergy) and/or poor DC maturation through changes in IL-6/IL-10/VEGF/granulocyte monocyte-colony stimulating factor (GM-CSF) (Biagi et al., 2007; Brenner and Heslop, 2010; Copier et al., 2009; Leen et al., 2007).

To overcome these problems, many approaches have been developed. One of the most attractive is genetic engineering of T cells. It dates backs to the middle of 1980, when scientists observed a spontaneous transcription of an aberrantly joined immunoglobulin variable heavy (IgVH) gene and a T cell receptor (TCR) JaCa gene resulting from site-specific Chromosome 14 inversion occur in human T-cell tumours (Gross et al., 1989). In another study in 1987, Gascoigne and co-workers reported a chimeric protein consists of T cell receptor variable domain and the immunoglobulin constant domain synthesized in myeloma cells. This protein with normal L chains formed a secreted tetramer (Gross et al., 1989). These reports have been inspirational for scientists to generate the supernatural T cells which possess both antibody and T cell abilities. These well-known T cells express T-bodies. In designing of T-bodies or chimeric antigen receptor (CAR), two humoral and cellular arms of immune system were exploited. CARs are typically composed of an extracellular antibody recognition domain (usually scFv) specific for tumour antigen that is linked by a hinge region to transmembrane and intracellular signaling domains. The use of antibody-binding regions in CARs enables T cell not only bind to TAA through their scFv in a non-MHC-restricted manner, but also respond to epitopes formed by protein, specially carbohydrate and lipid which are not recognized by conventional TCRs. After recognition site (scFv), second site belongs to hinge regions or spacers including the CH2–CH3 domains of the immunoglobulin heavy chain (IgG1, IgG3 and IgG4). Spacers lead to optimize function of CARs (by extending the distance between scFv and the T-cell membrane). The momentous parts of CAR, transmembrane and intracellular signaling domains, are derived from the cytoplasmic region of TCR complex (CD3ζ) or Fc-receptor-γ chain. The signaling domains of CARs are so crucial and determine functionality of genetically engineered T-cells. Many studies have been done and examined several motifs such as protein tyrosine kinase (PTK); ZAP70 and LCK to increase the power of TCR signaling (Eshhar, 2010; Sadelain, 2009). Stancovsky and co-workers constructed two anti-HER2 scFv, N29CD3ζ and N29CD3γ, chimeric genes that were expressed in cytotoxic T cell. Their study proved IL2 secretion and HER2-overexpressing cells lysis by redirected T cells (Stancovski et al., 1993). These simple structures were the first generation CARs with acceptable cytotoxicity but didn't show appropriate proliferation and prolonged survival after repeated exposure to Antigen. To achieve T cells with optimal activation and function, the signaling domain of co-stimulatory

molecules CD28, 4-1BB (CD137) and OX40 (CD134) were incorporated into the earlier structure and led to the second and third generations, respectively. In these tripartite constructs, in addition to a primary TCR-mediated signal, a secondary co-stimulatory signal is provided for T cells and results in highly efficient target cell lysis, proliferation, cytokine secretion, prolonged survival and rescue from apoptosis (Hombach and Abken, 2007; Zhong et al., 2010). The first generation of CAR have only been tested in phase I clinical trials in cancers such as ovarian, renal, lymphoma, and neuroblastoma, that have not shown significant results (Sadelain et al., 2003; Weisser and Hall, 2009). In the field of T cell therapy, scientists of ATTACK group (Adoptive engineered T cell Targeting to Activate Cancer Killing group) have focused on improving and optimizing the gene-redirected T cells activity (proliferation, secretion and cytotoxicity) in clinical applications. For these purposes, considerable progress in adoptive T cell therapy has been made in recent years.

2.3.1 Control of unwanted response of redirected T cells

Whereas usually retroviral or lentiviral gene transduction are utilized to constitutively express chimeric receptors in T cells, for the first time mRNA electroporation was applied to achieve transient immunoreceptor expression, to avoid of unintended auto-aggression. In this research, transfection of CD4 (+) and CD8 (+) T cells was efficiently performed with immunoreceptors specific for HER2 and carcino embryonic antigen (CEA). The immunoreceptor expression was transient with half-maximal expression at second day and no detectable immunoreceptor expression at nine days after electroporation. Immunoreceptor-transfected T cells were specifically activated upon co-incubation with HER2 (+) and CEA (+) tumour cells, respectively, resulting in secretion of interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumour necrosis factor-alpha (TNF-alpha). Furthermore, immunoreceptor-transfected CD8 (+) T cells specifically lysed HER2 (+) and CEA (+) tumour cells. The RNA-transfected T cells retained their cytotoxic function after two days of activation and exhibited cytolytic activities similar to T cells that have been transduced retrovirally (Birkholz et al., 2009).

Another approach to prevent unwanted response of redirected T cells on healthy tissues (Graft Versus Host), is incorporating the suicide genes such as herpes simplex viral thymidine kinase or caspase 9, into the CAR construct (Abken et al., 2003; Park et al., 2007). The herpes simplex viral thymidine kinase (HSV-tk) gene is widely used. Its product phosphorylates ganciclovir or acyclovir to the active moiety and interferes with DNA synthesis. Reprogrammed T cells with (HSV-tk) sucide gene are sensitive to the cytotoxic effects of gancyclovir, and even if GVHD (Graft Versus Host Disease) happened, by administering gancyclovir, cytotoxic T cells will be inactive. Unfortunately, there is a problem when HSV-tk is used in reprogrammed T cells in adoptive immunotherapy. Since the HSV-tk marker has viral origin and the viral antigens on transduced cells may be recognized by the host's native immune system, transferred T cells are eliminated before they have a chance to provide any therapeutic benefits (Berry et al., 2009; June, 2007; Leen et al., 2007).

2.3.2 Employing various strategies for reinforcing gene manipulated T cells against breast cancer cells

Several constructs have been designed to target three members of HER family HER2, HER3 and HER4, such as: scFv-CD3ζ (Altenschmidt et al., 1997), scFv-CD3γ (Li et al., 2008), scFv-CD28- CD3ζ (Moulder and Hortobagyi, 2008), heregulin-CD3ζ (Muniappan et al., 2000), ScFv-CD28-CD3ζ "infuluenza" (Dual-specific T cells were generated by gene modification of

influenza virus-specific mouse T cells with a chimeric gene-encoding reactivity against the HER2) (Murphy et al., 2007). In a study intravenously administration of primary mouse T cells with CAR against HER2 post tumour inoculation caused the rejection of established metastatic breast carcinoma (Berry et al., 2009; Moulder and Hortobagyi, 2008). Preclinical studies for investigating the engineered T cells with different constructs such as scFv-CD3γ and scFv-CD28- CD3ζ against HER2 on breast cancer cells are ongoing.

In a different study, the ability of T cells expressing an anti-HER2 chimeric receptor in eradicating tumour in HER2 transgenic mice that express human HER2 as a self-antigen in brain and mammary tissues was evaluated. After the administration of T cells expressing CAR with anti-HER2 as a recognition domain, remarkable enhancement in the survival of mice bearing HER2 (+) - 24JK tumour was observed in comparison with control T cells. Prior to adoptive transfer of T cells with CAR against HER2, mice lymphodepleted and IL-2 administrated that led to further enhance survival. This study highlighted the therapeutic potential of using T cells as a safe and effective treatment of cancer (Wang et al., 2010). In another study two constructs with a scFv derived from the humanized mAb 4D5 Herceptin (Trastuzumab) were designed to generate a CAR against HER2. In the first construct, scFv derived from 4D5 was linked to CD28 and CD3zeta. Expression of it on human peripheral blood lymphocytes (PBLs) led to Ag-specific activities against $HER2^{(+)}$ tumours. Also, this study demonstrated that CD3zeta signaling caused the transgene decrease; T cells expressing 4D5 CARs with mutations in their CD3 immunoreceptor tyrosine-based activation motif (ITAM) were less prone to apoptosis. In the second structure, 4-1BB cytoplasmic domains were added to the CD28-CD3zeta signaling moieties that led to increased transgene persistence, cytokine secretion and lytic activity in 4D5 CAR-transduced T cells (Zhao et al., 2009). Also, T cells were engineered to target MUC1 on breast cancer cells and exhibited considerable results (Wilkie et al., 2008).

B7.1 (important receptor in co-stimulation process) by binding to cytotoxic T-lymphocyte antigen 4 (CTLA4) anergy in T cells. A monoclonal antibody that blocks CTLA-4 binding has been developed to break tolerance. CTLA-4 blockade has entered clinical trials for patients with breast cancer. OX40 signaling on T-cells results in increased survival. OX40 has been targeted in several preclinical tumour models. For example, an agonist monoclonal antibody for OX40 on T-cells showed therapeutic activity. Hence, in a phase I clinical trial of an agonistic anti-OX40 monoclonal antibody has been begun for patients with advanced breast cancers who have failed standard cancer treatments (King et al., 2008; Ward and Kaufman, 2007).

2.3.3 Nanobody in CAR receptor: new insight in designing of the extracellular domain of CAR

As explained before, the recognition site of T-bodies is a scFv that includes the variable heavy (VH) and variable light (VL) domains of a specific antibody which are joined by a flexible linker. The scFvs specific for tumour antigens that are utilized in CARs, have murine origin and can be immunogenic in the host. Some studies were designed to develop chimeric receptor with a humanized scFv to reduce immunogenicity. An alternative approach has been explored that seems to be intellectual. scFvs with ideal properties, have still some drawbacks and must be improved in terms of stability, expression yield, protease resistance, and aggregation (because of its synthetic linker). Nanobodies with high affinity to a target antigen, small size and proper characteristics have been utilized to generate engineered T cells which express nanobody instead of scFv. Chimeric receptors with

nanobody as a recognition domain represent the fourth generation of chimeric T cell receptors. For first time, we replaced nanobody with scFv to target MUC1 on breast cancer cells. The final construct comprised of an anti-MUC1 nanobody as an extracellular domain which was linked via a hinge region to the intracellular domains of CD28 and CD3ζ. The results showed the specificity of modified T cells to tumour cells, IL2 secretion, proliferation and toxicity against breast cancer cells (Bakhtiari et al., 2009). In other study, the insertion of intracellular domain of OX40 to the previous chimeric receptor, examined and resulting in IL2 secretion in higher level. Also, several other studies are ongoing such as generation of redirected T cell against HER2 and TAG72 (Rahbarizadeh et al., 2011).

2.4 Nanoparticles for cancer therapy

Among several drug carriers and drug delivery systems, nanoparticles are very attractive particulate carrier systems under investigation (Kreuter, 2001). The body distribution of these carriers can be controlled by size and surface properties (Stayton et al., 2000). The particulate drug carrier systems have got characteristics such as considerable payload, controlled release of the drug and protection of the drug from degradation (Li et al., 1997). Following intravenous application, nanoparticles accumulate in the tissues of the mononuclear phagocyte system (MPS) and also in tumour tissue, which is often characterized by badly formed and leaky vasculature. This process which is due to an enhanced permeability and retention effect is called passive targeting (Maeda et al., 2000). To enhance the targeting of nanoparticles to specific cells or tissues, target-specific ligands should be linked to the nanoparticle surface (active targeting). Antibody-coupled liposomes (immunoliposomes) were first described in early 1980s (Leserman et al., 1980). Among several coupled homing devices, antibody-coupled nanoparticles can be regarded as an attractive drug-targeting system due to their advantageous properties such as stability (Weber et al., 2000).

In recent years, a variety of nanoparticles (NPs) functionalized with cancer-specific targeting ligands have been investigated to image tumours and detect peripheral metastases (Gao et al., 2004). Most of different types of nanoparticles can be classified into two major groups; (1) particles containing organic molecules as a major building material and (2) those that use inorganic elements, usually metals, as a core. Liposomes, dendrimers, carbon nanotubes, emulsions, and other polymers are a large and well-established group of organic particles (Duncan, 2003; Lee et al., 2005; Tasis et al., 2006; Yezhelyev et al., 2006). Most inorganic NPs share the same basic structure, consisting of a central core that defines the physical properties including fluorescence, optical, magnetic, and electronic features of the particle, with a protective organic coating on the surface which is usually responsible for the biological recognition and improvement of the particle solubility, for protecting the core from degradation in a physiologically aggressive environment and for evading the clearance action of the host immune system.

With the increasing use of targeted therapies in oncology, there is the requirement for the methods of molecular profiling to be optimized. The success of many targeted treatments depends on the expression of specific proteins or genes present in tumour cells. In breast cancer cells, the level of hormone-receptor expression correlates directly with the benefit of endocrine treatments, and the presence of HER2 protein overexpression and/or gene amplification is a prerequisite to benefit from target specific monoclonal antibodies (You et al., 2008). Some breast cancers produce protein biomarkers (such as estrogen receptor, progesterone receptor, and HER2), on which therapeutic decisions are made. The design of

methods that can detect *in vivo* the expression of such markers and monitor them during treatment is a real challenge.
Nanotechnology can be applied for the design of multifunctional nanoparticles that will be able to detect and image tumours and their metastases and meanwhile, it is used for therapy and monitor treatment progression. The application and efficiency of these nanoparticles *in vivo* will help enormously the pre and post cancer treatments (Scott et al., 2008). To design a diagnostic approach to breast carcinoma using nanoagents in humans, it is necessary to conclude several points for their utilization. These include high resolution, accuracy and sensitivity of detection, which may be provided by using NPs coated with specific monoclonal antibodies against protein biomarkers overexpressed by breast cancer cells. In addition, they must ideally have no toxicity, and be able to interact in a physiological way with biological tissues. In particular, they should have a good safety profile and not aggregate when delivered to biological tissues. Finally, since membrane receptors are endocytosed as part of their normal response to ligand binding, functionalized NPs have to follow physiological pathways when internalized. The ultimate challenge is represented by the development of efficient strategies for the good conjugation of targeting biomolecules on the NP surface. In fact, a major issue is the reliable conservation of the biological activity of immobilized macromolecules.
Among the various molecular targets explored for the treatment of human breast carcinoma, NPs conjugated with the anti-HER2 monoclonal antibody (Trastuzumab/Herceptin) is explained here.

2.4.1 Active targeting

There are numerous investigations for finding efficient systems for site specific delivery of drugs. One strategy involves usage of tumour-specific antibodies against overexpressed tumour associated antigens or receptors as targeting moieties which can be conjugated onto the nanoparticular surface for efficient delivery of drug . In active targeting of nanoparticles to specific sites in the body, targeting ligands are attached at the surface of the nanocarriers for binding specifically to appropriate receptors or exposed cellular biomolecules expressed with some degree of uniqueness at the target site (Mo and Lim, 2005). The ligand is chosen to bind to a receptor overexpressed by tumour cells or tumour vasculature and not expressed by normal cells. Moreover, targeted receptors should be expressed homogeneously on all targeted cells. Targeting ligands are either monoclonal antibodies (mAbs) and antibody fragments or non-antibody ligands such as, growth factors, transferrin, cytokines, folate and low-density lipoprotein (LDL) (Kocbek et al., 2007). Using tumour-specific antigens or antibodies as targeting moieties, cytotoxic drugs can be selectively delivered to tumour cells, thereby reducing the drug concentration in normal tissues and its toxic side effect (Smith et al., 2008).

2.4.2 Nanoparticles under investigation for breast cancer

Nanoparticles can be used to treat tumours in three different ways; (1) specific antibodies can be conjugated to the magnetic nanoparticles (MNPs) to selectively bind to related receptors and inhibit tumour growth; (2) targeted MNPs can be used for hyperthermia for tumour therapy; and (3) drugs can be loaded onto the MNPs for targeted therapy (Fernandez-Pacheco et al., 2007; Ghosh et al., 2008; Subramani et al., 2009).
Quantom dots (QDs) exhibit extraordinary photo-stable fluorescent signals and resistance to photo-bleaching. These NPs consist of a typical core/shell structure composed of heavy

metals (Lu et al., 2007). In many cases, QDs include a cadmium selenide or cadmium sulfide core, coated with a zinc sulfide shell. It is possible to modulate their size or change the nature of their metal core in order to vary their emission area in the range 450–850 nm. They are generally synthesized in high-boiling non-polar organic solvents. Thus, to be solubilized in aqueous buffers, their hydrophobic surface ligands must be replaced with suitable amphipathic ligands.

Superparamagnetic iron oxide NPs are useful for molecular imaging and thermal therapy. They are typically composed of magnetite (Fe_3O_4) nanocrystals; they have a spinel crystal structure with oxygen ions forming a close-packed cubic lattice and iron ions located at interstices. In recent years, several methods focusing on the synthesis of MNPs have been developed either in aqueous or organic phases (Peng et al., 2008). Many surfactants or polymers are usually employed in the synthesis to avoid aggregation and to reduce phagocytosis by macrophages and to increase circulation time in blood vessels. Among the most largely used MNP coating materials, PEG (poly-ethylene glycol) is highly water soluble and biologically inert, which renders MNPs immunologically stealth. Hence, significant efforts have focused on the possibility to functionalize their surface with ligands in order to create multifunctional NPs.

Gold nanoparticles (GNPs) and gold nanorods (GNRs) are under exploration in biomedicine since gold has been approved for optical detection and thermal therapy of tumours. These NPs are rapidly synthesized and their surface can be easily functionalized with targeting molecules and ligands by thiol chemistry (Chen et al., 2008). Many surfactants have been described, including citric acid and PEG, which are able to maintain the post-synthetic colloidal stability in aqueous physiological solutions.

2.4.3 Targeting of nanoparticle to breast cancer

One of the most commonly used strategies for targeted delivery of drugs to breast cancer utilizes the HER2, which is overexpressed in breast cancer. The surface of NPs may be coated with different functionalities, depending on the coating material and the functional groups present on the targeting ligand. Most commonly, amines or carboxylic acids are present on the NP surface. For this reason, the most largely employed method to attach Trastuzumab to NPs is the amide coupling involving carboxyl activation via the highly water soluble sulfo-NHS ester (Eghtedari et al., 2009). Two different targeting approaches have been reported by Nobs and co-workers for immunotargeting with Trastuzumab conjugated to nanoparticles (Nobs et al., 2006). One of the targeting procedure is a direct method using Trastuzumab-labeled poly lactic acid (PLA) nanoparticles and the other is a pretargeting method using the avidin-biotin technology. These experiments have shown that NPs covalently coupled with antibodies or neutr-avidin-rhodamine Red-X (NAR) can specifically and efficiently bind to cancer cells, suggesting that antibody conjugated NPs may be a useful drug carrier for tumour targeting. In other research nanoparticles based on human serum albumin (HSA) were developed. In this approach nanoparticles were covalently attached to thiolated trastuzumab (Steinhauser et al., 2006).

Anhorn and co-workers for the first time reported the specific targeting of HER2 overexpressing breast cancer cells with Doxorubicin-Loaded Trastuzumab-modified human serum albumin nanoparticles. HER2 overexpressing breast cancer cells showed a good cellular binding and uptake of these nanoparticles. The results indicate that these cell-type specific drug-loaded nanoparticles could achieve an improvement in cancer therapy (Anhorn et al., 2008).

Origin	Target	Fusion partner	Potential application
Immunized llama	EGFR	^{99m}TC	SPECT/micro-CT imaging
Immunized llama	EGFR	^{99m}TC	SPECT imaging
Immunized llama	Immunized or non immunized DC	^{99m}TC	*In vivo* imaging
Immunized llama	EGFR	PEG-liposome	EGFR down regulation
Immunized llama	EGFR	Pantabody-Fc	Tumour Targeting
Immunized llama	EGFR	(mPEG-*b*-p(HPMAm-Lacn)) core crosslinked thermosensitive polymeric micelles	Drug targeting
Immunized llama	EGFR	PEGylated quantum dots	Cancer imaging and detecting
Immunized dromedaries	CEA	^{99m}TC	Cancer imaging

Table 1. Some of nanobody-based fusions for diagnosis applications and therapy.

Cationic micellar nanoparticles were employed as carriers to co-deliver paclitaxel and Herceptin in order to targeted delivery of Paclitaxel to HER2 overexpressing human breast cancer cells. The co-delivery of Herceptin increased the cytotoxicity effect of Paclitaxel and this was dependent upon the level of HER2 expression on different cell lines used in this study. Targeting ability of this co-delivery system was demonstrated through confocal images, which showed significantly higher cellular uptake in HER2 overexpressing cells as compared to HER2 negative cells. This co-delivery system could be an important therapeutic tool against HER2 overexpressing breast cancers (Lee et al., 2009).

2.4.4 Nanobody targeted nanoparticle as a cancer therapeutic tool

An important obstacle in the use of antibodies for therapeutic purposes is the immunogenicity of these molecules. In the challenge to reduce the size and immunogenicity of antibodies, different modifications have been performed on the existing antibodies.

In another study, an anti- carcinoembryonic antigen (anti-CEA) VHH was used for targeting the genetically fused β-lactamase to tumour cells. This enzyme then converts an injected nontoxic prodrug into a toxic drug in the vicinity of the targeted tumour cells, leading to their killing (Cortez-Retamozo et al., 2004). More recently researchers presented a multivalent platform, consisting of nanobodies recognizing the ectodomain of EGFR (EGa1) coupled to poly (ethylene glycol)-liposomes, and the *in vitro* and *in vivo* effects of this system on EGFR internalization and downregulation were investigated (Oliveira et al., 2010). In another study Talelli and co-workers developed poly(ethylene glycol)-b-poly[N-(2-hydroxypropyl) methacrylamide-lactate] (mPEG-b-p(HPMAm-Lacn)) core-cross-linked thermosensitive biodegradable polymeric micelles suitable for active tumour targeting, by

coupling the anti-EGFR (EGa1 nanobody) to their surface (Roovers et al., 2007). In other research for the first time CdSe/ZnS quantum dots were biolabeled by a camelid single domain antibody (EG2), which is raised against epidermal growth factor receptor and these nanobody-conjugated quantum dot used as a specific labeling agent of EGFR expressing human breast cancer cells (Zaman et al., 2009).

2.4.5 Nanobody targeted nanoparticle for targeted cancer gene therapy

Cancer gene therapy is another approach in the treatment of breast cancer and it involves different strategies. An important issue for gene therapy is the choice of the delivery vehicle, which is able to successfully reach the nucleus of the tumour cells. In addition, the vector should be able to condense DNA from a large micrometer scale to a smaller nanometer scale suitable for endocytosis and to promote the escape of the gene from the endosomal compartment into the cytosol. Furthermore, the vector should be designed and synthesized to be recognized by specific receptors on the target cells and then, easily internalized. Following these concepts, nanoparticle-based DNA and RNA have been envisaged as advantageous delivery systems, using either viral or nonviral vectors for the gene transfection (Leuschner et al., 2006). In 2006, Hayes and co-workers published their preliminary results on using a DNA plasmid coupled with cationic lipids, to form lipid-nucleic acid-NPs, called Genopsheres (Eghtedari et al., 2009). To increase the delivery of the nanosystem into the cells of interest, genospheres were immunotargeted to selectively transfect HER2 overexpressing cells, by insertion of an anti-HER2 human single-chain monoclonal antibody (scFv)-PEG conjugate. Developing cancer gene therapy constructs based on transcriptional targeting strategy of genes to cancer cells is a new and promising modality for treatment of cancer. Induction of apoptosis in cancer cells could be an endogenous mechanism for cell death. By cloning and targeted expression of the pro-apoptotic gene in cancer cells, an anti-cancer gene therapy approach could be achieved. In 2006, tBid (a 15kDa protein cleaved from the cytoplasmic protein Bid) was announced as a suitable pro-apoptotic gene because it doesn't need any modification to become fully active and also because of its small size (Kazhdan et al., 2006). In order to limit the tBid expression to cancerous cells transcriptional targeted pro-apoptotic gene strategies in combination with tumour microenvironment factors are considered as efficient ways in cancer gene therapy approaches. Hypoxia and estrogen are microenvironment features of breast cancer cells which limit the action of constructs only to cancerous tissues. In these circumstances cells express hypoxia inducible transcription factor (HIF) that activates several genes. HIF bound to hypoxia responsive elements (HRE) in promoter of these genes and caused transcription.
Breast cancer cells also maintain the expression of intracellular estrogen receptors that act as a transcription factor to stimulate the expression of genes in the presence of estrogen and they bind to estrogen response elements (ERE) to activate transcription. We constructed two hybrid promoters which consisted of hypoxia responsive elements, estrogen response elements and MUC1 promoter (HEM) and also, Survivin promoter accompanied with hypoxia responsive elements and estrogen response elements (HES). tBid gene expression under the control of these two hybrid promoters were evaluated in normal and cancer cell lines with and without various treatments of hypoxia and estrogen. MUC1 promoter directs efficient expression of tBid gene under the control of the hybrid promoters which results in cell destruction. This study provides a significant advance in controlling lethal gene expression by using genetic characteristics and microenvironment elements in cancer cells

(Farokhimanesh et al., 2010). But the main hurdles in this type of treatment is finding appropriate vectors for the targeted delivery of genes *in vivo* and making sure that the apoptotic/killer gene is delivered to the target tumour cells. Polyplexes nanovectors are gaining more attention, mostly due to concerns regarding the safety and immunogenicity of vectors derived from viruses. Polycationic polymers, namely the highly cytotoxic poly-L-lysine (PLL) and polyethylenimine (PEI), are among the most widely used in gene therapy. PEI has shown to be efficient in gene delivery to eukaryotic cells both *in vitro* and *in vivo* (Boussif et al., 1995; Wightman et al., 2001). The high positive charge of the polyplexes (PEI/DNA complex) results in non-specific attachment of polyplexes to any negative charge surface, including plasma proteins and cellular membrane phospholipids. Free PEI, when administered systematically, precipitates in large clusters and adheres to cell surface which might result in destabilizing of the plasma membrane, inducing immediate toxicity. To overcome immediate toxicity, stealth nanoparticles were produced through coating the polyplexes with FDA approved polyethylene glycol (PEG) (Owens and Peppas, 2006). So we have used anti-MUC1 nanobody with high specificity for a MUC1 antigen to make PEGylated PEI conjugates for successful compaction of the tBid killer gene and its selective delivery into MUC1 expressing cell lines. Our attempt has provided a powerful proof of concept in combining nanobody-based targeting with transcriptional targeting as a safe way to deliver transgenes to specific cells.

3. Conclusion

With more than 20 therapeutic mAb products currently on the market and more than 100 in clinical trials, it is comprehensible that engineered antibodies have come of age as biopharmaceuticals (Reichert, 2008). In fact, in this decade, engineered antibodies are predicted to account for more than 30% of all revenues in the biotechnology market. Despite various beneficial characteristics of the conventional antibodies, the low inherent toxicity of the nanobodies together with their size characteristics and their high specificity and affinity for the antigen render them more promising candidates for delivery of pro-drugs, therapeutic genes (anti-angiogenesis, growth inhibitors and toxins) and chemotherapeutic agents. Moreover, nanobodies lack an antibody Fc tail, thus causing less immunological side effects. Nanobodies are anticipated to significantly expand the repertoire of antibody-based reagents against the vast range of novel biomarkers. Although few investigations have been conducted to use nanobodies against tumor markers, it is becoming increasingly clear that these potential immunotherapy nanosystems show omen in cancer therapy, and their successful use in treatment protocols is expected to be widely reported. As spelled out above, all these characteristics imply that there exists remarkable promise for nanobodies to be exploited as robust tumour diagnostic and cancer therapeutic reagents while presenting superior biological properties in comparison with conventional strategies.

4. References

Abken, H., Hombach, A. & Heuser, C. (2003). Immune response manipulation: recombinant immunoreceptors endow T-cells with predefined specificity. *Current pharmaceutical design*. 9:1992-2001.

Altenschmidt, U., Klundt, E. & Groner, B. (1997). Adoptive transfer of in vitro-targeted, activated T lymphocytes results in total tumor regression. *Journal of immunology*. 159:5509-15.

Anhorn, M. G., Wagner, S., Kreuter, J., Langer, K. & von Briesen, H. (2008). Specific targeting of HER2 overexpressing breast cancer cells with doxorubicin-loaded trastuzumab-modified human serum albumin nanoparticles. *Bioconjug Chem*. 19:2321-31.

Arbabi-Ghahroudi, M., Tanha, J. & MacKenzie, R. (2005). Prokaryotic expression of antibodies. *Cancer metastasis reviews*. 24:501-19.

Arbabi Ghahroudi, M., Desmyter, A., Wyns, L., Hamers, R. & Muyldermans, S. (1997). Selection and identification of single domain antibody fragments from camel heavy-chain antibodies. *FEBS letters*. 414:521-6.

Bakhtiari, S. H., Rahbarizadeh, F., Hasannia, S., Ahmadvand, D., Iri-Sofla, F. J. & Rasaee, M. J. (2009). Anti-MUC1 nanobody can redirect T-body cytotoxic effector function. *Hybridoma (Larchmt)*. 28:85-92.

Bazl, M. R., Rasaee, M. J., Foruzandeh, M., Rahimpour, A., Kiani, J., Rahbarizadeh, F., Alirezapour, B. & Mohammadi, M. (2007). Production of chimeric recombinant single domain antibody-green fluorescent fusion protein in Chinese hamster ovary cells. *Hybridoma*. 26:1-9.

Bell, A., Wang, Z. J., Arbabi-Ghahroudi, M., Chang, T. A., Durocher, Y., Trojahn, U., Baardsnes, J., Jaramillo, M. L., Li, S., Baral, T. N., O'Connor-McCourt, M., Mackenzie, R. & Zhang, J. (2010). Differential tumor-targeting abilities of three single-domain antibody formats. *Cancer letters*. 289:81-90.

Bender, E., Woof, J. M., Atkin, J. D., Barker, M. D., Bebbington, C. R. & Burton, D. R. (1993). Recombinant human antibodies: linkage of an Fab fragment from a combinatorial library to an Fc fragment for expression in mammalian cell culture. *Human antibodies and hybridomas*. 4:74-9.

Berry, L. J., Moeller, M., Darcy, P. K. (2009). Adoptive immunotherapy for cancer: the next generation of gene-engineered immune cells. *Tissue Antigens*. 74:277-89.

Biagi, E., Marin, V., Giordano Attianese, G. M., Dander, E., D'Amico, G. & Biondi, A. (2007). Chimeric T-cell receptors: new challenges for targeted immunotherapy in hematologic malignancies. *Haematologica*. 92:381-8.

Birkholz, K., Hombach, A., Krug, C., Reuter, S., Kershaw, M., Kampgen, E., Schuler, G., Abken, H., Schaft, N. & Dorrie, J. (2009). Transfer of mRNA encoding recombinant immunoreceptors reprograms CD4+ and CD8+ T cells for use in the adoptive immunotherapy of cancer. *Gene Therapy*. 16:596-604.

Bond, C. J., Marsters, J. C. & Sidhu, S. S. (2003). Contributions of CDR3 to VHH domain stability and the design of monobody scaffolds for naive antibody libraries. *Journal of Molecular Biology*. 332:643-655.

Bosch, A., Eroles, P., Zaragoza, R., Vina, J. R. & Lluch, A. (2010). Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research. *Cancer Treatments Reviews*. 36:206-15.

Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., Behr, J. P. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *The Proceedings of the National Academy of Sciences of the United States of America*. 92:7297-301.

Brenner, M. K. & Heslop, H. E. (2010). Adoptive T cell therapy of cancer. *Current opinion in immunology*. 22:251-7.

Cartellieri, M., Bachmann, M., Feldmann, A., Bippes, C., Stamova, S., Wehner, R., Temme, A. & Schmitz, M. (2010). Chimeric antigen receptor-engineered T cells for immunotherapy of cancer. *Journal of biomedicine & biotechnology*. 2010:956304.

Carter, P. (2001). Improving the efficacy of antibody-based cancer therapies. *Nature reviews. Cancer*. 1:118-29.

Chapman, A. P. (2002). PEGylated antibodies and antibody fragments for improved therapy: a review. *Advanced drug delivery reviews*. 54:531-45.

Chen, H., Gao, J., Lu, Y., Kou, G., Zhang, H., Fan, L., Sun, Z., Guo, Y. & Zhong, Y. (2008). Preparation and characterization of PE38KDEL-loaded anti-HER2 nanoparticles for targeted cancer therapy. *Journal of Controlled Release*. 128:209-16.

Chothia, C., Novotny, J., Bruccoleri, R. & Karplus, M. (1985). Domain association in immunoglobulin molecules. The packing of variable domains. *Journal of molecular biology*. 186:651-63.

Conrath, K. E., Lauwereys, M., Galleni, M., Matagne, A., Frere, J. M., Kinne, J., Wyns, L. & Muyldermans, S. (2001). Beta-lactamase inhibitors derived from single-domain antibody fragments elicited in the camelidae. *Antimicrobial agents and chemotherapy*. 45:2807-12.

Copier, J., Dalgleish, A. G., Britten, C. M., Finke, L. H., Gaudernack, G., Gnjatic, S., Kallen, K., Kiessling, R., Schuessler-Lenz, M., Singh, H., Talmadge, J., Zwierzina, H. & Hakansson, L. (2009). Improving the efficacy of cancer immunotherapy. *Europian Journal of Cancer*. 45:1424-31.

Cortez-Retamozo, V., Backmann, N., Senter, P. D., Wernery, U., De Baetselier, P., Muyldermans, S. & Revets, H. (2004). Efficient cancer therapy with a nanobody-based conjugate. *Cancer Research*. 64:2853-7.

De Groeve, K., Deschacht, N., De Koninck, C., Caveliers, V., Lahoutte, T., Devoogdt, N., Muyldermans, S., De Baetselier, P. & Raes, G. (2010). Nanobodies as Tools for In Vivo Imaging of Specific Immune Cell Types. *Journal of Nuclear Medicine*. 51:782-789.

Decanniere, K., Desmyter, A., Lauwereys, M., Ghahroudi, M. A., Muyldermans, S. & Wyns, L. (1999). A single-domain antibody fragment in complex with RNase A: non-canonical loop structures and nanomolar affinity using two CDR loops. *Structure*. 7:361-70.

Decanniere, K., Muyldermans, S. & Wyns, L. (2000). Canonical antigen-binding loop structures in immunoglobulins: more structures, more canonical classes? *Journal of molecular biology*. 300:83-91.

Desmyter, A., Spinelli, S., Payan, F., Lauwereys, M., Wyns, L., Muyldermans, S. & Cambillau, C. (2002a). Three camelid VHH domains in complex with porcine pancreatic alpha-amylase. Inhibition and versatility of binding topology. *The Journal of biological chemistry*. 277:23645-50.

Desmyter, A., Spinelli, S., Payan, F., Lauwereys, M., Wyns, L., Muyldermans, S. & Cambillau, C. (2002b). Three camelid VHH domains in complex with porcine pancreatic alpha-amylase. Inhibition and versatility of binding topology. *Journal of Biological Chemistry*. 277:23645-50.

Desmyter, A., Transue, T. R., Ghahroudi, M. A., Thi, M. H., Poortmans, F., Hamers, R., Muyldermans, S. & Wyns, L. (1996). Crystal structure of a camel single-domain VH antibody fragment in complex with lysozyme. *Nature Structural & Molecular Biology*. 3:803-11.

Dumoulin, M., Conrath, K., Van Meirhaeghe, A., Meersman, F., Heremans, K., Frenken, L. G., Muyldermans, S., Wyns, L. & Matagne, A. (2002). Single-domain antibody fragments with high conformational stability. *Protein Sciences*. 11:500-15.

Duncan, R. (2003). The dawning era of polymer therapeutics. *Nat Rev Drug Discov*. 2:347-60.

Eghtedari, M., Liopo, A. V., Copland, J. A., Oraevsky, A. A. & Motamedi, M. (2009). Engineering of hetero-functional gold nanorods for the in vivo molecular targeting of breast cancer cells. *Nano Letters*. 9:287-91.

Elbakri, A., Nelson, P. N. & Abu Odeh, R. O. (2010). The state of antibody therapy. *Human Immunology*. 71:1243-50.

Emde, A., Kostler, W. J. & Yarden, Y. (2010). Therapeutic strategies and mechanisms of tumorigenesis of HER2-overexpressing breast cancer. *Critical Review in Oncology and Hematology*.

Eshhar, Z. (2010). Adoptive cancer immunotherapy using genetically engineered designer T-cells: First steps into the clinic. *Current Opinion in Molecular Therapeutics*. 12:55-63.

Ewert, S., Huber, T., Honegger, A. & Pluckthun, A. (2003). Biophysical properties of human antibody variable domains. *Journal of molecular biology*. 325:531-53.

Farokhimanesh, S., Rahbarizadeh, F., Rasaee, M. J., Kamali, A. & Mashkani, B. (2010). Hybrid promoters directed tBid gene expression to breast cancer cells by transcriptional targeting. *Biotechnology Progress*. 26:505-11.

Ferlay, J., Shin, H. R., Bray, F., Forman, D., Mathers, C. & Parkin, D. M. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International journal of cancer. Journal international du cancer*. 127:2893-917.

Fernandez-Pacheco, R., Marquina, C., Valdivia, J. G., Gutierrez, M., Romero, M. S., Cornudella, R., Laborda, A., Viloria, A., Higuera, T., Garcia, A., de Jalon, J. A. G. & Ibarra, M. R. (2007). Magnetic nanoparticles for local drug delivery using magnetic implants. *Journal of Magnetism and Magnetic Materials*. 311:318-322.

Frenken, L. G. J., van der Linden, R. H. J., Hermans, P. W. J. J., Bos, J. W., Ruuls, R. C., de Geus, B. & Verrips, C. T. (2000). Isolation of antigen specific Llama V-HH antibody fragments and their high level secretion by Saccharomyces cerevisiae. *Journal of Biotechnology*. 78:11-21.

Gainkam, L. O., Huang, L., Caveliers, V., Keyaerts, M., Hernot, S., Vaneycken, I., Vanhove, C., Revets, H., De Baetselier, P. & Lahoutte, T. (2008). Comparison of the biodistribution and tumor targeting of two 99mTc-labeled anti-EGFR nanobodies in mice, using pinhole SPECT/micro-CT. *Journal of NuclearMedicine*. 49:788-95.

Gao, X., Cui, Y., Levenson, R. M., Chung, L. W. & Nie, S. (2004). In vivo cancer targeting and imaging with semiconductor quantum dots. *Nature Biotechnology*. 22:969-76.

Ghosh, P., Han, G., De, M., Kim, C. K. & Rotello, V. M. (2008). Gold nanoparticles in delivery applications. *Advances in Drug Delivery Reviews*. 60:1307-15.

Gross, G., Waks, T. & Eshhar, Z. (1989). Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *The Proceedings of the National Academy of Sciences of the United States of America.* 86:10024-8.

Guinn, B. A., Kasahara, N., Farzaneh, F., Habib, N. A., Norris, J. S. & Deisseroth, A. B. (2007). Recent advances and current challenges in tumor immunology and immunotherapy. *Molecular Therapy*. 15:1065-71.

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E. B., Bendahman, N. & Hamers, R. (1993). Naturally occurring antibodies devoid of light chains. *Nature*. 363:446-8.

Harmsen, M. M. & De Haard, H. J. (2007). Properties, production, and applications of camelid single-domain antibody fragments. *Applied Microbiolgy Biotechnology*. 77:13-22.

Harmsen, M. M., Ruuls, R. C., Nijman, I. J., Niewold, T. A., Frenken, L. G. & de Geus, B. (2000). Llama heavy-chain V regions consist of at least four distinct subfamilies revealing novel sequence features. *Molecular immunology*. 37:579-90.

Harmsen, M. M., Van Solt, C. B., Fijten, H. P. & Van Setten, M. C. (2005). Prolonged in vivo residence times of llama single-domain antibody fragments in pigs by binding to porcine immunoglobulins. *Vaccine*. 23:4926-34.

Hawkins, R. E., Gilham, D. E., Debets, R., Eshhar, Z., Taylor, N., Abken, H., Schumacher, T. N. & Consortium, A. (2010). Development of adoptive cell therapy for cancer: a clinical perspective. *Human Gene Therapy*. 21:665-72.

Hombach, A. & Abken, H. (2007). Costimulation tunes tumor-specific activation of redirected T cells in adoptive immunotherapy. *Cancer Immunology Immunotherapy*. 56:731-7.

Huang, X. Z., Wang, J., Huang, C., Chen, Y. Y., Shi, G. Y., Hu, Q. S. & Yi, J. (2008). Emodin enhances cytotoxicity of chemotherapeutic drugs in prostate cancer cells: the mechanisms involve ROS-mediated suppression of multidrug resistance and hypoxia inducible factor-1. *Cancer Biology and Therapy*. 7:468-75.

Ismaili, A., Jalali-Javaran, M., Rasaee, M. J., Rahbarizadeh, F., Forouzandeh-Moghadam, M. & Memari, H. R. (2007). Production and characterization of anti-(mucin MUC1) single-domain antibody in tobacco (Nicotiana tabacum cultivar Xanthi). *Biotechnology and Applied Biochemistry*. 47:11-9.

June, C. H. (2007). Adoptive T cell therapy for cancer in the clinic. *J Clin Invest*. 117:1466-76.

Kazhdan, I., Long, L., Montellano, R., Cavazos, D. A. & Marciniak, R. A. (2006). Targeted gene therapy for breast cancer with truncated Bid. *Cancer Gene Therapy*. 13:141-9.

King, J., Waxman, J. & Stauss, H. (2008). Advances in tumour immunotherapy. *QJM: An International Journal of Medicine*. 101:675-83.

Kocbek, P., Obermajer, N., Cegnar, M., Kos, J. & Kristl, J. (2007). Targeting cancer cells using PLGA nanoparticles surface modified with monoclonal antibody. *J Controlled Release*. 120:18-26.

Kreuter, J. (2001). Nanoparticulate systems for brain delivery of drugs. *Advanced drug delivery reviews*. 47:65-81.

Kruser, T. J. & Wheeler, D. L. (2010). Mechanisms of resistance to HER family targeting antibodies. *Experimental cell research*. 316:1083-100.

Lee, A. L. Z., Wang, Y., Cheng, H. Y., Pervaiz, S. & Yang, Y. Y. (2009). The co-delivery of paclitaxel and Herceptin using cationic micellar nanoparticles. *Biomaterials*. 30:919-927.

Lee, C. C., MacKay, J. A., Frechet, J. M. & Szoka, F. C. (2005). Designing dendrimers for biological applications. *Nature Biotechnology*. 23:1517-26.

Leen, A. M., Rooney, C. M. & Foster, A. E. (2007). Improving T cell therapy for cancer. *Annual Reviews in Immunology*. 25:243-65.

Leserman, L. D., Barbet, J., Kourilsky, F. & Weinstein, J. N. (1980). Targeting to cells of fluorescent liposomes covalently coupled with monoclonal antibody or protein A. *Nature*. 288:602-4.

Leuschner, C., Kumar, C. S., Hansel, W., Soboyejo, W., Zhou, J. & Hormes, J. (2006). LHRH-conjugated magnetic iron oxide nanoparticles for detection of breast cancer metastases. *Breast Cancer Research and Treatment*. 99:163-76.

Li, J. K., Wang, N. & Wu, X. S. (1997). A novel biodegradable system based on gelatin nanoparticles and poly(lactic-co-glycolic acid) microspheres for protein and peptide drug delivery. *Journal of Pharmacocutical Sciences*. 86:891-5.

Li, S., Yang, J., Urban, F. A., MacGregor, J. N., Hughes, D. P., Chang, A. E., McDonagh, K. T. & Li, Q. (2008). Genetically engineered T cells expressing a HER2-specific chimeric receptor mediate antigen-specific tumor regression. *Cancer Gene Therapy*. 15:382-92.

Lu, A. H., Salabas, E. L. & Schuth, F. (2007). Magnetic nanoparticles: synthesis, protection, functionalization, and application. *Angewandte Chemie International Edition*. 46:1222-44.

Maeda, H., Wu, J., Sawa, T., Matsumura, Y. & Hori, K. (2000). Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *Journal of Controlled Release*. 65:271-84.

Mo, Y. & Lim, L. Y. (2005). Paclitaxel-loaded PLGA nanoparticles: potentiation of anticancer activity by surface conjugation with wheat germ agglutinin. *Journal of Controlled Release*. 108:244-62.

Moulder, S. & Hortobagyi, G. N. (2008). Advances in the treatment of breast cancer. *Clinical Pharmacology and Therapeutics*. 83:26-36.

Muniappan, A., Banapour, B., Lebkowski, J. & Talib, S. (2000). Ligand-mediated cytolysis of tumor cells: use of heregulin-zeta chimeras to redirect cytotoxic T lymphocytes. *Cancer gene therapy*. 7:128-34.

Murphy, A., Westwood, J. A., Brown, L. E., Teng, M. W., Moeller, M., Xu, Y., Smyth, M. J., Hwu, P., Darcy, P. K. & Kershaw, M. H. (2007). Antitumor activity of dual-specific T cells and influenza virus. *Cancer Gene Therapy*. 14:499-508.

Muyldermans, S., Atarhouch, T., Saldanha, J., Barbosa, J. & Hamers, R. (1994). Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. *Protein Engineering*. 7:1129 - 35.

Nguyen, V. K., Hamers, R., Wyns, L. & Muyldermans, S. (2000). Camel heavy-chain antibodies: diverse germline V(H)H and specific mechanisms enlarge the antigen-binding repertoire. *The EMBO journal*. 19:921-30.

Nielsen, D. L., Andersson, M. & Kamby, C. (2009). HER2-targeted therapy in breast cancer. Monoclonal antibodies and tyrosine kinase inhibitors. *Cancer Treatment Reviews*. 35:121-36.

Nobs, L., Buchegger, F., Gurny, R. & Allemann, E. (2006). Biodegradable nanoparticles for direct or two-step tumor immunotargeting. *Bioconjugate Chemistry*. 17:139-45.

Oliveira, S., Schiffelers, R. M., van der Veeken, J., van der Meel, R., Vongpromek, R., van Bergen En Henegouwen, P. M., Storm, G. & Roovers, R. C. (2010). Downregulation of EGFR by a novel multivalent nanobody-liposome platform. *Journal of Controlled Release*.

Owens, D. E., 3rd & Peppas, N. A. (2006). Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. *International Journal of Pharmacology*. 307:93-102.

Park, J. R., Digiusto, D. L., Slovak, M., Wright, C., Naranjo, A., Wagner, J., Meechoovet, H. B., Bautista, C., Chang, W. C., Ostberg, J. R. & Jensen, M. C. (2007). Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. *Molecular Therapy*. 15:825-33.

Park, S., Jiang, Z., Mortenson, E. D., Deng, L., Radkevich-Brown, O., Yang, X., Sattar, H., Wang, Y., Brown, N. K., Greene, M., Liu, Y., Tang, J., Wang, S. & Fu, Y. X. (2010). The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. *Cancer Cell*. 18:160-70.

Peng, X. H., Qian, X., Mao, H., Wang, A. Y., Chen, Z. G., Nie, S. & Shin, D. M. (2008). Targeted magnetic iron oxide nanoparticles for tumor imaging and therapy. *International Journal of Nanomedicine*. 3:311-21.

Rahbarizadeh, F., Ahmadvand, D. & Sharifzadeh, Z. (2011). Nanobody; an old concept and new vehicle for immunotargeting. *Immunological Investigations*. 40:299-338.

Rahbarizadeh, F., Rasaee, M. J., Forouzandeh-Moghadam, M. & Allameh, A. A. (2005). High expression and purification of the recombinant camelid anti-MUC1 single domain antibodies in Escherichia coli. *Protein Expression and Purification*. 44:32-8.

Rahbarizadeh, F., Rasaee, M. J., Forouzandeh, M. & Allameh, A. A. (2006). Over expression of anti-MUC1 single-domain antibody fragments in the yeast Pichia pastoris. *Molecular Immunology*. 43:426-35.

Rahbarizadeh, F., Rasaee, M. J., Forouzandeh Moghadam, M., Allameh, A. A. & Sadroddiny, E. (2004a). Production of novel recombinant single-domain antibodies against tandem repeat region of MUC1 mucin. *Hybrid Hybridomics*. 23:151-9.

Rahbarizadeh, F., Rasaee, M. J., Moghadam, M. F., Allameh, A. A., Narang, S. A. & Sadeghizadeh, M. (2004b). Induction of immune response in Camelus bactrianus and Camelus dromedarius against MUC1 - Peptide produced heavy-chain antibodies with efficient combining properties. *Journal of Camel Practice and Research*. 11:1-9.

Rajabi-Memari, H., Jalali-Javaran, M., Rasaee, M. J., Rahbarizadeh, F., Forouzandeh-Moghadam, M. & Esmaili, A. (2006). Expression and characterization of a recombinant single-domain monoclonal antibody against MUC1 mucin in tobacco plants. *Hybridoma*. 25:209-15.

Reichert, J. M. (2008). Monoclonal antibodies as innovative therapeutics. *Current Pharmaceutical Biotechnology*. 9:423-30.

Roovers, R. C., Laeremans, T., Huang, L., De Taeye, S., Verkleij, A. J., Revets, H., de Haard, H. J. & van Bergen en Henegouwen, P. M. (2007). Efficient inhibition of EGFR signaling and of tumour growth by antagonistic anti-EFGR Nanobodies. *Cancer Immunology and Immunotherapy*. 56:303-317.

Rosebrough, S. F. & Hashmi, M. (1996). Galactose-modified streptavidin-GC4 antifibrin monoclonal antibody conjugates: application for two-step thrombus/embolus imaging. *Journal of Pharmacology and Experimental Therapeutic*s. 276:770-5.

Sadelain, M. (2009). T-cell engineering for cancer immunotherapy. *Cancer journal*. 15:451-5.

Sadelain, M., Riviere, I. & Brentjens, R. (2003). Targeting tumours with genetically enhanced T lymphocytes. *Nature Reviews Cancer*. 3:35-45.

Saerens, D., Kinne, J., Bosmans, E., Wernery, U., Muyldermans, S. & Conrath, K. (2004). Single domain antibodies derived from dromedary lymph node and peripheral blood lymphocytes sensing conformational variants of prostate-specific antigen. *Journal of Biological Chemistry*. 279:51965-72.

Scott, C. J., Marouf, W. M., Quinn, D. J., Buick, R. J., Orr, S. J., Donnelly, R. F. & McCarron, P. A. (2008). Immunocolloidal targeting of the endocytotic siglec-7 receptor using peripheral attachment of siglec-7 antibodies to poly(lactide-co-glycolide) nanoparticles. *Pharmacoceutical Research*. 25:135-46.

Skerra, A. & Pluckthun, A. (1988). Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. *Science*. 240:1038-41.

Smith, A. M., Duan, H., Mohs, A. M. & Nie, S. (2008). Bioconjugated quantum dots for in vivo molecular and cellular imaging. *Advances in Drug Delivery Reviews*. 60:1226-40.

Smith, B. J., Popplewell, A., Athwal, D., Chapman, A. P., Heywood, S., West, S. M., Carrington, B., Nesbitt, A., Lawson, A. D. G., Antoniw, P., Eddelston, A. & Suitters, A. (2001). Prolonged in vivo residence times of antibody fragments associated with albumin. *Bioconjugate Chemistry*. 12:750-756.

Spinelli, S., Frenken, L. G., Hermans, P., Verrips, T., Brown, K., Tegoni, M. & Cambillau, C. (2000). Camelid heavy-chain variable domains provide efficient combining sites to haptens. *Biochemistry*. 39:1217-22.

Spinelli, S., Tegoni, M., Frenken, L., van Vliet, C. & Cambillau, C. (2001). Lateral recognition of a dye hapten by a llama VHH domain. *Journal of Molecular Biology*. 311:123-9.

Stancovski, I., Schindler, D. G., Waks, T., Yarden, Y., Sela, M. & Eshhar, Z. (1993). Targeting of T lymphocytes to Neu/HER2-expressing cells using chimeric single chain Fv receptors. *Journal of Immunology*. 151:6577-82.

Stauss, H. J., Cesco-Gaspere, M., Thomas, S., Hart, D. P., Xue, S. A., Holler, A., Wright, G., Perro, M., Little, A. M., Pospori, C., King, J. & Morris, E. C. (2007). Monoclonal T-cell receptors: new reagents for cancer therapy. *Molecular Therapy*. 15:1744-50.

Stayton, P. S., Hoffman, A. S., Murthy, N., Lackey, C., Cheung, C., Tan, P., Klumb, L. A., Chilkoti, A., Wilbur, F. S. & Press, O. W. (2000). Molecular engineering of proteins and polymers for targeting and intracellular delivery of therapeutics. *Journal of Controlled Release*. 65:203-20.

Steinhauser, I., Spankuch, B., Strebhardt, K. & Langer, K. (2006). Trastuzumab-modified nanoparticles: optimisation of preparation and uptake in cancer cells. *Biomaterials*. 27:4975-83.

Subramani, K., Hosseinkhani, H., Khraisat, A., Hosseinkhani, M. & Pathak, Y. (2009). Targeting Nanoparticles as Drug Delivery Systems for Cancer Treatment. *Current Nanoscience*. 5:135-140.

Sundaresan, G., Yazaki, P. J., Shively, J. E., Finn, R. D., Larson, S. M., Raubitschek, A. A., Williams, L. E., Chatziioannou, A. F., Gambhir, S. S. & Wu, A. M. (2003). 124I-labeled engineered anti-CEA minibodies and diabodies allow high-contrast, antigen-specific small-animal PET imaging of xenografts in athymic mice. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine*. 44:1962-9.

Tanha, J., Dubuc, G., Hirama, T., Narang, S. A. & MacKenzie, C. R. (2002). Selection by phage display of llama conventional V(H) fragments with heavy chain antibody V(H)H properties. *Journal of Immunol Methods*. 263:97-109.

Tasis, D., Tagmatarchis, N., Bianco, A. &Prato, M. (2006). Chemistry of carbon nanotubes. *Chemical Reviews*. 106:1105-36.

Taylor-Papadimitriou, J., Burchell, J. M., Plunkett, T., Graham, R., Correa, I., Miles, D. & Smith, M. (2002). MUC1 and the immunobiology of cancer. *Journal of Mammary Gland Biology and Neoplasia*. 7:209-21.

Vu, K. B., Ghahroudi, M. A., Wyns, L. & Muyldermans, S. (1997). Comparison of llama VH sequences from conventional and heavy chain antibodies. *Molecular immunology*. 34:1121-31.

Wang, F., Cai, Y. & Korotkova, O. (2010). Degree of paraxiality of a partially coherent field. *The Journal of the Optical Society of America A*. 27:1120-6.

Ward, R. C. & Kaufman, H. L. (2007). Targeting costimulatory pathways for tumor immunotherapy. *Intenational Reviews of Immunology*. 26:161-96.

Weber, C., Reiss, S. & Langer, K. (2000). Preparation of surface modified protein nanoparticles by introduction of sulfhydryl groups. *Int J Pharm*. 211:67-78.

Weisser, N. E. & Hall, J. C. (2009). Applications of single-chain variable fragment antibodies in therapeutics and diagnostics. *Biotechnol Adv*. 27:502-20.

Whitlow, M., Bell, B. A., Feng, S. L., Filpula, D., Hardman, K. D., Hubert, S. L., Rollence, M. L., Wood, J. F., Schott, M. E. & Milenic, D. E., et al. (1993). An improved linker for single-chain Fv with reduced aggregation and enhanced proteolytic stability. *Protein engineering*. 6:989-95.

Wightman, L., Kircheis, R., Rossler, V., Carotta, S., Ruzicka, R., Kursa, M. & Wagner, E. (2001). Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J Gene Med*. 3:362-72.

Wilkie, S., Picco, G., Foster, J., Davies, D. M., Julien, S., Cooper, L., Arif, S., Mather, S. J., Taylor-Papadimitriou, J., Burchell, J. M. & Maher, J. (2008). Retargeting of human T cells to tumor-associated MUC1: the evolution of a chimeric antigen receptor. *J Immunol*. 180:4901-9.

Wu, T. T., Johnson, G. & Kabat, E. A. (1993). Length distribution of CDRH3 in antibodies. *Proteins*. 16:1-7.

Yan, L., Hsu, K., Beckman, R. A. (2008). Antibody-based therapy for solid tumors. *Cancer J*. 14:178-83.

Yezhelyev, M. V., Gao, X., Xing, Y., Al-Hajj, A., Nie, S. & O'Regan, R. M. (2006). Emerging use of nanoparticles in diagnosis and treatment of breast cancer. *Lancet Oncol*. 7:657-67.

You, J., Li, X., de Cui, F., Du, Y. Z., Yuan, H., Hu & F. Q. (2008). Folate-conjugated polymer micelles for active targeting to cancer cells: preparation, in vitro evaluation of targeting ability and cytotoxicity. *Nanotechnology*. 19:-.

Zaman, M. B., Baral, T. N., Zhang, J. B., Whitfield, D. & Yu, K. (2009). Single-Domain Antibody Functionalized CdSe/ZnS Quantum Dots for Cellular Imaging of Cancer Cells. *Journal of Physical Chemistry C*. 113:496-499.

Zhao, Y., Wang, Q. J., Yang, S., Kochenderfer, J. N., Zheng, Z., Zhong, X., Sadelain, M., Eshhar, Z., Rosenberg, S. A. & Morgan, R. A. (2009). A herceptin-based chimeric antigen receptor with modified signaling domains leads to enhanced survival of transduced T lymphocytes and antitumor activity. *J Immunol*. 183:5563-74.

Zhong, X. S., Matsushita, M., Plotkin, J., Riviere, I. & Sadelain, M. (2010). Chimeric antigen receptors combining 4-1BB and CD28 signaling domains augment PI3kinase/AKT/Bcl-XL activation and CD8+ T cell-mediated tumor eradication. *Mol Ther*. 18:413-20.

Zuckier LS, D. G. (1997). Trials and tribulations: oncological antibody imaging comes to the fore. *Semin. Nucl. Med.* 27:10 - 29.

18

Experimental Therapeutics for the Treatment of Triple Negative Breast Cancer

Julian Dzeyk, Babasaheb Yadav and Rhonda J. Rosengren
University of Otago
New Zealand

1. Introduction

Triple negative breast cancers (TNBCs) are defined as tumors that do not express the estrogen receptor (ER), the progesterone receptor (PR) and the HER2 isoform of the epidermal growth factor receptor (EGFR)(Foulkes et al., 2010). They account for approximately 10-17% of all breast cancers (Reis-Filho & Tutt, 2008). Clinically, they are more prevalent among young African and African-American women (Reis-Filho & Tutt, 2008). TNBCs are poorly differentiated, highly malignant, more aggressive and have a poor outcome (Chen & Russo, 2009). They are also characterized by early recurrence and a high rate of visceral metastasis (Conte & Guarneri, 2009). Moreover, the peak risk of recurrence occurs within three years of diagnosis and the mortality rates are increased for five years after diagnosis (Kwan et al., 2009). The molecular changes associated with TNBCs have been characterized by various immunohistochemistry and gene expression profiling studies. Specifically, they include *p53* mutation, overexpression of Ki67 and EGFR, and dysfunction in the BRCA1 pathway (Rowe et al., 2009). It is estimated that EGFR is expressed in 60% of TNBCs (Arslan et al., 2009). In addition, TNBCs have an over expression of cytokeratins 5, 6, 14, and 17, smooth muscle actin, P-cadherin and c-kit (Irvin & Carey, 2008, Venkitaraman, 2010).

2. Naturally-derived experimental therapies

2.1 Epigallocatechin gallate

TNBCs have limited treatment options due to the lack of a specific therapeutic target, such as hormonal or antibody therapy as well as a diverse biology and treatment sensitivity (Arslan et al., 2009). Therefore, there is an urgent need for novel therapeutic agents for the management of TNBC. Accordingly, naturally-derived compounds are under investigation and provide a source of experimental drugs from which novel therapies could develop. One of these natural agents is epigallocatechin gallate (EGCG, Figure 1). It is the most abundant and active catechin obtained from green tea (*Camellia sinensis*) (Graham, 1992), as it has shown anti-tumorigenic activity in a variety of cancer models including TNBC cell lines such as MDA-MB-231 and MDA-MB-468 cells. Specifically, EGCG inhibited the proliferation of MDA-MB-468 and MDA-MB-231 cells with inhibitory concentrations (IC_{50}) of 30 and 80 μg/ml, respectively (Kavanagh et al., 2001, Masuda et al., 2002). Mechanisms for the cytotoxic effect of EGCG include induction of apoptosis as well as the modulation of various

cell signaling proteins involved in cell survival, proliferation and death. Specifically, EGCG (20-60 μg/ml) caused 20-54% of MDA-MB-468 cells to become apoptotic after 72 h (Roy et al., 2005), while 25 μM caused 12% of MDA-MB-231 cells to undergo apoptosis after 36 h (Stuart et al., 2007). Even though EGCG causes cells to undergo cell cycle arrest in the G1 phase, this is not a mechanistic driver of apoptosis in TNBC cells, as the significant increase in apoptosis precedes the increase in G1 arrest (Stuart et al, 2007). Therefore, other mechanisms drive apoptosis.

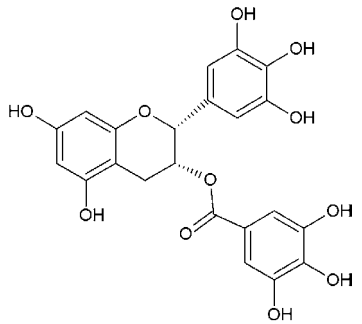


Fig. 1. Chemical structure of (-)-epigallocatechin gallate.

The cell surface epidermal growth factor receptor is over-expressed in 45-70% of TNBC cells and is associated with poor prognosis of patients (Bosch et al., 2010, Koenders et al., 1991). EGFR activation via growth fator binding causes dimerization and subsequent auto-phosphorylation of specific tyrosine residues at the intracellular C-terminal end of the receptor. Through conserved protein binding domains (SH2, SH3) that interact with the phospho-tyrosine residues of EGFR, intracellular signaling cascades such as the mitogen activated kinase (MAPK) pathway, C-Jun N-terminal kinase (JNK) pathway and the phosphoinositol-3-kinase/Akt (PI3K/Akt) pathway can be activated (Casalini et al., 2004). Of particular importance for breast cancer is the PI3K/Akt pathway which is associated with phosphatase and tensin homolog (PTEN) inactivation, commonly found to be dysregulated in cancers (DeGraffenried et al., 2004). The protein product (phosphatase) of the tumor suppressor gene PTEN is involved in cell cycle regulation, preventing cells from growing and dividing excessively (Chu & Tarnawski, 2004). In a gene expression analysis of 106 breast cancer patients it was shown that there was a 34% decrease in PTEN and a corresponding 29% increase in EGFR expression, but only in patients with triple-negative breast tumors (Andre et al., 2009). A further study with 11 TNBC patients provided evidence that low PTEN expression is associated with increased activation of Akt. Although it should be noted that the Spearman correlation between Akt and PTEN expression was 0.593, and may therefore suggest that Akt could be activated and affected by multiple mechanisms (Berrada et al., 2010). In MDA-MB-468 cells, EGCG treatment (30 μg/ml) for 72 h inhibited the phosphorylation and therefore the activation of EGFR, Akt and STAT3 in the presence and absence of TGF-α (Masuda *et al.* 2007).

Using scintillation proximity assays, EGCG inhibited all four (PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ) class I PI3K isoforms (Van Aller et al., 2011). In particular EGCG was potent towards the PI3Kα isoform with a K_i value of 380 nM. Additionally, mTOR was inhibited with a K_i of 320 nM. Further analysis showed inhibition of both PI3K and mTOR occurred via competition with ATP binding to these proteins (Van Aller et al., 2011). This was confirmed

by molecular modeling studies where the structure of PI3Kγ complexed with myricetin (PDB:1E90) was used to dock with EGCG. Myricetin, a structurally related flavonoid, is an ATP competitive inhibitor of PI3K with an IC_{50} of 1.8 μM (Walker et al., 2000). Except for the chromandiol moiety of EGCG which was flipped by 180° compared to the chromone moiety of myricetin, the binding mode of EGCG is similar to that of myricetin, further supporting the notion that EGCG is a PI3K inhibitor by competing with ATP binding (Van Aller et al., 2011). Since the mTOR (C2 isoform) is one of the 3-phosphoinositide-dependent kinases responsible for the activation/phosphorylation of Akt at Ser473, the effects of EGCG on phospho-Akt was examined in MDA-MB-231 cells. EGCG inhibited Akt phosphorylation in a concentration-dependent manner with IC_{50} values below 1 μM, which was consistent with a direct inhibition of mTOR and PI3K by EGCG (Van Aller et al., 2011).

The anti-apoptotic Bcl-2 protein family is important for mitochondrial and endoplasmic reticulum membrane permeability as well as transduction and integration of apoptotic signals upon homo- and heterodimerization (Adams & Cory, 1998). Another protein from the Bcl-2 family, Bax, is classified as pro-apoptotic protein, which directs the release of cytochrome c. The expression ratio of Bax/Bcl-2 is a determining factor for apoptosis in biochemical studies (Adams & Cory, 1998). In MDA-MB-468 cells, EGCG (20-60 μg/ml) for 48 h caused a dose-dependent 1-to-3-fold increase in the expression ratio of Bax and Bcl-2 (Roy et al., 2005). This indicated that the apoptosis inducing effects of EGCG are transmitted via reductions in anti-apoptotic signals by reducing Bcl-2 protein and increasing pro-apoptotic signals mediated by Bax (Roy et al., 2005). Western blotting of other important pro-apoptotic signaling proteins provided further verification of the underlying mechanism by which EGCG induces apoptosis. It was shown that treatment of MDA-MB-468 cells with EGCG (20 μg/ml) for 48 h elevated the expression of cytochrome c (2-fold), Apaf-1 (7-fold), caspase 3 as well as poly(ADP-ribose) polymerase (PARP) (Roy et al., 2005). Similar observations were also made in MDA-MB-231 cells, which were treated with 50 or 80 μg/ml for 24 h. The protein expression ratio of Bax and Bcl-2, as visualized by Western blotting, showed a dose-dependent relationship. The full length PARP protein (116 kDa) was also degraded into the cleaved, inactive 85 kDa form by the proteolytic caspase-3, which is an integral part of mitochondrial-regulated apoptosis (Thangapazham et al., 2007a).

Another potential target for cancer chemoprevention is the ribonucleoprotein telomerase (synthesizes the cap-telomere-end, 5'-TTAGGG-3', of eukaryotic chromosomes), as it is expressed in ~85% of human cancers (75% of breast carcinoma *in situ* and 88% in ductal and lobulal breast carcinomas (Shay & Bacchetti, 1997)). In contrast, after embryonic development, telomerase is barely detectable in normal human somatic cells (Cunningham et al., 2006). The action of EGCG on telomerase activity has also been assessed in MDA-MB-231 cells and MCF10A non-cancerous breast cells. Using a conventional gel-based PCR method, the relative mRNA levels of human telomerase reverse transcriptase (*hTERT*; the key catalytic subunit of telomerase (Cunningham et al., 2006)) were measured in both cell lines after treatment with 40 μM of EGCG for 3, 6, 9 or 12 days. EGCG was found to time-dependently inhibit *hTERT* expression (~40 and ~50% decrease after 9 and 12 days, respectively) in MDA-MB-231 but not MCF10A cells (Meeran et al., 2011). Since *hTERT* is an epigenetically regulated gene (Cunningham et al., 2006), the activity of epigenetic-modulating enzymes such as DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), and histone deacetylases (HDACs) was assessed in order to determine the mechanism under which EGCG influences *hTERT* mRNA levels. Specifically, in MDA-MB-231 cells EGCG (40 μM) treatment for 9 days decreased both DNMT and HAT activity by

~40%, while HDAC activity was unaffected (Meeran et al., 2011). The authors suggested that the observed effects might be due to direct binding of EGCG to the active site of DNMTs and inhibition of HATs. Additionally, treatment with EGCG (40 μM) reduced methylation to ~60%. This indicated that the inhibition of DNMT expression following EGCG treatment could be a contributing factor in the facilitation of *hTERT* promoter demethylation, which would lead to transcriptional repression of *hTERT* expression (Meeran et al., 2011).

An intricate part of tumor growth, invasion and metastasis is the process of new blood vessel formation, referred to as angiogenesis. A key mediator required for this process is vascular endothelial growth factor (VEGF), which is expressed at 34% higher levels in TNBC patients compared to hormone-sensitive tumors (Andre et al., 2009). Angiogenesis is also stimulated by other pro-angiogenic factors such as basic fibroblast growth factor and hypoxia-inducible factor (Schneider & Miller, 2005). Therefore, VEGF targeting anti-angiogenic agents such as bevacizumab have been beneficial in the treatment of TNBC patients (Carey et al., 2010). Matrix metalloproteinases (MMPs) also play an important role in the progression of invasive and metastatic breast cancer (Schneider & Miller, 2005). Targeting various markers such as MMPs that inhibit the rapid growth and metastasis has emerged as one of the strategies for treatment of highly proliferative TNBCs (Greenberg & Rugo, 2010). The inhibition of angiogenesis is yet another critical component to the plethora of effects elicited by EGCG. Specifically, EGCG (40 μg/ml) significantly decreased VEGF peptide levels by 85% and VEGF mRNA levels by 75% compared to control in MDA-MB-231 cells (Sartippour et al., 2002). Effects of the drug were also assessed on VEGF promoter activity and results showed that EGCG (40 μg/ml) reduced promoter activity by ~30% (Sartippour et al., 2002). EGCG also decreased VEGF production in MDA-MB-468 cells by 55% as well as NFκB activity by 4-fold compared to control (Masuda et al., 2007). The effect of protein kinase C has also been examined, as this protein has been shown to be a modulator of VEGF expression (Hossain et al., 2000). Using Western blotting, it was shown that protein kinase C levels decreased by ~70% upon EGCG treatment compared to control (Sartippour et al., 2002). In a Boyden chamber assay, to assess the anti-metastatic potential of EGCG using MDA-MB-231, cells it was shown that treatment with 80 μg/ml for 24 h caused a 28% reduction in cell invasiveness. Furthermore, EGCG decreased the expression of matrix metalloproteinase-9 (MMP-9) 5-fold using microarray experiments. This was confirmed using RT-PCR, which also showed a down-regulation of MMP-9 at the transcriptional level (Thangapazham et al., 2007a). Thus, EGCG suppresses angiogenesis in TNBC via a variety of mechanisms.

An important prognostic markers for breast cancer is Met, a transmembrane receptor for the hepatocyte growth factor (HGF). Importantly, it also has been assessed as one of the targets of EGCG. High levels of Met are correlated with a lower patient survival rate, which led researchers to postulate that EGCG may affect HGF signaling via Met. Using Western blotting in MDA-MB-231 cells, it was shown that 1 h pre-treatment with EGCG (0.6-30 μM) followed by 15 min of exposure to HGF (30 ng/ml), significantly blocked HGF-induced Met, AKT and ERK phosphorylation (Bigelow & Cardelli, 2006). A Boyden chamber assay using MDA-MB-231 cells showed that the observed ~7-fold increase in invading cells by HGF was decreased to ~2-fold by EGCG (5 μM) (Bigelow & Cardelli, 2006). Therefore the modulation of Met by EGCG also contributes to its anti-cancer action in TNBC cells.

The anticancer activity of EGCG is also evident *in vivo*, as isolated EGCG and/or a mixture of green tea polyphenols have suppressed TNBC growth *in vivo*. Specifically, in a MDA-MB-231 xenograft model tumor volume after 10 weeks of treatment was reduced by 45% in EGCG treated mice and by 61% in green tea polyphenol (GTP) treated mice compared to

control (Thangapazham et al., 2007b). GTP and EGCG treatment increased the number of apoptotic cells by 3.5 and 2.6-fold, respectively. This study showed that GTP was slightly more effective than pure EGCG in reducing the tumor incidence, volume and number of apoptotic cells. However, this may be related to the dose given (~3 mg/mouse GTP vs. 1 mg/mouse EGCG) (Thangapazham et al., 2007b). These findings are supported by earlier *in vivo* studies with EGCG or green tea extracts (GTE) (Kavanagh et al. 2001, Sartippour et al., 2001). Specifically, 0.62, 1.25, 2.5 g/l of GTE dose-dependently prevented tumor growth, with a 90% reduction in tumor volume in the 2.5g/l treatment group compared to control (Sartippour et al., 2001). Using immunohistochemistry it was also shown that GTE decreased the overall microvessel density by ~50% in the treated animals compared to control (Sartippour et al., 2001). Importantly, these studies and many others demonstrated that the *in vitro* anticancer effects of EGCG translate into tumor suppression *in vivo*.

2.2 Curcumin

Curcumin (Figure 2), obtained from the roots and rhizomes of the perennial plant *Curcuma longa*, is cytotoxic towards both ER positive and TNBC cells (Verma et al., 1997). For example, curcumin (10 μM) inhibited the proliferation of MDA-MB-468 and MDA-MB-231 cells, with IC_{50} values of 1 μM and 16.25 μg/ml, respectively (Squires et al., 2003). Mechanisms for the cytotoxic effect of curcumin include G2/M cell cycle arrest, induction of apoptosis as well as the modulation of various cell signaling proteins involved in cell survival, proliferation and death. Specifically, cell cycle studies demonstrated that curcumin (20 μM) treatment for 24 h increased the proportion of MDA-MB-231 cells in the G2/M phase by 164% (Chiu & Su, 2009, Fang et al., 2011). Furthermore, curcumin (20 μM) increased the proportion of MDA-MB-468 cells in the G2/M phase by 143% (Squires et al., 2003). Thus, in contrast to EGCG, G2/M phase arrest is one of the drivers of curcumin-mediated apoptosis.

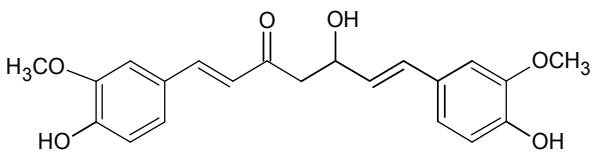


Fig. 2. Chemical structure of curcumin.

The cell cycle is promoted by activation of cyclin dependent kinases, which are positively regulated by cyclins and negatively by cyclin dependent kinase inhibitors (CDKIs) (Malumbres & Barbacid, 2009). Cyclin D1 regulates cell cycle progression through G1-phase of the cell cycle by activating CDK4 and CDK6. Cyclin D1 is a proto-oncogene which is overexpressed in ER negative breast cancers and is a predictor of poor prognosis (Umekita et al., 2002), while cyclin E along with CDK2 regulates the entry of cells from late G1 to S phase. Cyclin E overexpression is associated with poor prognosis and high proliferation in ER negative breast cancer patients (Potemski et al., 2006). CDKIs, p21 and p27 belong to Cip/Kip family of proteins and their decreased expression has been correlated with poor prognosis in breast cancer patients (Catzavelos et al., 1997, Pellikainen et al., 2003). It is reported that altered expression of proteins regulating the cell cycle makes TNBC more sensitive to cytotoxic therapy (Rouzier et al., 2005). Studies have demonstrated that curcumin modulates the expression of cyclins, CDKs and CDKIs in breast cancer cells.

Specifically, curcumin decreased the expression of cyclin D1 and increased levels of p21 expression in MDA-MB-231 cells (Liu et al., 2009) and this was followed by the induction of apoptosis (Chiu & Su, 2009).

Curcumin induces apoptosis in most, if not all, breast cancer cell lines and this occurs mainly via a mitochondrial-dependent pathway (Karunagaran et al., 2005). In most cells, curcumin induces a loss of mitochondrial membrane potential, opening of the transition pore, release of cytochrome c, caspase-9 activation, caspase-3 activation and subsequent cleavage of PARP all of which lead to DNA fragmentation and apoptosis (Aggarwal et al., 2003, Ravindran et al., 2009). Down regulation of anti-apoptotic proteins (Bcl-2 and Bcl-XL) and upregulation of pro-apoptotic proteins (Bad and Bax) also leads to curcumin induced apoptosis in many cancer cells including breast cancer (Ravindran et al., 2009). Curcumin-induced apoptosis via inhibition of reactive oxygen species (ROS) has also been shown. ROS regulate intracellular signaling pathways in various cancer cells including breast cancer (Waris & Ahsan, 2006). Higher production of ROS and glutathione depletion cause oxidative stress, loss of cell function and ultimately leads to apoptosis. Curcumin causes rapid depletion of glutathione (GSH) which results in an increase in the production of ROS and induction of apoptosis (Shehzad et al., 2010). Additionally, curcumin induced apoptosis in MDA-MB cells through the generation of ROS originating from glutathione depletion by buthioninesulfoximine thereby further sensitizing the tumor cells to curcumin (Syng-Ai et al., 2004).

The induction of apoptosis and modulation of the cell cycle result from the effect of curcumin on various intracellular pathways. Curcumin inhibits epidermal growth factor stimulated phosphorylation of EGFR and further inhibits downstream ERK1/2, JNK, and Akt activity in MDA-MB-468 cells (Squires et al., 2003). NFκB is a transcription factor that regulates various genes involved in both proliferation and apoptosis and aberrant NFκB expression is implicated in different types of breast cancers (Wu & Kral, 2005). Furthermore, it is constitutively activated in ER negative breast cancers and its inhibition suppresses cell growth and induces cell death (Biswas et al., 2001, Schlotter et al., 2008). For example, 5 μg/ml of curcumin reduced the expression of nuclear NFκB in MDA-MB-231 cells (Liu et al., 2009). Also, curcumin abolished paclitaxel induced NFκB activation in MDA-MB-435 breast cancer cells (Aggarwal et al., 2005). Modulation of the Wnt/β-catenin pathway by curcumin has been shown to play a role in the inhibition of cell proliferation and induction of apoptosis in MDA-MB-231 breast cancer cells (Prasad et al., 2009). The Wnt/β-catenin pathway is an important pathway as it is associated with worse overall survival in TNBC (Khramtsov et al., 2010).

Curcumin is an inhibitor of angiogenesis, as 50 μM suppressed the transcription levels of VEGF and b-FGF in MDA-MB-231 cells (Shao et al., 2002). Additionally, curcumin downregulated post-transcriptional levels of both HIF-1□α and HIF-1□β in MDA-MB-231 cells (Thomas et al., 2008). Curcumin also inhibited the invasive potential of MDA-MB-231 cells via down regulation of MMP-2, MMP-3 and MMP-9 and up regulation of tissue inhibitor metalloproteinase (TIMP-1, 2) which regulates tumor cell invasion (Boonrao et al., 2010, Shao et al., 2002). In another study, the anti-invasive properties of curcumin were mediated through inhibition of RON tyrosine kinase receptor (Narasimhan & Ammanamanchi, 2008). Curcumin also inhibits integrin α (6) β (4), a laminin adhesion receptor in MDA-MB-231 cells and thus inhibits cell motility and invasion (Kim et al., 2008). Recently, it was shown that curcumin induces upregulation of maspin, a serine protease inhibitor and thus inhibits invasion of MDA-MB-231 cells (Prasad et al., 2009). Furthermore, curcumin inhibited migration of MDA-MB-231 cells through the down regulation of protein

expression of the transcription factor, NFκB (Chiu & Su, 2009). Lastly, curcumin also reduced the expression of the two prometastatic cytokines, CXCL1 and -2, which in turn reduces expression of the chemotactic receptor CXCR4 along with other metastasis-promoting genes (Bachmeier et al., 2008).

The anti-metastatic effect of curcumin has also been studied in various *in vivo* models. Dietary administration of curcumin (2%) significantly decreased the incidence of breast cancer metastasis to the lung in an MDA-MB-231 xenograft model. They also observed that curcumin significantly suppressed the expression of NFκB, COX2 and MMP-9 (Aggarwal et al., 2005). In another study, curcumin decreased lung metastasis in a mouse xenograft model. MDA-MB-231 cells were inoculated into nude mice by intercardiac injection and the treatment group was fed with 1% dietary curcumin. After 5 weeks of treatment, 21% of animals from the curcumin treatment group were metastasis free compared with the control group who all had metastasis (Bachmeier et al., 2007). Many other studies have used chemical carcinogens such as dimethylbenzanthracene or diethylstilbestrol to show that curcumin inhibits mammary carcinogenesis. However, none of these models are representative of TNBC. Nevertheless, curcumin elicits a plethora of effects in TNBC and importantly, as with EGCG, anticancer activity is retained *in vivo*. Both compounds also modulated many different cell signaling pathways that cumulate in a strong apoptotic response (Figure 3).

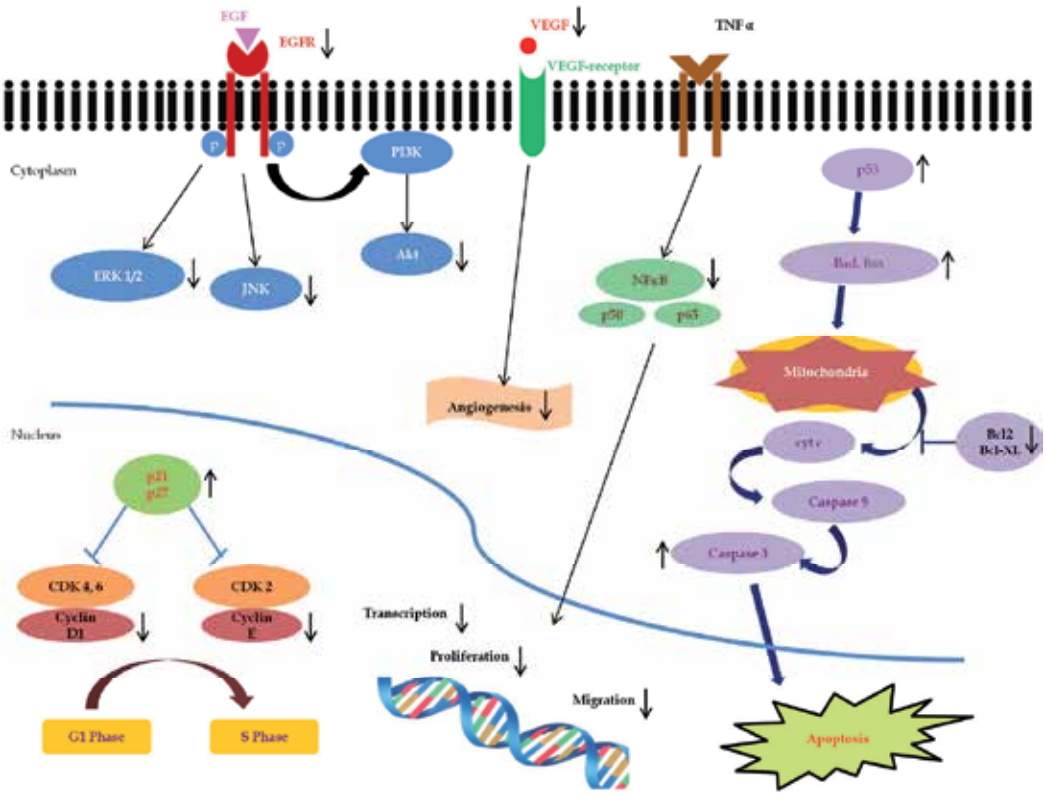


Fig. 3. Schematic diagram of intracellular cell signaling cascades activated following auto-phosphorylation of the EGFR. Arrows indicate an increase or decrease in the protein expression/activity following treatment with either EGCG or curcumin in models of TNBC.

3. Improving naturally derived compounds

3.1 Combination studies

Initial studies with EGCG or curcumin demonstrated that these natural compounds had promise as potential treatments for TNBC. However, both compounds have also been used in various combination studies in order to improve their efficacy. This was achieved in MDA-MB-231 cells where EGCG (20 μM) in combination with paclitaxel (1 μM) reduced cell viability by ~80% after 24 h compared to control (Luo et al., 2010). Treatment of just paclitaxel reduced cell viability by ~40%, whereas EGCG on its own had no effect compared to control. These results suggested that EGCG may act synergistically with paclitaxel to enhance its anti-carcinogenic effects (Luo et al., 2010). Other studies have also shown synergistic effects by using EGCG in combination with other known breast cancer treatments. One such study showed that the selective estrogen receptor modulator (SERM), tamoxifen in combination with EGCG elicited synergistic cytotoxicity as well as earlier and enhanced apoptosis in MDA-MB-231 cells (Chisholm et al., 2004, Stuart et al., 2007). This effect was further analyzed in an *in vivo* xenograft model. Specifically, mice were treated with either tamoxifen (75 μg/kg), EGCG (25 mg/kg) or a combination of the two for 10 weeks. Results showed that the tumor volume in the EGCG + tamoxifen treatment group was 75% smaller compared to vehicle or tamoxifen (Scandlyn et al., 2008). Combination treated mice also had significantly smaller tumors compared to EGCG treatment. Tumor suppression was not through the up-regulation of the ER in MDA-MB-231 cells (Scandlyn et al., 2008). Instead EGFR and its activated form were reduced by ~78% compared to control. Similar reductions in mTOR and CYP1B1 expression were also found in tumors from EGCG + tamoxifen treated mice, indicating that the reductions of EGFR, mTOR as well as CYP1B1 expression are likely to be important mechanistic contributors to the suppression of tumor growth (Scandlyn et al., 2008).

Highly interesting was the finding that EGCG at the specific concentration of 10 μM in combination with 10 ng/ml of trichostatin A (TSA; a histone deacetylase inhibitor) increased estrogen receptor-α expression 6-fold in MDA-MB-231 cells (Li et al., 2010). Using CHiP assays it was confirmed that EGCG and TSA increased histone acetyl transferase activity 5-fold compared control. Furthermore, the combination of EGCG, TSA and tamoxifen in MDA-MB-231 cells, caused a significant ~60% reduction in cell viability, which was more effective than combining tamoxifen with either EGCG or TSA (Li et al., 2010). These results showed that epigenetic modifications induced by EGCG + TSA may be useful in sensitizing ER-negative tumors to anti-hormonal therapy. However, it should be noted that the EGCG concentration required for the observed 6-fold increase in ER-α expression was exactly 10 μM, any concentration above or below did not have any effect on ER-α expression (Li et al., 2010). Additionally, a similar effect *in vivo* has not been reported.

Combining EGCG with another SERM, raloxifene, has also shown synergistic cytotoxicity. In MDA-MB-231 cells the combination of EGCG (25 μM) and raloxifene (5 μM) decreased the cell number by ~28% compared to control. Individually, both compounds at the same concentrations were not cytotoxic towards the cancer cells (Stuart & Rosengren, 2008). Additionally, the number of apoptotic cells after 48 h was 34% higher compared to control following combination treatment. The mechanism of action was further assessed by protein expression analysis and the results showed that the protein expression of EGFR, Akt, mTOR and S-6-kinase were decreased by 22, 31, 41 and 46%, respectively (Stuart et al., 2010) after 18 h of treatment. Thus these two compounds elicit a strong response in pathways downstream of the EGFR.

Interestingly, combination of the two naturally derived compounds curcumin and EGCG was effective in both *in vitro* and *in vivo* models of TNBC. In MDA-MB-231 cells these two compounds (EGCG at 25 μM and curcumin at 3 μM) increased apoptotic cells and G2 arrest 2.6-fold compared to curcumin alone (Somers-Edgar et al., 2008). This effect was only observed in TNBC cells and not in MCF-7 cells. Importantly, this *in vitro* effect translated to tumor suppression *in vivo*. Specifically, curcumin (200 mg/kg) and EGCG (25 mg/kg) significantly suppressed MDA-MB-231 xenograft tumor volume by 49% compared to vehicle control after 10 weeks of treatment (Somers-Edgar et al., 2008). This was in part driven by a 78% decrease in VEGFR-1 protein expression in tumors. Tumor suppression has also been shown in other combination studies with curcumin. Specifically, when curcumin was combined with paclitaxel in an MDA-MB-231 xenograft model, there was a significant reduction in tumor growth following combination treatment compared to either agent alone. Mechanistic studies showed that the combination decreased the expression of MMP-9 and increased apoptosis in the tumors of treated mice. Interestingly, the dose of paclitaxel was much lower (7 mg/kg) than previously reported as a single treatment (Kang et al., 2009). Success has also been shown using *in vitro* combination studies. Specifically, when curcumin was combined with piperine the two drugs worked synergistically to inhibit breast cancer stem cell self-renewal without affecting normal cells. The authors showed that this effect was mediated by the inhibition of mammosphere formation via the Wnt signaling pathway (Kakarala et al., 2010). Synergistic growth inhibition and the induction of apoptosis in MDA-MB-231 cells also occurred following the combination of curcumin and xanthorrhizol (Cheah et al., 2009). These studies all illustrate that the naturally derived compounds EGCG and curcumin can also be used in combination in order to increase the potency of these compounds and/or potentially sensitize cancer cells to the effects of other chemotherapeutic agents.

3.2 Novel drug delivery of natural compounds

Various novel drug delivery systems such as nanoparticles, liposomes, micells, adjuvants and phospholipid complexes have been developed in order to specifically target cancer cells in order to improve efficacy and bioavailability, while reducing toxicity. (Anand et al., 2008). Nanoparticles can improve the biodistribution of drugs, as they are able to act as carriers of anti-cancer drugs by selectively using the unique pathophysiology of tumors, such as their enhanced vascular permeability and extensive angiogenesis (Figure 4) (Cho et al., 2008). The term nanochemoprevention was recently introduced, combining nanotechnology with chemoprevention using EGCG as an encapsulated agent in polylactic acid–polyethylene glycol nanoparticles. The safety and improved efficacy of such gelantin nanoparticles has resulted in increased accumulation within the tumor and prolonged *in vivo* circulation (Vlerken et al., 2007). This is very important, as a downfall of both EGCG and curcumin is their low bioavailability (Siddiqui et al., 2009). To ensure that EGCG contained in such nanoparticles was functionally similar to free EGCG, it was essential to first test these nanoparticles *in vitro*. To accomplish this, MDA-MB-231 cells were treated with empty gelatin nanoparticles (5 μM), nanoparticles with EGCG (1 and 5 μM), or free EGCG (5 μM) for 30 min or 5 h. This was followed by the addition of HGF (30 ng/ml) and lysate preparation for Western blotting. The analysis showed that 5 μM free EGCG was able to block HGF-induced Met, AKT and ERK activation. On the other hand both the nanoparticles with and without EGCG did not inhibit HGF-induced signalling after pre-incubation of 30

min. However, at the 5 h time-point the gelatin coated nanoparticle containing EGCG blocked HGF-induced signalling, therefore demonstrating that EGCG activity was retained but released slowly (Shutava et al., 2009).

Recently, curcumin nanoparticles have shown enhanced bioavailability and greater cytotoxicity against breast cancer cells. For example, silk fibroin-derived curcumin nanoparticles (< 100 nm) exhibited higher uptake, intracellular residence time and efficacy against HER2 positive MDA-MB-453 breast cancer cells (Gupta et al., 2009). In another study, curcumin-PLGA nanoparticle formulation elicited an enhanced inhibitory effect on the growth of MDA-MB-231 cells compared with curcumin alone (Yallapu et al., 2010). Furthermore, curcumin-PLGA-PEG nanoparticles showed a concentration-dependent anti-proliferative effect toward MDA-MB-231 cells. It was observed that curcumin nanoparticle formulation had a higher bioavailability and longer half-life in rats compared to curcumin. Specifically, after intravenous administration of curcumin or curcumin-nanoparticle (2.5 mg/kg), the serum levels of curcumin were almost twice as high in the curcumin-nanoparticle treated rats (Anand et al., 2010). Curcumin (Cur-OEG) nanoparticles have also been studied for their anticancer effect in both *in vitro* and *in vivo* models of breast cancer. Curc-OEG nanoparticles showed broad *in vitro* antitumor activity toward several human cancer cells with an IC_{50} value of 1.4 μg/ml in MDA-MB-468 cells. These particles showed safety and efficacy *in vivo*, as a single 25 mg/kg intravenous injection of Curc-OEG nanoparticles was non-toxic to the mice and exhibited improved bioavailability and significant tumor suppression after 48 h in an MDA-MB-468 xenograft model.

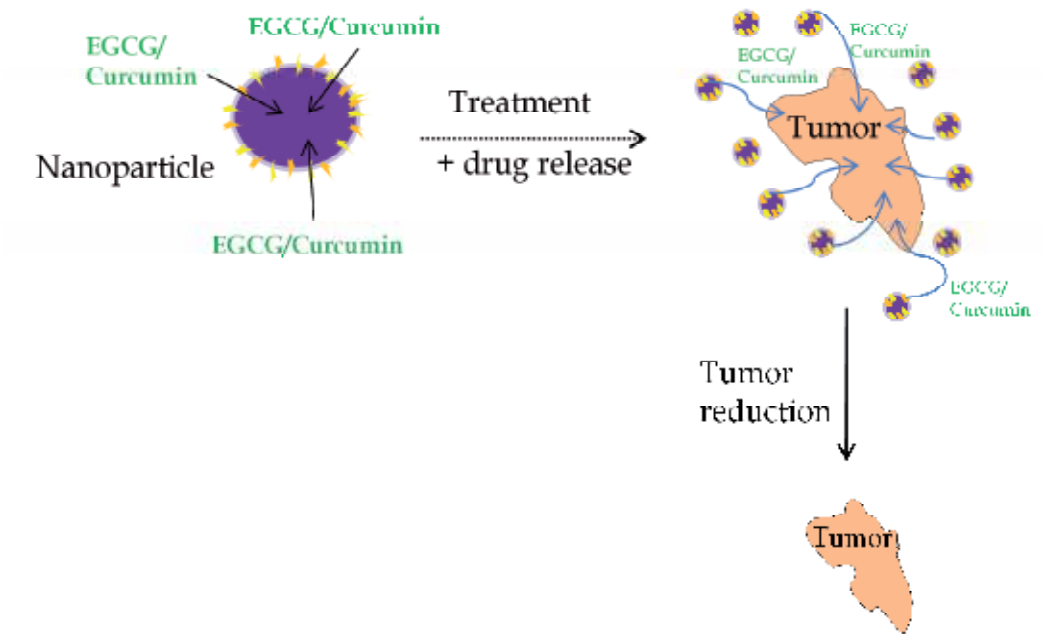


Fig. 4. Schematic of the principles of nanoparticle drug delivery. Nanoparticles, commonly consist of various different polymers and polyelectrolytes, encapsulate the drug of interest and following administration show enhanced bioavailability and accumulation within the tumor. Subsequently the drug is released (blue errors) from the nanoparticle and taken up by the tumor.

Injectable sustained release poly(D,L-lacctide-co-glycolide (PLGA)-microparticles of curcumin for breast cancer chemoprevention have also been formulated. These PLGA-microparticles exhibited enhanced bioavailability compared to curcumin in mice. Specifically, a single dose of subcutaneously injected PLGA-microparticles sustained curcumin levels in the blood for a month whereas single or multiple i.p. injections of curcumin resulted in a shorter half-life (Shahani et al., 2010). In addition, the curcumin concentration was 10–30-fold higher in the lungs and brain than in the blood. Furthermore, curcumin inhibited the growth of tumors in an MDA-MB-231 mouse xenograft model by 49% compared to the mice treated with blank PLGA-microparticles (Shahani et al., 2010). Mechanisms for this effect were attributed to the down regulation of the markers of angiogenesis, metastasis and proliferation. Specifically, the curcumin PLGA-microparticles treated group showed smaller and less well developed CD31 positive microvessels compared to curcumin alone. Furthermore, treatment with curcumin PLGA-microparticles decreased the relative VEGF expression in tumors by 78%, compared with control (Shahani et al., 2010). Additionally, the relative expression of MMP-9 in tumors from the curcumin PLGA-microparticle treated group was decreased 57% compared to control, while Ki-67 and cyclin D1 were decreased by 45% and 52%, respectively. There was also a 2.5-fold increase in the number of apoptotic cells compared to blank PLGA-microparticle treatment (Shahani et al., 2010). Since repeated systemic dosing of curcumin had no effect on tumor cell proliferation, apoptosis, or the relative cyclin D1 expression, the study concluded that sustained release microparticles of curcumin are more effective than repeated systemic injections of curcumin for breast cancer chemoprevention. Thus, significant improvement in the selectivity and potency of both EGCG and curcumin can be achieved through the use of nanomedicine.

3.3 Prodrugs, analogues and synthetic derivatives

Even though preclinical research shows promising results *in vitro* and *in vivo* with natural polyphenolic compounds such as EGCG and curcumin, clinical trials have shown limited success commonly due to inefficient delivery and bioavailability of these agents (Siddiqui et al., 2009). Thus, bioavailability is one of the major downfalls of these compounds. Additional strategies for improving these compounds include the synthesis of prodrugs, analogues and synthetic derivatives. All of these techniques aim to produce a compound with greater stability, bioavailability and ultimately efficacy.

Various studies have shown that biotransformation reactions, in particular methylation but potentially also glucuronidation and sulfate conjugation, modify the hydroxyl groups of EGCG, resulting in reduced biological activities (Landis Piwowar et al., 2007, Okushio et al., 1999). To prevent this, one group has synthesized novel fluoro-substituted EGCG pro-drug analogues by eliminating the reactive hydroxyl groups and replacing them with either peracetate groups (Pro-EGCG) or one or two fluorine(s) at the meta-position (Pro-F-EGCG2) or the meta- and para-positions on the phenyl ring (Pro-F-EGCG4) (Figure 5)(Yang et al., 2010). These analogues (50 mg/kg) were given daily via subcutaneous injections for 31 days to mice bearing MDA-MB-231 xenografts. Tumor growth was suppressed by ~63% by Pro-EGCG compared to control, whereas Pro-F- EGCG2 and Pro-F-EGCG4 were slightly more effective as tumor growth was reduced by ~67% and ~70%, respectively, compared to control (Yang et al., 2010). As an indicator of apoptosis, PARP cleavage (65 kDa) was found to a greater extent in tumors from mice treated with the fluorosubstituted analogues. Furthermore, the TUNEL assay showed apoptotic cells in the tumors of the animals treated with Pro-F-EGCG2 or Pro-F-EGCG4 but not in untreated control group. Cells with apoptotic

nuclei were also shown in the treated animals. The proteasomal chymotrypsin-like activity was reduced by 33% and 42% in animals treated with Pro-F-EGCG2 or Pro-F-EGCG4, respectively, compared to control. Additionally, it was shown using Western blotting, that the proteasome substrates p27 and Bax were increased in EGCG-analogue treated animals indicating that the proteasome activation may be a cellular target of the EGCG analogues (Yang et al., 2010). Therefore, prodrugs of EGCG are emerging as an experimental therapy with potential for clinical translation.

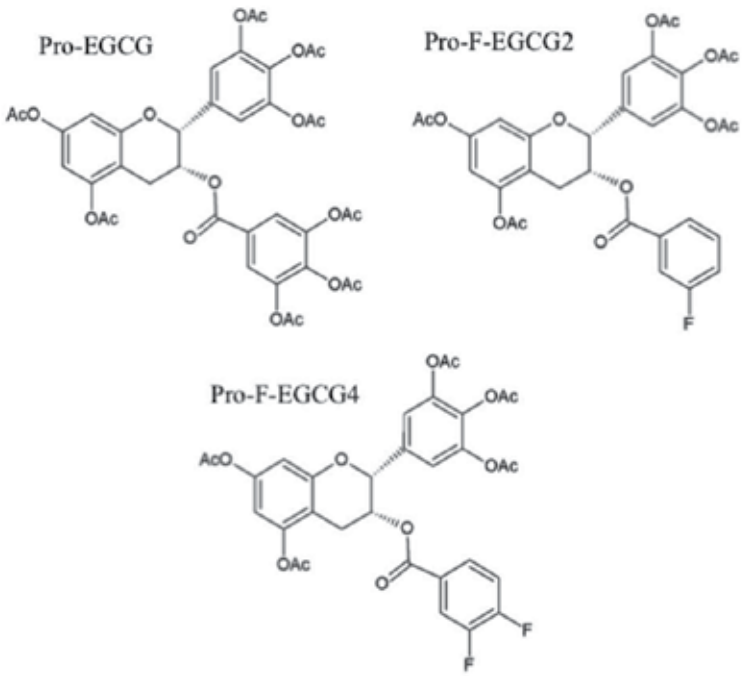


Fig. 5. Chemical structures of EGCG analogues (Yang et al., 2010).

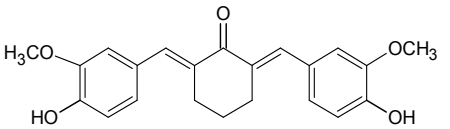
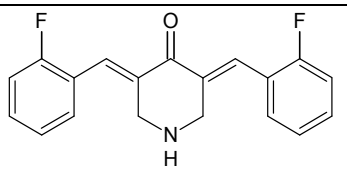
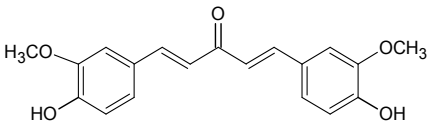
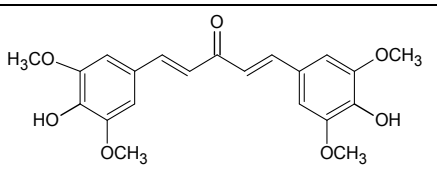
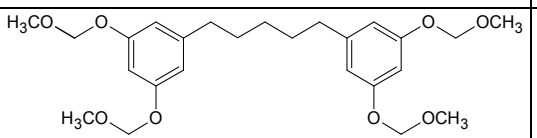
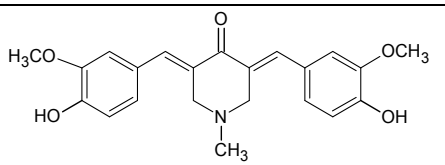
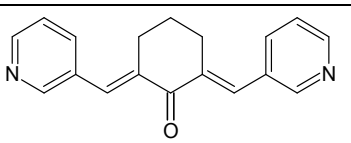
Development of curcumin analogues has developed as a strategy to enhance bioavailability and selectivity towards cancer cells. Modification of the aromatic rings and β-diketone moiety of curcumin has led to different curcumin analogues with improved activities (Table 1)(Mosley et al., 2007). The first-generation curcumin derivatives were the cyclohexanones, which exhibited enhanced activity and stability in biological medium compared to curcumin. For example, the cyclohexanone-containing curcumin derivative 2,6-bis ((3-methoxy-4-hydroxyphenyl) methylene)-cyclohexanone (BMHPC) was cytotoxic toward ER-negative breast cancer cells (IC50 of 5.0 μM) and displayed anti-angiogenic properties in human and murine endothelial cell lines (Adams et al., 2004). These results led the authors to further synthesize several fluorinated derivatives, one of which (EF-24)(Table 1) exhibited potent cytotoxicity toward MDA-MB-231 cells (IC50 of 0.8 μM) (Adams et al., 2005). Moreover, EF-24 induced breast tumor regression in athymic nude mice. Specifically, tumor weight following 20 mg/kg was decreased by ~30% compared to control, whereas 100 mg/kg decreased tumor weight by 55%. Interestingly, no toxicity was observed at a dose of 100 mg/kg, which was well below maximum tolerated dose (MTD) of 200 mg/kg (Adams et al., 2005). Mechanistic studies were also performed in MDA-MB-231 cells. Specifically, EF-24

(10 μM) inhibited cell proliferation by 70-80% and arrested cells in the G2/M phase of the cell cycle. Additionally, EF24 (20 μM) increased the percentage of early apoptotic cells to 25.3 % after 72 h. Similarly, the cell population in late apoptosis increased to 45.6%. In addition, EF-24 increased intracellular ROS levels by 55% at 48 h (Adams et al., 2005). Further mechanistic studies demonstrated that EF-24 inhibited the pro-angiogenic transcription factor, HIF-1□α □at the posttranscriptional level by a VHL-dependent but proteasome-independent mechanism in MDA-MB-231 cells (Thomas et al., 2008). The therapeutic potential of EF-24 by using coagulation factor VIIa (fVIIa) as a carrier for targeted delivery of EF-24 to the tissue factor (TF) expressed in tumor cells and vascular endothelial cells and thus showed its anti-angiogenic and anti-cancer activity in breast cancer cells. They demonstrated that EF-24-FFRck-fVIIa conjugate significantly decreased the viability of the TF-expressing MDA-MB-231 and HUVEC cells in a concentration dependent manner. Furthermore, the administration of 5 intravenous injections of the EF-24-FFRck-fVIIa conjugate (containing 50 μM of EF-24) for two weeks significantly reduced the tumor size in MDA-MB-231 breast cancer xenografts. Moreover, the tumor cells showed activation of caspase 3 as a marker of apoptosis (Shoji et al., 2008).

Another set of curcumin analogues (FLLL 11 and FLLL 12)(Table 1) produced by exchanging the β-diketone moiety for an α β unsaturated ketone, exhibited more potent antitumor activity than curcumin in various ER positive and ER negative human breast cancer cells (Lin et al., 2009). The IC_{50} values for FLLL11 and FLLL12 ranged from 9 to 48 fold lower than curcumin. Furthermore, both the analogues at 10 μM inhibited STAT3, Akt and HER2/Neu pathways and induced apoptosis. The apoptosis was mediated via activation of cleaved PARP and caspase 3. These analogues were also effective in combination with doxorubicin as they exhibited a synergistic anti-proliferative effect in MDA-MB-231 breast cancer cells. In addition, the compounds inhibited anchorage independent growth and cell migration in MDA-MB-231 cells (Lin et al., 2009). In another study, Li et al, synthesized two series of monoketone curcumin analogues namely, heptadienone and pentadienone series and investigated their anti-cancer properties *in vitro*. Among the 24 compounds that were synthesized, compound 23 (Table 1) was the most potent analogue with IC_{50} values in sub-micromolar range in MDA-MB-231 cells (Fuchs et al., 2009). Various 1,5-diarylpentadienon containing curcumin analogues with an alkoxy substitution on aromatic rings at each of the positions 3 and 5 have also been synthesized (Ohori et al., 2006). One of the analogues, GO-YO30 (Table 1) showed substantially higher cytotoxicity and anchorage independency compared to curcumin in MDA-MB-231 cells. Furthermore, it also inhibited STAT activity in a dose-dependent manner. Interestingly, GO-YO30 induced apoptosis in MDA-MB-231 at concentrations far lower than those required to elicit a comparable effect following curcumin treatment (Hutzen et al., 2009).

Monoketo curcumin analogues with a piperidone ring impart a rigid confirmation that has led to a broad spectrum of antitumor activity. Compound, 8 and 18 (Table 1) bearing the n-alkyl piperidone group showed potent cytotoxic activity towards various breast cancer cells (Youssef & El-Sherbeny, 2005). This structure was recently further modified by replacing the methylene groups and the two carbonyl groups in curcumin by N-methyl-4 piperidone. The resulting compound 5-bis (4-hydroxy-3- methoxybenzylidene)- N - methyl-4-piperidone (PAC) (Table 1) was 5 times more effective than curcumin in inducing apoptosis in ER negative breast cancer cells (MDA-MB-231, BEC114) (Al-Hujaily et al., 2010). Also, it's pro-apoptotic effect was 10 times higher against ER negative breast cancer cells than against ER positive cells (MCF-7, T-47D). Cell cycle analysis revealed that PAC (10 μM) treatment of

MDA-MB-231 cells increased the proportion of cells undergoing G2/M phase arrest by 185%. Furthermore, at 10 μM, PAC induced apoptosis in 55% of MDA-MB-231 cells (Al-Hujaily et al., 2010). PAC exhibited its cytotoxic effect by down regulating the expression of NFκB, survivin and its downstream effectors cyclin D1 and Bcl-2 and subsequently showed up-regulation of $p21^{WAF1}$ expression both *in vitro* and *in vivo*. Interestingly, PAC (100 mg/kg/day) suppressed the growth of MDA-MB-231 xenografts (Al-Hujaily et al., 2010). Importantly, the solubility of PAC was 27-fold higher than curcumin and 1 h after the injection, the levels of ^{18}F-PAC in the blood was 5-fold higher than the levels ^{18}F-curcumin. These studies suggested better pharmacokinetics and tissue bio-distribution of PAC compared to curcumin in mice (Al-Hujaily et al., 2010).

Compound Name	Structure	Cell line	IC 50 (μM)
BMHPC		MDA-MB-231	5
EF-24		MDA-MB-231	0.8
FLLL11		MCF-7 MDA-MB-231 MDA-MB-468 MDA-MB-453 SKBr3	2.4 2.8 0.3 4.7 5.7
FLLL12		MCF-7 MDA-MB-231 MDA-MB-468 MDA-MB-453 SKBr3	1.7 2.7 0.3 1.3 3.8
GO-YO30		MDA-MB-231	1.2
PAC		Not known	
RL90		MDA-MB-231 SKBr3	1.54 0.51

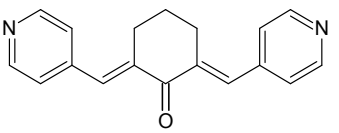
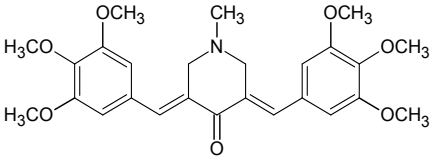
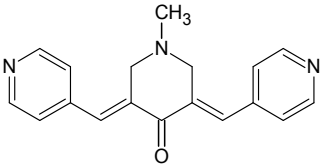
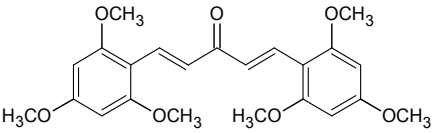
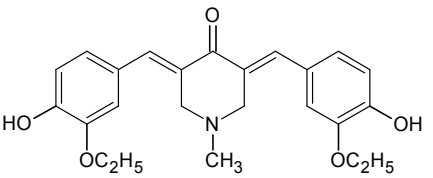
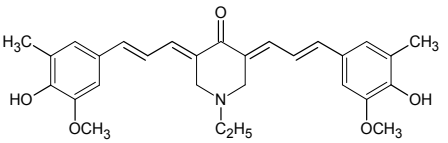
Compound Name	Structure	Cell line	IC 50 (μM)
RL91		MDA-MB-231 SKBr3	1.10 0.23
B10		MDA-MB-231 MDA-MB-468 SKBr3	0.3 0.3 0.4
B1		MDA-MB-231 MDA-MB-468 SKBr3	0.8 0.5 0.6
Compound 23		MCF-7 MDA-MB-231	0.4 0.6
Compound 8		MCF-7 MDA-MB-31 MDA-MB-435 HS-578T BT-549 T-47D	2.3 17.9 6.8 5.4 32.8 15.1
Compound 18		MCF-7 MDA-MB-31 MDA-MB-435 HS-578T BT-549 T-47D	3.3 2.8 3.7 3.8 2.6 2.7

Table 1. Chemical structures of curcumin derivatives and their relative *in vitro* potency.

Second generation curcumin analogues have been synthesized by replacing the phenyl group of cyclohexanone curcumin derivatives with heterocyclic rings. Two analogues, 2,6-bis(pyridin-3-ylmethylene)-cyclohexanone (RL90) and 2,6-bis(pyridin-4-ylmethylene)-cyclohexanone (RL91)(Table 1) showed potent cytotoxic towards ER negative breast cancer cells (MDA-MB-231, SKBr3) and modulated the expression of variety of cell signaling proteins such as EGFR, Akt, HER2, β-catenin and NFκB. Treatment with RL90 and RL91 also showed activation of stress kinases, as evidenced by phosphorylation of both JNK1/2 and p38 MAPK. Furthermore, RL90 and RL91 produced cell cycle arrest at G2/M phase in MDA-MB-231 and SKBr3 cells. Specifically, treatment of MDA-MB-231 cells with RL90 (3 μM) or RL91 (2.5 μM) significantly increased the proportion of cells in G2/M phase by 52 and 49% compared to control, respectively. RL90 and RL91 also increased the proportion of

apoptotic cells by 164% and 406% of control, respectively (Somers-Edgar et al., 2011). Thus, these second-generation curcumin derivatives are more potent *in vitro* than first generation derivatives such as BMHPC.

Another set of cyclohexanone analogues of curcumin included modification resulting in N-methylpiperidone, tropinone or cyclopentanone core groups. The aromatic substitutions on these compounds included pyrrole, imidazole, indole flouro-pyridines as well as trimethoxyphenyl and dimethoxyphenyl groups. The resulting compounds were screened for their cytotoxicity in TNBC cells. Among 18 analogues examined, 3,5-bis (pyridine-4-yl)-1-methylpiperidin-4-one (B1) and 3,5-bis (3,4,5-trimethoxybenzylidene)-1-methylpiperidin-4-one (B10) (Table 1) showed potent cytotoxicity towards MBA-MB-231 and MDA-MB-468 cells with IC_{50} values below 1 μM (Yadav et al., 2010). Furthermore, B1 and B10 induced apoptosis in MDA-MB-231 cells. Specifically, B1 (2 μM) significantly increased the proportion of apoptotic cells by 4-fold compared to control at 12 h whereas B 10 (1 μM) was more potent as there was a 10-fold increase in the proportion of apoptotic MDA-MB-231 cells after 24 h (Yadav et al., 2010).

The last set of compounds involves a series of mono-carbonyl analogues of curcumin using three different 5-carbon linkers, namely cyclopentanone, acetone, and cyclohexanone with various substituents on aryl rings. They reported that all the analogues had enhanced stability *in vitro* and improved pharmacokinetic profile *in vivo*. In this study, 500 mg/kg of compound B02 and B33 (Table 1) were orally administered to male Sprague-Dawley rats. The peak plasma concentrations of B02 and B33 were increased to 0.82 μg/ml and 4.1 μg/ml, respectively, compared to curcumin (0.091 μg/ml). This correlated with a decrease in the plasma clearance of both drugs (B02 was 125.4 l/kg/h and B33 38.98 l/kg/h) compared to curcumin (835.2 l/Kg/h). Furthermore, the half-life of B02 was increased 2-fold greater than curcumin and absorption of B33 was rapid. In addition, the analogues with acetone or cyclohexanone spacer groups showed increased cytotoxicity against several tumor cell lines. Interestingly, the analogues in which the benzene ring was replaced by a hetero aromatic ring enhanced the cytotoxic activity of these mono-carbonyl analogues (Liang et al., 2009). Overall, these studies all demonstrated that significant improvement occurs with each successive generation of synthetic analogues.

4. Conclusion

There is a large body of evidence demonstrating that the natural compounds EGCG and curcumin have potent actions in both *in vitro* and *in vivo* models of triple negative breast cancer. While these compounds both have many beneficial actions, they are hindered by poor bioavailability. However, their knowledge base of mechanistic actions have allowed them to be improved by various methods such as; 1) use in combination therapies, 2) imported via specific targeting via nanomedine and 3) improved via chemical modification into prodrugs or new structural analogues. Therefore, it is likely that a novel therapy for triple negative breast cancer will emerge as a synthetic derivative of one of these natural compounds that may ultimately be delivered to the tumor via nanomedicine.

5. References

Adams, B. K., Cai, J., Armstrong, J., Herold, M., Lu, Y. J., Sun, A., Snyder, J. P., Liotta, D. C., Jones, D. P. & Shoji, M. (2005). EF24, a novel synthetic curcumin analog, induces

apoptosis in cancer cells via a redox-dependent mechanism. *Anticancer Drugs,* Vol. 16, No. 3, pp. 263-275, 0959-4973

Adams, B. K., Ferstl, E. M., Davis, M. C., Herold, M., Kurtkaya, S., Camalier, R. F., Hollingshead, M. G., Kaur, G., Sausville, E. A., Rickles, F. R., Snyder, J. P., Liotta, D. C. & Shoji, M. (2004). Synthesis and biological evaluation of novel curcumin analogs as anti-cancer and anti-angiogenesis agents. *Bioorg Med Chem,* Vol. 12, No. 14, pp. 3871-3883, 0968-0896

Adams, J. & Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. *Science,* Vol. 281, No. 5381, pp. 1322-1326, 0036-8075

Aggarwal, B. B., Kumar, A. & Bharti, A. C. (2003). Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res,* Vol. 23, No. 1A, pp. 363-398, 0250-7005

Aggarwal, B. B., Shishodia, S., Takada, Y., Banerjee, S., Newman, R. A., Bueso-Ramos, C. E. & Price, J. E. (2005). Curcumin suppresses the paclitaxel-induced nuclear factor-kappaB pathway in breast cancer cells and inhibits lung metastasis of human breast cancer in nude mice. *Clin Cancer Res,* Vol. 11, No. 20, pp. 7490-7498, 1078-0432

Al-Hujaily, E. M., Mohamed, A. G., Al-Sharif, I., Youssef, K. M., Manogaran, P. S., Al-Otaibi, B., Al-Haza'a, A., Al-Jammaz, I., Al-Hussein, K. & Aboussekhra, A. (2010). PAC, a novel curcumin analogue, has anti-breast cancer properties with higher efficiency on ER-negative cells. *Breast Cancer Res Treat,* Vol. No. pp. 1573-7217

Anand, P., Nair, H. B., Sung, B., Kunnumakkara, A. B., Yadav, V. R., Tekmal, R. R. & Aggarwal, B. B. (2010). Design of curcumin-loaded PLGA nanoparticles formulation with enhanced cellular uptake, and increased bioactivity in vitro and superior bioavailability in vivo. *Biochemical Pharmacology,* Vol. 79, No. 3, pp. 330-338, 1873-2968

Anand, P., Thomas, S. G., Kunnumakkara, A. B., Sundaram, C., Harikumar, K. B., Sung, B., Tharakan, S. T., Misra, K., Priyadarsini, I. K., Rajasekharan, K. N. & Aggarwal, B. B. (2008). Biological activities of curcumin and its analogues (Congeners) made by man and Mother Nature. *Biochem Pharmacol,* Vol. 76, No. 11, pp. 1590-1611, 1873-2968

Andre, F., Job, B., Dessen, P., Tordai, A., Michiels, S., Liedtke, C., Richon, C., Yan, K., Wang, B. & Vassal, G. (2009). Molecular characterization of breast cancer with high-resolution oligonucleotide comparative genomic hybridization array. *Clinical Cancer Research,* Vol. 15, No. 2, pp. 441-451, 1078-0432

Arslan, C., Dizdar, O. & Altundag, K. (2009). Pharmacotherapy of triple-negative breast cancer. *Expert Opin Pharmacother,* Vol. 10, No. 13, pp. 2081-2093, 1744-7666

Bachmeier, B., Nerlich, A. G., Iancu, C. M., Cilli, M., Schleicher, E., Vene, R., Dell'Eva, R., Jochum, M., Albini, A. & Pfeffer, U. (2007). The chemopreventive polyphenol Curcumin prevents hematogenous breast cancer metastases in immunodeficient mice. *Cell Physiol Biochem,* Vol. 19, No. 1-4, pp. 137-152, 1015-8987

Bachmeier, B. E., Mohrenz, I. V., Mirisola, V., Schleicher, E., Romeo, F., Hohneke, C., Jochum, M., Nerlich, A. G. & Pfeffer, U. (2008). Curcumin downregulates the inflammatory cytokines CXCL1 and -2 in breast cancer cells via NFkappaB. *Carcinogenesis,* Vol. 29, No. 4, pp. 779-789, 1460-2180

Berrada, N., Delaloge, S. & André, F. (2010). Treatment of triple-negative metastatic breast cancer: toward individualized targeted treatments or chemosensitization? *Annals of Oncology,* Vol. 21, No. Suppl 7, pp. vii30-vii35, 0923-7534

Bigelow, R. & Cardelli, J. (2006). The green tea catechins,(-)-epigallocatechin-3-gallate (EGCG) and (-)-Epicatechin-3-gallate (ECG), inhibit HGF/Met signaling in immortalized and tumorigenic breast epithelial cells. *Oncology,* Vol. 25, No. 13, pp. 1922-1930, 0950-9232

Biswas, D. K., Dai, S. C., Cruz, A., Weiser, B., Graner, E. & Pardee, A. B. (2001). The nuclear factor kappa B (NF-kappa B): a potential therapeutic target for estrogen receptor negative breast cancers. *Proc Natl Acad Sci U S A,* Vol. 98, No. 18, pp. 10386-10391, 0027-8424

Boonrao, M., Yodkeeree, S., Ampasavate, C., Anuchapreeda, S. & Limtrakul, P. (2010). The inhibitory effect of turmeric curcuminoids on matrix metalloproteinase-3 secretion in human invasive breast carcinoma cells. *Arch Pharm Res,* Vol. 33, No. 7, pp. 989-998, 0253-6269

Bosch, A., Eroles, P., Zaragoza, R., Vina, J. R. & Lluch, A. (2010). Triple-negative breast cancer: molecular features, pathogenesis, treatment and current lines of research. *Cancer Treat Rev,* Vol. 36, No. 3, pp. 206-215, 1532-1967

Carey, L., Winer, E., Viale, G., Cameron, D. & Gianni, L. (2010). Triple-negative breast cancer: disease entity or title of convenience? *Nat Rev Clin Oncol,* Vol. 7, No. 12, pp. 683-692, 1759-4782

Casalini, P., Iorio, M., Galmozzi, E. & Ménard, S. (2004). Role of HER receptors family in development and differentiation. *Journal of Cellular Physiology,* Vol. 200, No. 3, pp. 343-350, 1097-4652

Catzavelos, C., Bhattacharya, N., Ung, Y. C., Wilson, J. A., Roncari, L., Sandhu, C., Shaw, P., Yeger, H., Morava-Protzner, I., Kapusta, L., Franssen, E., Pritchard, K. I. & Slingerland, J. M. (1997). Decreased levels of the cell-cycle inhibitor p27Kip1 protein: prognostic implications in primary breast cancer. *Nat Med,* Vol. 3, No. 2, pp. 227-230, 1078-8956

Cheah, Y. H., Nordin, F. J., Sarip, R., Tee, T. T., Azimahtol, H. L., Sirat, H. M., Rashid, B. A., Abdullah, N. R. & Ismail, Z. (2009). Combined xanthorrhizol-curcumin exhibits synergistic growth inhibitory activity via apoptosis induction in human breast cancer cells MDA-MB-231. *Cancer Cell Int,* Vol. 9, No. pp. 1, 1475-2867

Chen, J. Q. & Russo, J. (2009). ERalpha-negative and triple negative breast cancer: Molecular features and potential therapeutic approaches. *Biochim Biophys Acta,* Vol. 1796, No. 2, pp. 162-175, 0006-3002

Chisholm, K., Bray, B. & Rosengren, R. (2004). Tamoxifen and epigallocatechin gallate are synergistically cytotoxic to MDA-MB-231 human breast cancer cells. *Anti-Cancer Drugs,* Vol. 15, No. 9, pp. 889-897, 0959-4973

Chiu, T. L. & Su, C. C. (2009). Curcumin inhibits proliferation and migration by increasing the Bax to Bcl-2 ratio and decreasing NF-kappaBp65 expression in breast cancer MDA-MB-231 cells. *Int J Mol Med,* Vol. 23, No. 4, pp. 469-475, 1107-3756

Cho, K., Wang, X., Nie, S., Chen, Z. G. & Shin, D. M. (2008). Therapeutic nanoparticles for drug delivery in cancer. *Clin Cancer Res,* Vol. 14, No. 5, pp. 1310-1316, 1078-0432

Chu, E. & Tarnawski, A. (2004). PTEN regulatory functions in tumor suppression and cell biology. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research,* Vol. 10, No. 10, pp. 235-241, 1234-1010

Conte, P. & Guarneri, V. (2009). Triple-negative breast cancer: current management and future options. *EJC Supplements*, Vol. 7, No. 1, pp. 14-18, 1359-6349

Cunningham, A., Love, W., Zhang, R., Andrews, L. & Tollefsbol, T. (2006). Telomerase inhibition in cancer therapeutics: molecular-based approaches. *Current Medicinal Chemistry,* Vol. 13, No. 24, pp. 2875–2888,

DeGraffenried, L., Fulcher, L., Friedrichs, W., Grünwald, V., Ray, R. & Hidalgo, M. (2004). Reduced PTEN expression in breast cancer cells confers susceptibility to inhibitors of the PI3 kinase/Akt pathway. *Annals of Oncology,* Vol. 15, No. 10, pp. 1510-1516, 0923-7534

Fang, H. Y., Chen, S. B., Guo, D. J., Pan, S. Y. & Yu, Z. L. (2011). Proteomic identification of differentially expressed proteins in curcumin-treated MCF-7 cells. *Phytomedicine,* Vol. No. pp. 1618-095X

Foulkes, W. D., Smith, I. E. & Reis-Filho, J. S. (2010). Triple-negative breast cancer. *N Engl J Med,* Vol. 363, No. 20, pp. 1938-1948, 1533-4406

Fuchs, J. R., Pandit, B., Bhasin, D., Etter, J. P., Regan, N., Abdelhamid, D., Li, C., Lin, J. & Li, P. K. (2009). Structure-activity relationship studies of curcumin analogues. *Bioorg Med Chem Lett,* Vol. 19, No. 7, pp. 2065-2069, 1464-3405

Graham, H. (1992). Green tea composition, consumption, and polyphenol chemistry* 1. *Preventive Medicine,* Vol. 21, No. 3, pp. 334-350, 0091-7435

Greenberg, S. & Rugo, H. S. (2010). Challenging clinical scenarios: treatment of patients with triple-negative or basal-like metastatic breast cancer *Clin Breast Cancer,* Vol. 10, No. pp. S20-29, 1938-0666

Gupta, V., Aseh, A., Rios, C. N., Aggarwal, B. B. & Mathur, A. B. (2009). Fabrication and characterization of silk fibroin-derived curcumin nanoparticles for cancer therapy. *Int J Nanomedicine,* Vol. 4, No. pp. 115-122, 1178-2013

Hossain, M., Bouton, C., Pevsner, J. & Laterra, J. (2000). Induction of vascular endothelial growth factor in human astrocytes by lead: involvement of a protein kinase C/activator protein-1 complex-dependent and hypoxia-inducible factor 1-independent signaling pathway. *Journal of Biological Chemistry,* Vol. 8, No. 275, pp. 27874-27882, 0021-9258

Hutzen, B., Friedman, L., Sobo, M., Lin, L., Cen, L., De Angelis, S., Yamakoshi, H., Shibata, H., Iwabuchi, Y. & Lin, J. (2009). Curcumin analogue GO-Y030 inhibits STAT3 activity and cell growth in breast and pancreatic carcinomas. *Int J Oncol,* Vol. 35, No. 4, pp. 867-872, 1791-2423

Irvin, W. J., Jr. & Carey, L. A. (2008). What is triple-negative breast cancer? *Eur J Cancer,* Vol. 44, No. 18, pp. 2799-2805, 1879-0852

Kakarala, M., Brenner, D. E., Korkaya, H., Cheng, C., Tazi, K., Ginestier, C., Liu, S., Dontu, G. & Wicha, M. S. (2010). Targeting breast stem cells with the cancer preventive compounds curcumin and piperine. *Breast Cancer Res Treat,* Vol. 122, No. 3, pp. 777-785, 1573-7217

Kang, H. J., Lee, S. H., Price, J. E. & Kim, L. S. (2009). Curcumin suppresses the paclitaxel-induced nuclear factor-kappaB in breast cancer cells and potentiates the growth

inhibitory effect of paclitaxel in a breast cancer nude mice model. *Breast J,* Vol. 15, No. 3, pp. 223-229, 1524-4741

Karunagaran, D., Rashmi, R. & Kumar, T. R. (2005). Induction of apoptosis by curcumin and its implications for cancer therapy. *Curr Cancer Drug Targets,* Vol. 5, No. 2, pp. 117-129, 1568-0096

Kavanagh, K., Hafer, L., Kim, D., Mann, K., Sherr, D., Rogers, A. & Sonenshein, G. (2001). Green tea extracts decrease carcinogen induced mammary tumor burden in rats and rate of breast cancer cell proliferation in culture. *Journal of Cellular Biochemistry,* Vol. 82, No. 3, pp. 387-398, 1097-4644

Khramtsov, A. I., Khramtsova, G. F., Tretiakova, M., Huo, D., Olopade, O. I. & Goss, K. H. (2010). Wnt/beta-catenin pathway activation is enriched in basal-like breast cancers and predicts poor outcome. *Am J Pathol,* Vol. 176, No. 6, pp. 2911-2920, 1525-2191

Kim, H. I., Huang, H., Cheepala, S., Huang, S. & Chung, J. (2008). Curcumin inhibition of integrin (alpha6beta4)-dependent breast cancer cell motility and invasion. *Cancer Prev Res (Phila),* Vol. 1, No. 5, pp. 385-391, 1940-6215

Koenders, P. G., Beex, L. V., Geurts-Moespot, A., Heuvel, J. J., Kienhuis, C. B. & Benraad, T. J. (1991). Epidermal growth factor receptor-negative tumors are predominantly confined to the subgroup of estradiol receptor-positive human primary breast cancers. *Cancer Res,* Vol. 51, No. 17, pp. 4544-4548,

Kwan, M. L., Kushi, L. H., Weltzien, E., Maring, B., Kutner, S. E., Fulton, R. S., Lee, M. M., Ambrosone, C. B. & Caan, B. J. (2009). Epidemiology of breast cancer subtypes in two prospective cohort studies of breast cancer survivors. *Breast Cancer Res,* Vol. 11, No. 3, pp. R31, 1465-542X (Electronic)

Landis Piwowar, K., Wan, S., Wiegand, R., Kuhn, D., Chan, T. & Dou, Q. (2007). Methylation suppresses the proteasome inhibitory function of green tea polyphenols. *Journal of Cellular Physiology,* Vol. 213, No. 1, pp. 252-260, 1097-4652

Li, Y., Yuan, Y., Meeran, S. & Tollefsbol, T. (2010). Synergistic epigenetic reactivation of estrogen receptor- (ER) by combined green tea polyphenol and histone deacetylase inhibitor in ER -negative breast cancer cells. *Molecular Cancer,* Vol. 9, No. 1, pp. 274-286, 1476-4598

Liang, G., Shao, L., Wang, Y., Zhao, C., Chu, Y., Xiao, J., Zhao, Y., Li, X. & Yang, S. (2009). Exploration and synthesis of curcumin analogues with improved structural stability both in vitro and in vivo as cytotoxic agents. *Bioorg Med Chem,* Vol. 17, No. 6, pp. 2623-2631, 1464-3391

Lin, L., Hutzen, B., Ball, S., Foust, E., Sobo, M., Deangelis, S., Pandit, B., Friedman, L., Li, C., Li, P. K., Fuchs, J. & Lin, J. (2009). New curcumin analogues exhibit enhanced growth-suppressive activity and inhibit AKT and signal transducer and activator of transcription 3 phosphorylation in breast and prostate cancer cells. *Cancer Sci,* Vol. 100, No. 9, pp. 1719-1727, 1349-7006

Liu, Q., Loo, W. T., Sze, S. C. & Tong, Y. (2009). Curcumin inhibits cell proliferation of MDA-MB-231 and BT-483 breast cancer cells mediated by down-regulation of NFkappaB, cyclinD and MMP-1 transcription. *Phytomedicine,* Vol. 16, No. 10, pp. 916-922, 1618-095X

Luo, T., Wang, J., Yin, Y., Hua, H., Jing, J., Sun, X., Li, M., Zhang, Y. & Jiang, Y. (2010). (-)-Epigallocatechin gallate sensitizes breast cancer cells to paclitaxel in a murine

model of breast carcinoma. *Breast Cancer Research,* Vol. 12, No. 1, pp. R8-18, 1465-5411

Malumbres, M. & Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer,* Vol. 9, No. 3, pp. 153-166, 1474-1768

Masuda, M., Suzui, M., Lim, J. T. E., Deguchi, A., Soh, J. W. & Weinstein, I. B. (2002). Epigallocatechin 3 gallate decreases VEGF production in head and neck and breast carcinoma cells by inhibiting EGFR related pathways of signal transduction. *Journal of Experimental Therapeutics and Oncology,* Vol. 2, No. 6, pp. 350-359, 1533-869X

Meeran, S., Patel, S., Chan, T. & Tollefsbol, T. (2011). A Novel Prodrug of Epigallocatechin-3-gallate: Differential Epigenetic hTERT Repression in Human Breast Cancer Cells. *Cancer Prevention Research,* epub ahead of print, 1940-6207

Mosley, C. A., Liotta, D. C. & Snyder, J. P. (2007). Highly active anticancer curcumin analogues. *Adv Exp Med Biol,* Vol. 595, No. pp. 77-103, 0065-2598

Narasimhan, M. & Ammanamanchi, S. (2008). Curcumin blocks RON tyrosine kinase-mediated invasion of breast carcinoma cells. *Cancer Res,* Vol. 68, No. 13, pp. 5185-5192, 1538-7445

Ohori, H., Yamakoshi, H., Tomizawa, M., Shibuya, M., Kakudo, Y., Takahashi, A., Takahashi, S., Kato, S., Suzuki, T., Ishioka, C., Iwabuchi, Y. & Shibata, H. (2006). Synthesis and biological analysis of new curcumin analogues bearing an enhanced potential for the medicinal treatment of cancer. *Mol Cancer Ther,* Vol. 5, No. 10, pp. 2563-2571, 1535-7163

Okushio, K., Suzuki, M., Matsumoto, N., Nanjo, F. & HARA, Y. (1999). Methylation of tea catechins by rat liver homogenates. *Bioscience, Biotechnology, and Biochemistry,* Vol. 63, No. 2, pp. 430-432, 0916-8451

Pellikainen, M. J., Pekola, T. T., Ropponen, K. M., Kataja, V. V., Kellokoski, J. K., Eskelinen, M. J. & Kosma, V. M. (2003). p21WAF1 expression in invasive breast cancer and its association with p53, AP-2, cell proliferation, and prognosis. *J Clin Pathol,* Vol. 56, No. 3, pp. 214-220, 0021-9746

Potemski, P., Kusinska, R., Watala, C., Pluciennik, E., Bednarek, A. K. & Kordek, R. (2006). Cyclin E expression in breast cancer correlates with negative steroid receptor status, HER2 expression, tumor grade and proliferation. *J Exp Clin Cancer Res,* Vol. 25, No. 1, pp. 59-64, 0392-9078

Prasad, C. P., Rath, G., Mathur, S., Bhatnagar, D. & Ralhan, R. (2009). Potent growth suppressive activity of curcumin in human breast cancer cells: Modulation of Wnt/beta-catenin signaling. *Chem Biol Interact,* Vol. 181, No. 2, pp. 263-271, 1872-7786

Ravindran, J., Prasad, S. & Aggarwal, B. B. (2009). Curcumin and cancer cells: how many ways can curry kill tumor cells selectively? *AAPS J,* Vol. 11, No. 3, pp. 495-510, 1550-7416

Reis-Filho, J. S. & Tutt, A. N. (2008). Triple negative tumours: a critical review. *Histopathology,* Vol. 52, No. 1, pp. 108-118, 1365-2559

Rouzier, R., Perou, C. M., Symmans, W. F., Ibrahim, N., Cristofanilli, M., Anderson, K., Hess, K. R., Stec, J., Ayers, M., Wagner, P., Morandi, P., Fan, C., Rabiul, I., Ross, J. S., Hortobagyi, G. N. & Pusztai, L. (2005). Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res,* Vol. 11, No. 16, pp. 5678-5685, 1078-0432

Rowe, D. L., Ozbay, T., O'Regan, R. M. & Nahta, R. (2009). Modulation of the BRCA1 protein and induction of apoptosis in triple negative breast cancer cell lines by the polyphenolic compound curcumin. *Breast Cancer,* Vol. 3, No. pp. 61-75, 1178-2234

Roy, A., Baliga, M. & Katiyar, S. (2005). Epigallocatechin-3-gallate induces apoptosis in estrogen receptor–negative human breast carcinoma cells via modulation in protein expression of p53 and Bax and caspase-3 activation. *Molecular cancer therapeutics,* Vol. 4, No. 1, pp. 81-90, 1535-7163

Sartippour, M., Heber, D., Ma, J., Lu, Q., Go, V. & Nguyen, M. (2001). Green tea and its catechins inhibit breast cancer xenografts. *Nutrition and Cancer,* Vol. 40, No. 2, pp. 149-156, 0163-5581

Sartippour, M., Shao, Z., Heber, D., Beatty, P., Zhang, L., Liu, C., Ellis, L., Liu, W., Go, V. & Brooks, M. (2002). Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. *Journal of Nutrition,* Vol. 132, No. 8, pp. 2307-2311, 0022-3166

Scandlyn, M., Stuart, E., Somers-Edgar, T., Menzies, A. & Rosengren, R. (2008). A new role for tamoxifen in oestrogen receptor-negative breast cancer when it is combined with epigallocatechin gallate. *British Journal of Cancer,* Vol. 99, No. 7, pp. 1056-1063, 0007-0920

Schlotter, C. M., Vogt, U., Allgayer, H. & Brandt, B. (2008). Molecular targeted therapies for breast cancer treatment. *Breast Cancer Research,* Vol. 10, No. 4, pp. 211, 1465-542X

Schneider, B. P. & Miller, K. D. (2005). Angiogenesis of breast cancer. *J Clin Oncol,* Vol. 23, No. 8, pp. 1782-1790, 0732-183X

Shahani, K., Swaminathan, S. K., Freeman, D., Blum, A., Ma, L. & Panyam, J. (2010). Injectable sustained release microparticles of curcumin: a new concept for cancer chemoprevention. *Cancer Research,* Vol. 70, No. 11, pp. 4443-4452, 1538-7445

Shao, Z. M., Shen, Z. Z., Liu, C. H., Sartippour, M. R., Go, V. L., Heber, D. & Nguyen, M. (2002). Curcumin exerts multiple suppressive effects on human breast carcinoma cells. *Int J Cancer,* Vol. 98, No. 2, pp. 234-240, 0020-7136 (

Shay, J. & Bacchetti, S. (1997). A survey of telomerase activity in human cancer. *European Journal of Cancer,* Vol. 33, No. 5, pp. 787-791, 0959-8049

Shehzad, A., Wahid, F. & Lee, Y. S. (2010). Curcumin in cancer chemoprevention: molecular targets, pharmacokinetics, bioavailability, and clinical trials. *Arch Pharm (Weinheim),* Vol. 343, No. 9, pp. 489-499, 1521-4184

Shoji, M., Sun, A., Kisiel, W., Lu, Y. J., Shim, H., McCarey, B. E., Nichols, C., Parker, E. T., Pohl, J., Mosley, C. A., Alizadeh, A. R., Liotta, D. C. & Snyder, J. P. (2008). Targeting tissue factor-expressing tumor angiogenesis and tumors with EF24 conjugated to factor VIIa. *J Drug Target,* Vol. 16, No. 3, pp. 185-197, 1061-186X

Shutava, T., Balkundi, S., Vangala, P., Steffan, J., Bigelow, R., Cardelli, J., O'Neal, D. & Lvov, Y. (2009). Layer-by-layer-coated gelatin nanoparticles as a vehicle for delivery of natural polyphenols. *ACS nano,* Vol. 3, No. 7, pp. 1877-1885, 1936-0851

Siddiqui, I., Adhami, V., Bharali, D., Hafeez, B., Asim, M., Khwaja, S., Ahmad, N., Cui, H., Mousa, S. & Mukhtar, H. (2009). Introducing nanochemoprevention as a novel approach for cancer control: proof of principle with green tea polyphenol epigallocatechin-3-gallate. *Cancer Research,* Vol. 69, No. 5, pp. 1712, 0008-5472

Somers-Edgar, T. J., Scandlyn, M. J., Stuart, E. C., Le Nedelec, M. J., Valentine, S. P. & Rosengren, R. J. (2008). The combination of epigallocatechin gallate and curcumin

suppresses ER alpha-breast cancer cell growth in vitro and in vivo. *Int J Cancer,* Vol. 122, No. 9, pp. 1966-1971, 1097-0215

Somers-Edgar, T. J., Taurin, S., Larsen, L., Chandramouli, A., Nelson, M. A. & Rosengren, R. J. (2011). Mechanisms for the activity of heterocyclic cyclohexanone curcumin derivatives in estrogen receptor negative human breast cancer cell lines. *Invest New Drugs,* Vol. 29, No. 1, pp. 87-97, 1573-0646

Squires, M. S., Hudson, E. A., Howells, L., Sale, S., Houghton, C. E., Jones, J. L., Fox, L. H., Dickens, M., Prigent, S. A. & Manson, M. M. (2003). Relevance of mitogen activated protein kinase (MAPK) and phosphotidylinositol-3-kinase/protein kinase B (PI3K/PKB) pathways to induction of apoptosis by curcumin in breast cells. *Biochem Pharmacol,* Vol. 65, No. 3, pp. 361-376, 0006-2952

Stuart, E., Jarvis, R. & Rosengren, R. (2010). In vitro mechanism of action for the cytotoxicity elicited by the combination of epigallocatechin gallate and raloxifene in MDA-MB-231 cells. *Oncology Reports,* Vol. 24, No. 3, pp. 779-785, 1021-335X

Stuart, E., Larsen, L. & Rosengren, R. (2007). Potential mechanisms for the synergistic cytotoxicity elicited by 4-hydroxytamoxifen and epigallocatechin gallate in MDA-MB-231 cells. *International Journal of Oncology,* Vol. 30, No. 6, pp. 1407-1412, 1019-6439

Stuart, E. & Rosengren, R. (2008). The combination of raloxifene and epigallocatechin gallate suppresses growth and induces apoptosis in MDA-MB-231 cells. *Life Sciences,* Vol. 82, No. 17, pp. 943-948, 0024-3205

Syng-Ai, C., Kumari, A. L. & Khar, A. (2004). Effect of curcumin on normal and tumor cells: role of glutathione and bcl-2. *Mol Cancer Ther,* Vol. 3, No. 9, pp. 1101-1108, 1535-7163

Thangapazham, R., Passi, N. & Maheshwari, R. (2007a). Green tea polyphenol and epigallocatechin gallate induce apoptosis and inhibit invasion in human breast cancer cells. *Cancer Biology & Therapy,* Vol. 6, No. 12, pp. 1938-1943, 4974

Thangapazham, R., Singh, A., Sharma, A., Warren, J., Gaddipati, J. & Maheshwari, R. (2007b). Green tea polyphenols and its constituent epigallocatechin gallate inhibits proliferation of human breast cancer cells in vitro and in vivo. *Cancer Letters,* Vol. 245, No. 1-2, pp. 232-241, 0304-3835

Thomas, S. L., Zhong, D., Zhou, W., Malik, S., Liotta, D., Snyder, J. P., Hamel, E. & Giannakakou, P. (2008). EF24, a novel curcumin analog, disrupts the microtubule cytoskeleton and inhibits HIF-1. *Cell Cycle,* Vol. 7, No. 15, pp. 2409-2417, 1551-4005

Umekita, Y., Ohi, Y., Sagara, Y. & Yoshida, H. (2002). Overexpression of cyclinD1 predicts for poor prognosis in estrogen receptor-negative breast cancer patients. *Int J Cancer,* Vol. 98, No. 3, pp. 415-418, 0020-7136

Van Aller, G., Carson, J., Tang, W., Peng, H., Zhao, L., Copeland, R., Tummino, P. & Luo, L. (2011). Epigallocatechin gallate (EGCG), a major component of green tea, is a dual phosphoinositide-3-kinase/mTOR inhibitor. *Biochemical and Biophysical Research Communications,* Vol. 406, No. 2, pp. 194-199, 0006-291X

Venkitaraman, R. (2010). Triple-negative/basal-like breast cancer: clinical, pathologic and molecular features. *Expert Rev Anticancer Ther,* Vol. 10, No. 2, pp. 199-207, 1744-8328

Verma, S. P., Salamone, E. & Goldin, B. (1997). Curcumin and genistein, plant natural products, show synergistic inhibitory effects on the growth of human breast cancer

MCF-7 cells induced by estrogenic pesticides. *Biochem Biophys Res Commun,* Vol. 233, No. 3, pp. 692-696, 0006-291X

Vlerken, L., Vyas, T. & Amiji, M. (2007). Poly (ethylene glycol)-modified nanocarriers for tumor-targeted and intracellular delivery. *Pharmaceutical Research,* Vol. 24, No. 8, pp. 1405-1414, 0724-8741

Walker, E., Pacold, M., Perisic, O., Stephens, L., Hawkins, P., Wymann, M. & Williams, R. (2000). Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Molecular Cell,* Vol. 6, No. 4, pp. 909-919, 1097-2765

Waris, G. & Ahsan, H. (2006). Reactive oxygen species: role in the development of cancer and various chronic conditions. *J Carcinog,* Vol. 5, No. pp. 14, 1477-3163

Wu, J. T. & Kral, J. G. (2005). The NF-kappaB/IkappaB signaling system: a molecular target in breast cancer therapy. *J Surg Res,* Vol. 123, No. 1, pp. 158-169, 0022-4804

Yadav, B., Taurin, S., Rosengren, R. J., Schumacher, M., Diederich, M., Somers-Edgar, T. J. & Larsen, L. (2010). Synthesis and cytotoxic potential of heterocyclic cyclohexanone analogues of curcumin. *Bioorg Med Chem,* Vol. 18, No. 18, pp. 6701-6707, 1464-3391

Yallapu, M. M., Gupta, B. K., Jaggi, M. & Chauhan, S. C. (2010). Fabrication of curcumin encapsulated PLGA nanoparticles for improved therapeutic effects in metastatic cancer cells. *J Colloid Interface Sci,* Vol. 351, No. 1, pp. 19-29, 1095-7103

Yang, H., Sun, D., Chen, D., Cui, Q., Gu, Y., Jiang, T., Chen, W., Wan, S. & Dou, Q. (2010). Antitumor activity of novel fluoro-substituted (-)-epigallocatechin-3-gallate analogs. *Cancer Letters,* Vol. 292, No. 1, pp. 48-53, 0304-3835

Youssef, K. M. & El-Sherbeny, M. A. (2005). Synthesis and antitumor activity of some curcumin analogs. *Arch Pharm (Weinheim),* Vol. 338, No. 4, pp. 181-189, 0365-6233

19

New Experimental Therapies Targetting Breast Cancer Cell

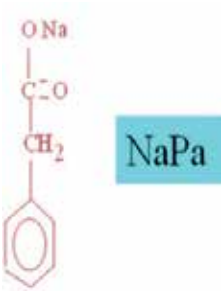
Di Benedetto Melanie
INSERM U940/CSPBAT, Université P13, France

1. Introduction

Proliferation/survival, migration and invasion are processes common to both primary tumor angiogenesis and metastases formation. Among the treatment approaches being investigated, the most developed is the use of genomics and proteomics research to assist the identification of unique targets involved in tumor angiogenesis or invasiveness. In addition, metastasis in breast cancer patients accounts for over 90% of the deaths. Preclinical studies reveal that many drugs used in the management of primary tumors are not or little effective against metastasis (Perret & Crepin, 2008). Although the mechanism by which metastases develop is still not fully understood, it is generally believed that tumor cells acquire features that affect their metastatic potential during the progression of the tumor; these features include increased survival, invasive and migratory abilities of breast cancer cells. Breast cancer progression is a complex cascade of sequential steps, none of which being fully understood. Many studies implicated stroma in the development of metastases. Stroma and cancer cell interactions were found to contribute to cell detachment from primary tumors, intravasation into the blood stream, and extravasation at distant sites where tumor cells can seed and form tumor metastases (Shekhar et al., 2003). Previously, fibroblasts, endothelial cells and macrophages and other stroma cells were reported to be implicated in the occurrence of metastases (Cunha et al., 1992; Haslam et al., 2001; Shekhar et al., 2003). In these conditions, new strategies as well as the identification of novel therapeutic targets will be needed to effectively target the interactions between stroma and tumor cells via growth factor. Thus, it is important to take into account not also the context near tumor cells (like growth factors as well as chemokines) but also the distribution of the tumor cells in the metastatic sites.

As of today, the poorly predictive preclinical models, lack of tumor target specificity, lack of effective cellular and intracellular delivery, development of resistance have slowed down the progress in anti-cancer therapy. Although the pharmaceutical industry prefers to develop small molecule therapy that can be orally administered to patients, it is now admitted that development of new drug delivery system strategy is essential to increase therapeutic index. Synthetic and natural polymers have an established role as in several biomedical applications, including their use as prosthesis or implants (Anderson, 2001). During the past decade, polymer implants have been used in cancer therapy to treat locally hormone-dependent tumors (Zoladex, Lupron depot) or brain tumors by implanting chemotherapy delivering polymer post-surgically. Over than 10 water-soluble polymer

drugs conjugates have entered in phase I/II clinical trials as I.V. administrated anticancer agents. These include six conjugates based on methacrylamide polymers (HPMA). These polymers have been developed in the basis of the achieved tumor–specific targeting by the enhanced permeability and retention effect (Ducan et al., 2005; Matsumura et al., 1986). This increasing tumor retention has been observed and attributed to the better extravasation in the blood vessel of macromolecules and the absence of their drainage release (Noguchi Y et al., 1998; Seymour et al., 1995). HPMA copolymer conjugate with chemotherapeutic agents as doxorubicin has shown high retention and efficacy toward tumors without side effects (Ducan et al., 2005). The specific tumor cell targeting can also be attributed to the interaction of copolymers and heparin growth factors which are highly expressed in the tumor cell environment. Better retention is also attributed to the endocytic internalisation of conjugates which allows also to bypassing MDR efflux responsible for drug resistance. Also, another type of natural polymer interesting to be evaluated is the glycoaminoglycans analogs since it is well-known that they importantly interact with growth factors and receptors on the cell membranes. In another hand, the different type of glycosaminoglycans on the cell surface support in relation with normal or tumoral statues supported their involvement. We have recently developed the two kinds of copolymers that show interesting results in basis of the possibility to functionalize them with active biomolecules. Among active biomolecules, the interesting ones are those which inhibited the ancrage of rho/ras signaling molecules to tumor cells since this pathway plays a key role in invasion, migration and proliferation of tumor cells. The inhibition of the prenylation of ras and rho leads to the blocking of ancrage to the membrane. We have focused our attention on two types of such small molecules. The first one, phenylacetate (NaPa), comes from the metabolism (Fig. 1). NaPa, which has been originally used for urea children disorders (Samid et al., 1992, 1993) has since been demonstrated to efficiently inhibits cancer cell lines proliferation *in vitro*. The second small molecules are bisphosphonates (PBs, fig 1), which are mostly known for their efficacy in the treatment of bone disorders and are also efficient *in vitro* in inhibiting cancer cell proliferation. For both molecules, the main obstacle with their use in cancer therapy stems



NaPa

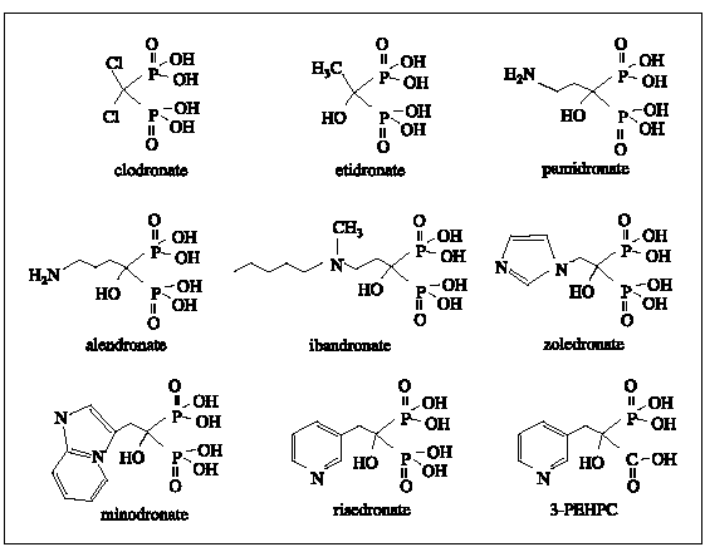


Fig. 1. Phenylacetate (NaPa) and bisphosphonates (BPs) molecules.

from the high concentrations (up to micromolars) that are needed to achieve efficacy in vivo. Since we think that these molecules remain of potential use in cancer because they target specific step involved in both primary tumor growth and metastasis formation, we are developing new strategies that aim to increase their efficacy using drug delivery systems in specific cancer cells localized in specific sites. Herein, we will present all these strategies, first by using polymers for NaPa, and secondly using chemical transformation of the compounds, in particular esterification for the bisphosphonates. Also we will present possible future directions, such as the use of new polymers as well as new delivery systems like nanotechnologies.

2. Glycoaminoglycan polymer strategy

2.1 Carboxybenzylamide dextran (CMDB) and NaPa combination

Carboxymethyl benzylamide dextran derivative (in particular CMDB7) inhibits breast cancer cell proliferation *in vitro* and in nude mice (Bagheri-Yarmand et al, 1992, 1997, 1998a, b, 1999). This *in vitro* effect is associated with a decrease in the S-phase cell population and with an accumulation of cells in G1 phase of cell cycle (Bagheri-Yarmand et al, 1992). CMDB7 disrupts the mitogenic effect of growth factors by preventing their binding to specific receptors as reported for Fibroblast Growth Factor-2 and -4 (FGF2, FGF4, Bagheri-Yarmand et al, 1998a), Platelet-Derived Growth Factor-BB and Transforming Growth Factor-β1 (PDGF-BB, TGFB Bagheri-Yarmand et al, 1998b). *In vivo*, CMDB7 treatment reduces the growth of MCF-7ras (Bagheri-Yarmand et al, 1998b) and FGF4-transfected HBL100 xenografts and decreases the tumor angiogenesis (Bagheri-Yarmand et al, 1998a). Sodium phenylacetate (NaPa), a physiological metabolite of phenylalanine, is normally found in human plasma at micromolar concentrations. At higher concentrations, NaPa is reported to induce the cytostasis and the reversion of malignant phenotype of different cancer cells *in vitro* (Samid et al, 1993, 1994, 1997, 2000; Adam et al, 1995). Furthermore, NaPa is described to modulate the synthesis and/or the release of some growth factors (Ferrandina et al, 1997; Thibout et al, 1998) and to increase, in synergistic manner, the effect of some molecules affecting the growth factor pathways (Prasanna et al, 1996; Samid et al, 1993). For example, NaPa potentiates the antitumor activity of tamoxifen by increasing apoptosis in breast cancer xenografts in nude mice. Finally, NaPa has been used in phase I and II clinical trials on patients with malignant tumors (Chang et al., 1999; Thibault et al, 1994). In basis of this data, we have evaluated *in vitro* and *in vivo* the efficacy of combined treatment with NaPa and an industrial dextran derivative LS4 (Sterilyo Laboratories) whose composition is similar to CMDB7 one, on breast cancer cell growth. We have used the MCF-7ras cell line obtained by transfection of MCF-7 cells, isolated from pleural metastasis of breast adenocarcinoma, with v-Ha-ras oncogene. The MCF-7ras cells secrete high quantities of TGFα, TGFβ, epithelial growth factor (EGF) and insulin growth factor (IGF) (Albini et al, 1986). This cell line represents an oestrogen-independent cellular model corresponding to some malignant breast tumors (Spandidos and Agnantis, 1984) and does no require oestrogen supplementation to induce a high incidence of tumors in nude mice (Sommers et al, 1990). The analysis of CMDBLS4-NaPa combination effect is performed by the isobole method.

NaPa enhances the dextran derivative CMDBLS4 antiproliferative effect on breast cancer MCF-7ras cells both *in vitro* and *in vivo*. Indeed, NaPa or CMDBLS4, delivered alone for 7 weeks, inhibits MCF-7ras tumor growth by 60% and 40%, respectively, while the CMDBLS4-NaPa combination decreases MCF-7ras tumor growth by 83% without any toxicity. The

effectiveness of the NaPa and CMDBLS4 combination can be explained by their distinct mechanisms of action. MCF-7ras breast cancer cells secrete an important amount of mitogenic growth factors such as TGFβ and PDGF (Bronzert et al, 1987; Dickson et al, 1987; Knabbe et al, 1987). The mitogenic effects of these growth factors can be reduced by inhibition of their synthesis or/and their action on target cells. Treatment of cells with NaPa decreases the mitogenic activity of MCF-7ras conditioned medium on BALBc/3T3. The possible mechanism is a modulation of the synthesis and the release of growth factors like TGFβ in MCF-7ras breast cancer cells (Thibout et al, 1998). CMDBLS4, when added to conditioned medium, inhibits conditioned medium mitogenic effect on BALBc/3T3 fibroblasts. This finding argues for CMDBLS4 interactions with growth factors contained in CM. Indeed, previous studies have shown that dextran derivatives interact with heparin-binding growth factors like TGFβ, PDGFBB or FGF-2 and inhibit their mitogenic effect (Bagheri-Yarmand et al, 1998a, b). All our and others' observations suggest that NaPa and CMDBLS4 act on distinct targets involved in the tumor development. NaPa alters the mitogenic growth factor production and renders the tumor cells quiescent in the G1 phase while CMDBLS4 interacts with MCF-7ras growth factors and inhibits their mitogenic activities.

aCMDBLS4 (Ac; mM)	**NaPa (Bc; mM)**	**% I**	**CMDBLS4 (Ae; mM)**	**NaPa (Be; mM)**	**D**
48 h					
3.7	0.75	33	18.5	17	0.44b
7.4	1.5	45.8	>18.5	30	0.45b
14.8	3	48.1	>18.5	32.1	0.89
18.5	4	50	>18.5	34	1.12
72 h					
3.7	0.75	51.4	>18.5	20.6	0.23b
7.4	1.5	54.8	>18.5	23	0.46b
14.8	3	53.3	>18.5	24	0.93
18.5	4	60.2	>18.5	29	1.13

Table 1. Effects of NaPa and CMDBLS4 combination with a ratio = 5 on MCF-7ras cell proliferation. [a] Ac and Bc, concentrations of agents A and B used in the combination treatment; Ae and Be,concentrations of agents A and B able to produce the same magnitude of effect if used individually. D combination index If D=1 the effect is additive, id D<1, the effect is synergistic.[b] $P < 0.05$. % of inhibition determined by MTT assay:[1–(absorbance of cells in medium containing agents/absorbance of cells in control culture medium)]× 100.(Di Benedetto et al., 2001).

2.2 Carboxybenzylamide –phenylacetate dextran (NaPaC)

NaPa molecule enhances, in a synergistic or additive manner, the inhibitory effect of CMDB on breast cancer cell growth *in vitro* and in nude mice when administrated at a CMDB/NaPa ratio of 4 (Di Benedetto et al., 2001). To obtain a new drug with the same properties but easier to use as a future anti-cancer molecule than the combined treatment, we have performed the esterification of CMDB by NaPa respecting the synergistic CMDB/NaPa ratio. We have then investigated the *in vitro* and *in vivo* effects of this new

dextran derivative, phenylacetate carboxymethyl benzylamide dextran, named NaPaC, on breast cancer cell proliferation as well as its apoptotic and anti-angiogenic effects.

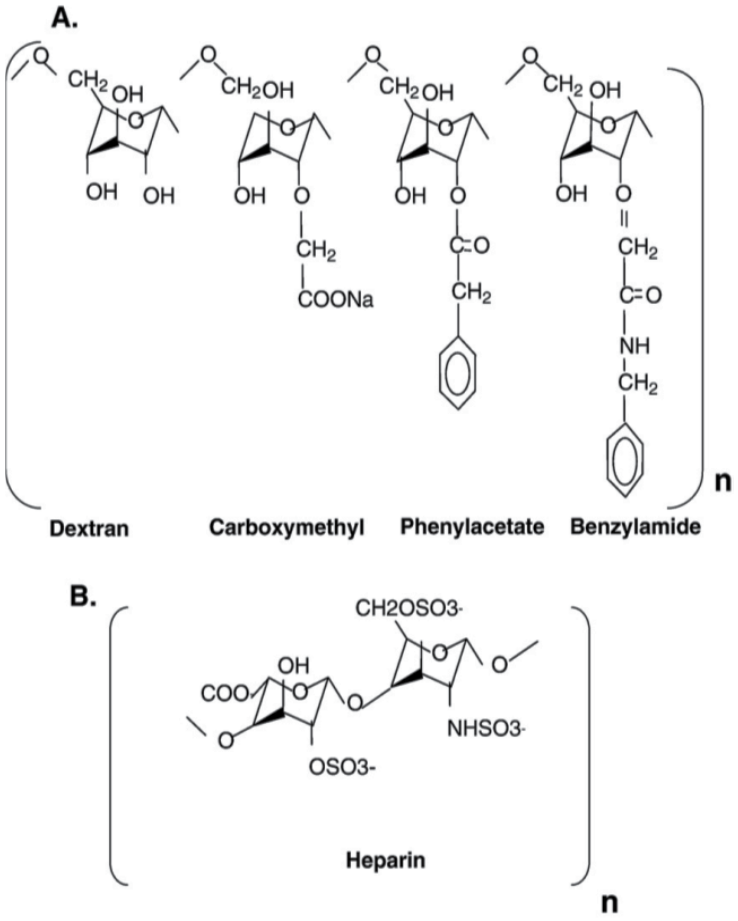


Fig. 2. Carboxymethylbenzylamide-NaPa (NaPac) (A) and heparin (B) molecules (Di Benedetto et al., 2002).

Interestingly, NaPaC inhibits the growth of breast cancer MCF-7ras cells at a concentration lower than CMDB or NaPa. The comparison of IC50 for three drugs supplies the additional evidence for the highly enhanced efficiency of NaPaC as compared to CMDB and NaPa (Table 2). Therefore, the hybrid molecule retains at least the additive effect of its two components observed previously (Di Benedetto et al., 2001). This effect is not only specifc to MCF-7 ras cells as similar results are obtained for other breast cancer cell lines, including MCF-7, MDA-MB-231 and MDA-MB-435. NaPaC inhibited in vivo MCF-7ras tumor growth more efficiently and at lower dose than CMDB or NaPa. This can be explained by the fact that NaPaC gathers the antiproliferative, aponecrotic and anti-angiogenic actions generally admitted to lead *in vivo* to concerted inhibition of tumor growth. The inhibition of the endothelium growth, causing the impaired delivery of nutrients and oxygen to tumor, leads to tumor cell death. Indeed, we observed *in vivo* that the inhibition of MCF-7ras tumor

growth by NaPaC is concomitant with a poor microvessel density (as compared to control) at short-time treatment and with multifocal necrotic areas at long-term administration. Therefore, NaPaC is more efficient than CMDB or NaPa and decreases the microvessel density at dose lower than its components.

Treatment	Tumor (volume) at Day 1 (mm^3)	Tumor (volume) at day 5(mm^3)	Tumour growth (%)
Control	221±90	2103±328	-
NaP4 40 m/ kg (0.25 mmol/ kg)	157±30	990±192	57
CMDB 150 mg/kg (1.85 µmol/kg)	199±70	1326±281	33
NaPaC 15 m/kg (0.18 mmol/ kg)	167±40	717±203	66

Table 2. Inhibition of MCF-7ras tumor growth by NaPaC and its components, CMDB and NaPa. MCF-7ras cells were inoculated s.c in nude mice near the fad pad. After tumor uptake, the animals were treated for 7 weeks with NaPa (n=10), CMDB (n=10) and NaPaC (n=10). Tumor volume (+s.e.m.) for different experimental groups were compared to control (Di Benedetto et al., 2002).

Of note, no necrotic areas were detected in tumors treated with CMDB or NaPa alone. In accord with our *in vivo* results on tumor neovascularization, NaPaC *in vitro* inhibits the growth of human endothelial cells more efficiently that CMDB or NaPa. The mechanisms involved in CMDB and NaPa actions on endothelial cell proliferation seem to be distinct. CMDB interacts directly with VEGF165 (Hamma-Kourbali et al., 2001), the most specific angiogenic factor (Plouet et al., 1989) and inhibits the VEGF165-induced HUVE-C-C growth.

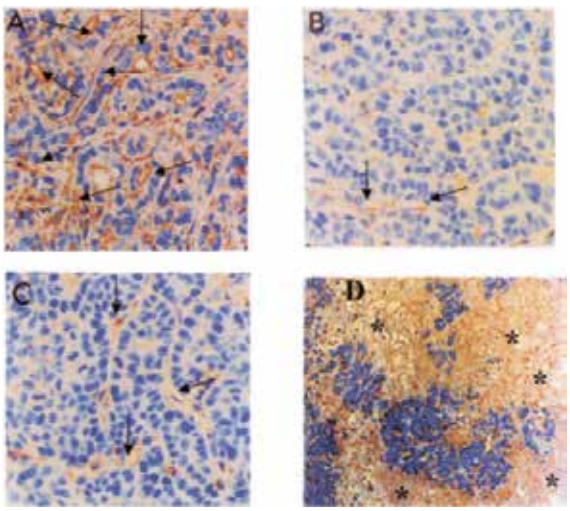


Fig. 3. Inhibition of MCF-7ras tumor angiogenesis by NaPaC, CMDB and NaPa. Microvessel staining of tumors untreated (A) and treated with 150 mg/kg CMDB (B), 40 mg/kg NaPa (C) or 15 mg/kg NaPaC (D), twice a week, for 7 weeks, was performed using GSL-1 lectin. Necrotic areas in panel d are indicated with the asterisks. Magnification X250 was used for panels A,B and C, and magnification X100 was applied for panel D. The representative microvessels in panels A±C are marked with the arrows (Di Benedetto et al., 2002).

In our laboratory, we observe that NaPa do not interact with VEGF 165 molecule, and has no effect on VEGF165-dependent cell growth. Up to date, the mechanism of NaPa action on HUVE-C-C growth is unknown. Concerning NaPaC, it inherits the CMDB ability to interact with angiogenic growth factor and blocks the VEGF165-induced endothelial cell proliferation at lower concentration than CMDB (Fig. 4). However, the involvement of the NaPa mechanism in NaPaC action on HUVE-C-C growth is still unknown.

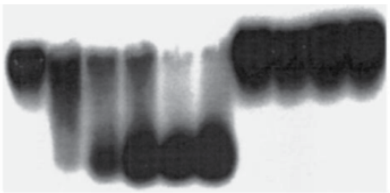


Fig. 4. NaPaC directly interacts with VEGF165. After a 1 h incubation of 125I-VEGF165 (105 c.p.m.=3 ng) with NaPaC or NaPa at 48C, the mixtures (10 ml) were electrophoretically analysed in non denaturant 1% agarose gel at pH 7.0. Lane 1 represents the migration of 125I-VEGF165 alone; lanes 2 ± 6 correspond to 0.6, 1.8, 5.5, 15 or 48 mM NaPaC; lanes 7 ± 10 represent the shifts after addition of 1.0, 5.0, 10 or 20 mM NaPa (Di Benedetto et al., 2003).

Finally, it is noteworthy that NaPa treatments at high concentrations can induce pathological effects (Thibault et al., 1994; Chang et al., 1999). The use of non-toxic new molecule, NaPaC, should limit side effects of NaPa and increase its therapeutic efficacy. In conclusion, NaPaC provides additional interesting clues in developing new anti-cancer drugs with specific triple activity: antiproliferative, aponecrotic and anti-angiogenic. The inhibition of angiogenesis is crucial for blocking tumor progression since tumor-associated high-density neovascularization is responsible for development of metastases.

3. Bisphosphonate esterifications

Bisphosphonates (BPs) have long been used in metabolic bone disease as osteoporosis, tumor-associated hypercalcaemia and metastases-induced osteolysis due to their ability to inhibit bone resorption. BPs are able to bind divalent cations like Ca^{2+} or zinc, constituting the basis of their bone-targeting property and their inhibition of the proteolytic activity of matrix metalloproteinases (MMP), respectively. The nature of their side chains gives rise to a variety of possible structures and stereochemistry determining their different potencies (Clezardin et al., 2005; Cleardin et al., 2003; Caraglia et al., 2006; Green, 2003). Non-nitrogen containing BPs (non N-BPs) act by forming non hydrolysable ATP-analogues and are less effective than nitrogen-containing BPs (N-BPs) in inhibiting bone metastasis (Roger et al., 2003). However, Zoledronate treatment of patients are reported to induce toxic side effect characterised by osteonecrosis of the jaw while non N-BP did not produce this effect (Van den Wingaert, 2006; Diel et al., 2007). N-BPs, such as zoledronate, act on the mevalonate pathway, inhibiting the farnesyl diphosphate synthase (FPP) and thereby depleting the cells of the farnesyl (FPP) or geranylgeranyl (GGPP) diphosphate isoprenoids (Roelofs et al., 2006). Isoprenoids are required for translocation and anchorage of small G proteins like Rho or Ras to the plasma membrane assuring their ultimate involvement in signal transduction during several important normal and tumor cellular pathways. However, *in vivo* efficacy of all BPs on extra-osseous sites or primary tumors is still debated. Only a small number of studies have demonstrated their *in vivo* antiproliferative activity on tumors or metastasis

present in soft tissues (Stresing et al., 2007). The reasons are the poor oral bioavaibility (0.3-7% in humans) due to chelation of metal ions by phosphonic acid group inside the digestive lumen, poor membrane permeability due to poor BP lipophilicity as well as strong uptake by bone tissue (Ezra & Colomb, 2000). Previously, new strategy to overcome BP hydrophilicity is masking the phosphonic acid with organic protecting groups and introducing hydrophobic functions in the side chain (Migianu et al., 2005). An esterified BP with methyl group displays antitumor growth and antiangiogenic activities on A431 tumors, being more effective *in vivo* than *in vitro* (Ledoux et al., 2006). In order to further increase the lipophilicity of BPs (and their entering into the cells), we have synthesized new aromatic 1-hydroxymethylene-1,1-bisphosphonic acids containing phenyl or halogen phenyl ring in their side chains. Interestingly, these compounds exhibit potent antiproliferative activities *in vitro* on human epidermoid A431 cells (Guenin et al., 2005). In parallel, recently crystallographic and computational investigation reveal that the presence of phenyl ring in the side chain permitted non N-BPs to interact with farnesyl enzyme (Mao et al., 2006). Based on these data, we have synthesized a class of BPs that contains bromobenzyl in their side chains (BP7033Br, Fig. 5), as well assymmetrically esterified one of each phosphonic acids with aromatic groups (BP7033Br ALK, Fig. 5).

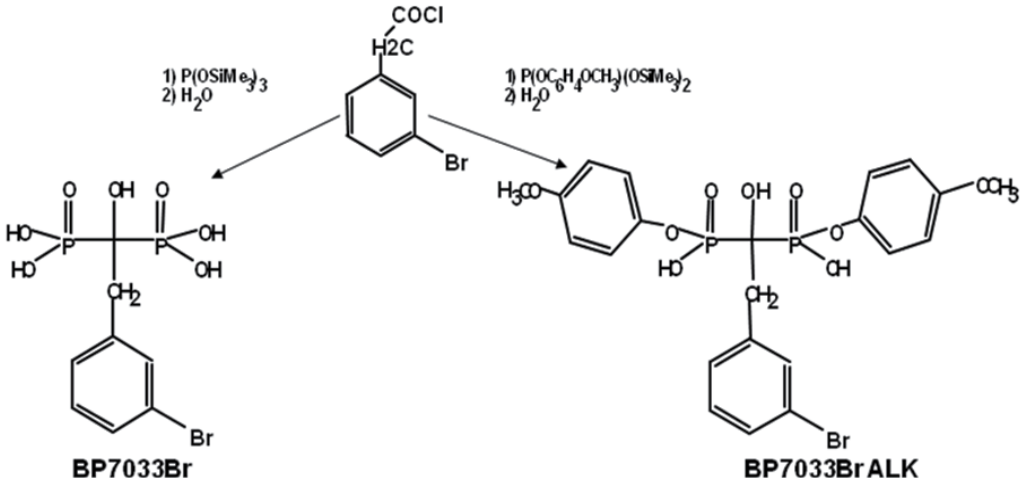


Fig. 5. Chemical structure of BP7033Br and BP7033Br ALK (Abdelkarim et al., 2009).

BPs represents an emerging class of drugs for cancer therapy and new class of non-N-BPs which exhibits higher antiproliferative activities on breast cancer cells compared to previously described non-N-BPs such as clodronate (Journe et al., 2004). Both types of m-bromobenzyl BPs inhibit the viability of several breast cancer cell lines with different estrogen-receptor statuses (Fig. 6). The esterified BP is the more effective on estrogen-responsive cells since the maximal inhibition is reached at 250 µM in contrast to non esterified BP that does not induce maximal inhibition even at 1 mM. In addition, at 250 µM, BP7033Br ALK is effective on cells independently from the estrogen-receptor status. Both types of our BP inhibits viability of estrogen non-responsive cells and particularly that of MDA-MB-231 and D3H2LN cells, the last cell line being the more aggressive ones. Indeed, it is worth to note that dramatic improvement of antiproliferative effect of non-N-BPs on breast cancer cells is reached since clodronate at the same concentration range (200 µM) and

the same time-treatment (72h) do not reduce MDA-MB-231 cell viability (Fromigue et al., 2000; Monkkonene et al., 2008; Senaratne et al., 2000). In addition, clodronate demonstrates mitogenic effects via MCF7 estrogenic receptor (Journe et al., 2004) and we never observe this effect with our BPs. Based on our results, it appears also that BP7033Br ALK antiproliferative effect is estrogen-receptor-independent. The occurrence of this new effect of non-N-BPs could result from the addition of aromatic functions in the side chain. Heterocycle in the side chain is implicated in the induction of cell apoptosis by preventing the prenylation of signalling proteins such as Ras or Rho (Luckman et al., 1998). Inhibition of Ras processing using non bromo-containing BP7033 is also reached (Hamma-kourbali et al., 2003). Also, the addition of phenyl function in the side chain of BPs rendered the catalytic pocket of geranyl and/or farnesyl synthase enzymes of the mevalonate pathway more accessible (Mao et al., 2006; Steeg et al., 2006). BP7033Br ALK reduces MDA-MB-231 and D3H2LN cell viabilities about 90% with a concentration 4-fold inferior to that of BP7033Br.

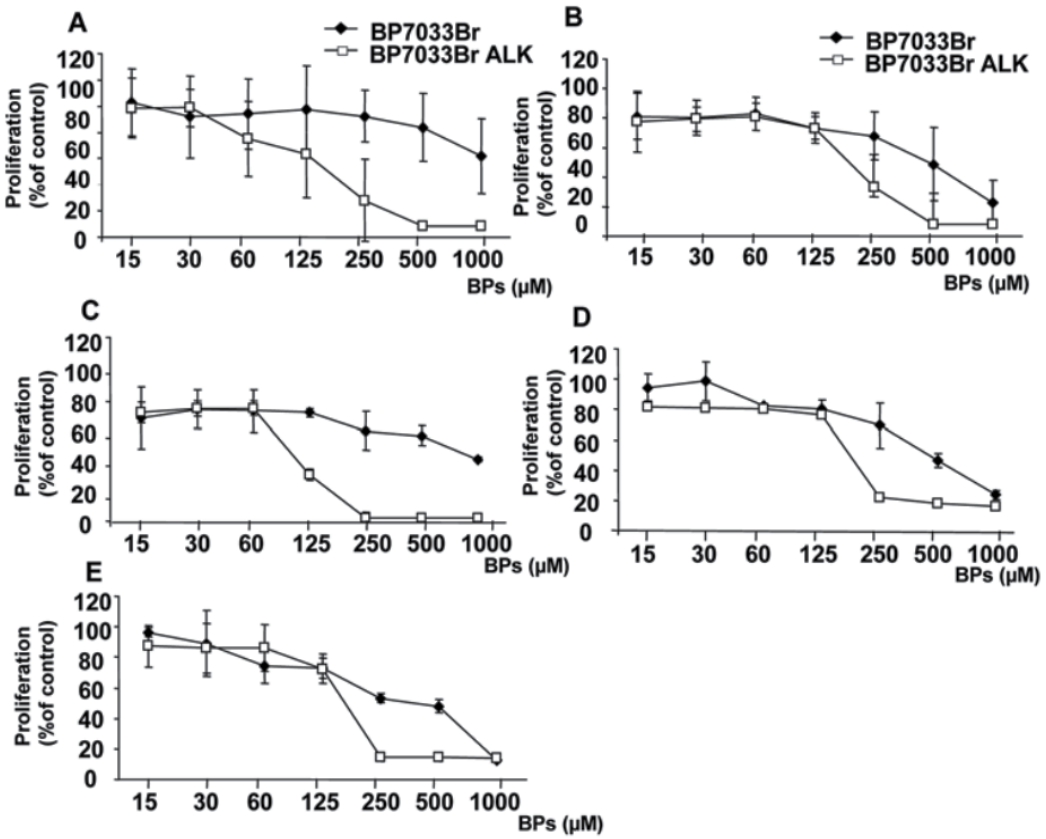


Fig. 6. BP7033Br and BP7033Br ALK inhibited viability of different breast cancer cells. T47D (A), MCF-7 (B), SKBR3 (C), MDA-MB-231 (D) or D3H2LN cells (E) ($1x10^5$) were treated with BP7033Br and BP7033Br ALK at increasing concentrations for 72h. Then, the cells were washed and incubated with 0.1 mL of MTT (2 mg/mL) for 4 h. Optical density was measured at 570 nm using a Labsystems Multiskan MS microplate reader. Data represents the mean value (± SD) of three independent experiments (Abdelkarim et al., 2009).

These data are in agreement with previous results on epidermoid A431 cell proliferation that show a beneficial effect of esterification of the phosphonic groups (Guenin et al., 2005; Ledoux et al., 2006). Our hypothesis is that such esterified compounds rendered BPs more hydrophobic increasing their cell uptake and could therefore act like prodrugs releasing active BP into the cells. In accordance, characterisation of the hydrophilicity demonstrated a shift toward lipophilicity of the BP7033Br ALK compound (Log P values of -0.75 versus -031, respectively). Alternatively, one could also hypothesize that esterified BPs have their own mechanisms since they block the cells into the S phase while non esterified BPs inhibits the G0/G1 cell phase transition. On the other hand, both type of BPs (esterified or not) induce cell death apoptosis of both MDA-MB-231 and D3H2LN cells. These results are interesting since D3H2LN cells have a greater metastatic potential than MDA-MB-231 and consequently could be more resistant to apoptosis, as it is described for metastatic cells (Wang et al., 2002; Winter et al., 2008). Both BPs induce strong D3H2LN metastatic cell apoptosis but the concentration of the esterified analogue used is 2-fold lower. Also, the two BPs inhibits migration of MDA-MB-231 cell lines with a more important effect obtained with BP7033Br ALK. In contrast, BP7033Br ALK is less effective in inhibiting D3H2LN cell lines invasion concomitant with a less important effect on MMP-9 and MMP-2 activities. BP7033Br strongly inhibits MMP-9 and MMP-2, the major form of metalloproteinases present in extracellular matrix. Since MMPs are zinc-dependent endopeptidases, we speculate that the reduction of BP7033Br ALK effect could be due to a reduction of available phosphonic acid groups able to chelate zinc and consequently inhibit MMPs. These results are in agreement with previous studies which show that MMPs inhibition activity by BPs is related to zinc chelation (Clezardin et al., 2003). However, we hypothesise that release of BP7033Br ALK with free phosphonic group could be more important in *in vivo* system because of the presence of phosphodiesterases in serum.

BPs antitumor effects have been mainly observed on D3H2LN xenografts growth and metastasis (Fig. 7). D3H2LN cells have been derived from a MDA-MB-231 subclone isolated from a lymph node metastases and induce an increased xenograft tumor growth as compared to parental cells when injected *in vivo (*Jenkins et al., 2005). Both BP7033Br and BP7033Br ALK inhibit D3H2LN tumor growth after intratumoral injection of about 286 µg BPs per mouse twice a week during 21 days. We establish that this new class of BPs is the most potent among the current non N-BPs since clodronate, even used at 1600 mg twice daily during several weeks (as compared to BP7033 ALK corresponding human dose of 770 mg twice a week during only 2 weeks) fails to reduce primary tumor growth (Winter et al., 2008). Also, BPs are 10-fold more potent than the non halogenated phenyl analogues (Sebbah-Louriki et al., 2002). In addition, BP7033Br ALK better inhibits D3H2LN vessel density than BP7033Br. This point is also supported by the large necrosis area not detected in BP7033Br treated tumors. In addition, we cannot exclude that these large necrosis areas could also be due to a greater amount of esterified BP penetrating into tumor to induce cell death. As compared to N-BPs, it is noteworthy that risedronate or ibandronate failed to inhibit MDA-MB-231 tumor growth in nude mice (Higara et al., 2001, Sasaki et al., 1995). Furthermore, no pre-clinical works demonstrate an antiproliferative effect of zoledronate on primary breast tumor growth in nude mice. The only study demonstrating an inhibition effect of zoledronate on primary tumor growth uses mesothelioma tumors which involves calcification that could uptake the drug (Wakchoure et al., 2006) In addition, the efficacy of zoledronate on bone metastasis seems to be supported by its affinity for osseous tissues rather than its direct antiproliferative effect on tumor cells (Winter et al., 2008).

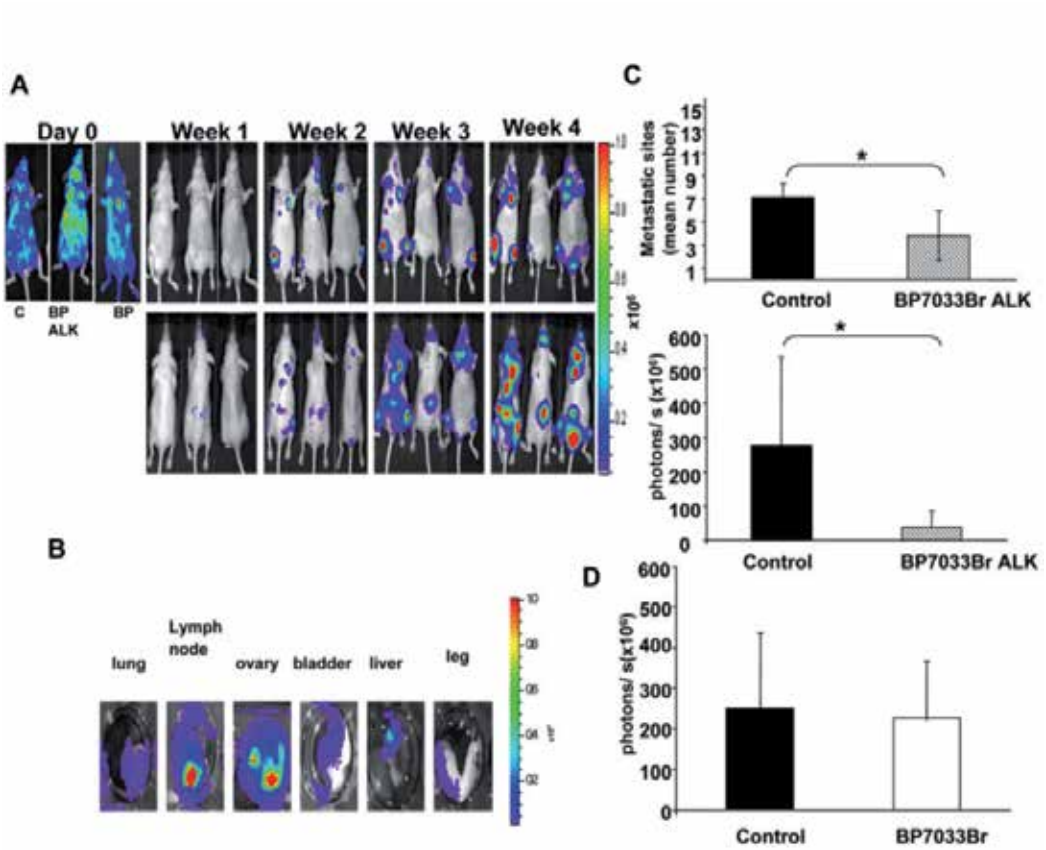


Fig. 7. Only BP7033Br ALK inhibited D3H2LN metastasis. D3H2LN cells were injected into the left ventricle of nude mice (n=7). Day 0 showed the successful intracardiac cells injection. Within 2 weeks, when metastasis were initiated, mice were treated with BP7033Br ALK or BP7033Br (A). At the indicated days, the bioluminescence images were acquired for control (c, left panel) and BPs treated mice (BP7033BrALK and BP7033Br middle and right panel, respectively). *Ex vivo* data confirm soft tissue metastasis from D3H2LN cells injection (B). Quantification of the mean metastatic sites and the photons/s after BP7033Br ALK treatment (C). Quantification the photons/s after BP7033Br treatment (D). Each column represents a mean (±SD) of three independent experiments. $*P_{\text{versus control}} < 0.05$ (Abdelkarim et al., 2009).

Also, zoledronate is a compound rapidly eliminated from plasma, resulting in renal excretion, rapid bone or calcified tissues uptake and accumulation partly due to its phosphonic functions. We have recently showed that the symmetrical esterification of the phosphonic groups may improved BPs soft tissue bioavaibility limiting osseous or calcified tissue uptake (Ref). Also, as their chemical structures are close to the apomine BP, which presents aliphatic ester group, their half-life are expected to be close to that found for this drug (156 h with micromolar plasma concentration, (Alberts et al., 2001). Thus, esterified BP7033Br ALK abrogates angiogenesis, both soft tissue and bone metastasis whereas BP7033Br does not. Noteworthy, in BP7033Br treated mice, luminescence signalling of leg osseous metastasis is not significantly reduced because 2/7 mice do not respond to the

treatment in contrast to BP7033Br ALK treatment that induces significant reduction (Ref). Indeed, the esterified functions seem to be important for the BPs distribution within the systemic system and less for local injection (subcutaneous tumors) since the two N-BPs studied both inhibit D3H2LN xenograft growth. Of note, N-BPs induced osteonecrosis of treated patients (Diel et al., 2007) whereas no apparent side effects have been observed with non N-BPs. Therefore, esterified m-bromobenzyl non N-BPs constitute a new class of drugs with potent direct antitumor, antiangiogenic and antimetastatic efficacy on breast tumors (Abdelkarim et al., 2009).

4. Future directions Polynass/nanotechnologies

4.1 PolyNASS polymers

Polymers display antiproliferative, aponecrotic and anti-angiogenic effects without cytotoxicity on endothelial and cancer cells. Indeed, after 4 days of incubation, only 0,6µM of 20MA/80NaSS is required to induce 85% of cell growth inhibition of MDA-MB-231 cell growth. In contrast and interestingly, in HUVECs cells, polymers 50MA/50NaSS is more efficient with 70% inhibition achieved with 0,06µM (Fig. 8). The effect of 20MA/80NaSS on breast tumor cell is not specific only to MDA-MB-231 cells since it also inhibits other breast cancer cell lines MCF-7 (Skhiri *et al*., 2008).The inhibition achieved with MA/NaSS polymers is comparable to that obtained in non invasive breast carcinoma cell MCF-7 (Skhiri *et al.*, 2008)and the inhibition effect varies in function of percentage of carboxylate and sufonate content in polymers. Interestingly, the strongly percentage of tumor cell proliferation inhibition was observed in presence of 20MA/80NaSS polymer. At 6µM, this polymer induces up to 80% of inhibition of the cellular MDA-MB-231cancer cell proliferation after 4 days of culture. On the other hand, at the same concentration, the polyNaSS effect on cellular proliferation was much lower (60%). Indeed, the presence of two groupings carboxylate and sufonate is necessary and plays an important role in breast cancer anti-proliferation inhibition activity. Also, it seems that similar proportion (50 MA/50 NASS) of functional subunits is rather an interesting chemical composition to obtain the optimal inhibition of endothelial cells proliferation. In addition, it seems that a strong proportion of sulfonate units in polymers is necessary for a high antiproliferative activity in cancer cells while the equal proportion of the functional groups are necessary for the endothelial cells. These results suggest that the mechanism implicated in the inhibition of the two types of cells is different. The effect of these two copolymers on HUVEC and MDA-MB231 cell cycle was also different as a decrease of cells in the S-phase and an accumulation of cells in G0/G1-phase were observed. In conclusion, these new type of polymers could lead to the development of new anti-cancer drugs with specific triple activity: antiproliferative, aponecrotic and anti-angiogenic. Since the development of resistant tumor cell clones can be a serious problem, the inhibition of angiogenesis is crucial for blocking tumour progression since tumor associated high density neovascularization is responsible for development of metastases. Therefore, polymers could be good candidate by selectively inhibiting tumor cells or endothelial cells in basis of their composition.

In addition, these kinds of molecule could be conjugated with specific drugs (copolymers) that will target genes specific to endothelial or tumors cell activities enhancing in this way, the benefit of the therapies. It is to note that in contrast to glycoaminoglycan like polymers, PolyNASS polymer synthesis is better controlled in basis of their chemical composition that is determined by titration of carboxylic groups and ^{1}H NMR.

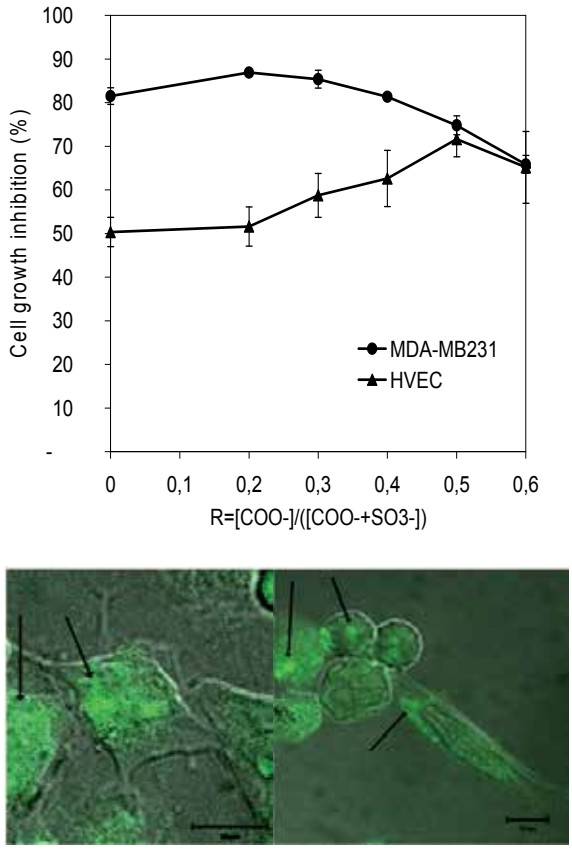


Fig. 8. Stucture-function relationship of PolyNASS copolymers and penetration into MDA-MB-231 tumor (left) and HUVEC endothelial cells (right).

4.2 Nanotechnologies

An innovative multimodal system, which combines magnetic targeting of therapeutic agents with both magnetic resonance and fluorescence imaging into one system has been recently described (Bennayou et al., 2011). This new magnetic nanoplatform consists of superparamagnetic γFe_2O_3 nanoparticles, used clinically as an MRI contrast agent, conjugated to therapeutic molecules of the hydroxylmethylene bisphosphonate family (HMBPs): alendronate with an amine function as the terminal group. *In vitro* tests with breast cancer cells show that the γFe_2O_3@alendronate hybrid nanomaterial reduces cell viability and acts as a drug delivery system. The presence of both γFe_2O_3@alendronate and a magnetic field significantly reduced the development of tumors. The amine functionalities can be used as precursor groups for the covalent coupling of peptides or monoclonal antibodies for specific biological targeting. The feasibility of this process is demonstrated by coupling rhodamine B, a fluorescence marker, to the γFe_2O_3@alendronate nanohybrid. The system shows fluorescent properties and high affinity for cells. Therefore, magnetic and fluorescent nanoparticles are potential candidates for smart drug-delivery systems. Also, the superparamagnetic behaviour of such nanoparticles may be exploited as MRI contrast agents to improve therapeutic diagnostics.

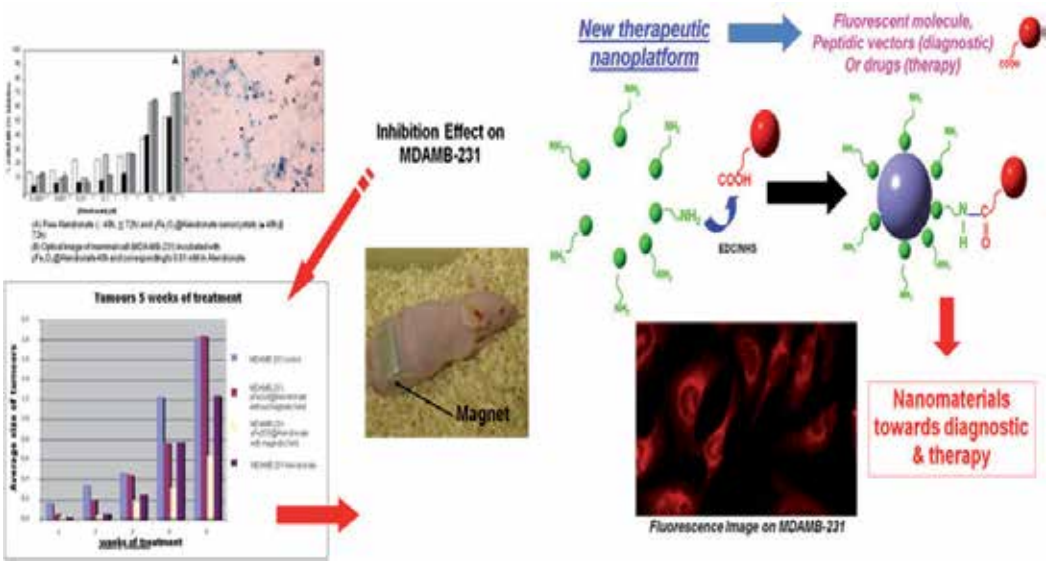


Fig. 9. Nanoplatform showing the possibility to graft BPs on superparamagnetic γFe_2O_3 nanoparticles.

5. References

Abdelkarim M, Guenin E, Sainte-Catherine O, Vintonenko N, Peyri N, Perret GY & Crepin M, Khatib AM, Lecouvey M, Di Benedetto M. (2009) *Plos one.* New symmetrically esterified m-bromobenzyl non- aminobisphosphonates inhibited breast cancer growth and metastases. 4(3):e4685.

Alberts S, Hallum AV, Stratton-Custis M, Garcia D J, Gleason-Guzman M, Salmon SE, Santabarbara P, Niesor EJ, Floret S &Bentzen CL. (2001) *Clin CancerRes.* Phase I pharmacokinetic trial and correlative in vitro phase II tumorkinetic study of apomine (SR-45023A), a novel oral biphosphonate anticancerdrug. 7: 1246–1250.

Anderson JM. (2001) *Annual Rev Mat Res*. Biological responses to materials. 31:81-110.

Bagheri-Yarmand R, Morere JF, Letouneur D, Jozefonvicz J, Israel L & Crepin M (1992) *Anticancer Res.* Inhibitory effect of dextran derivatives in vitro on the growth characteristics of premalignant and malignant human mammary epithelial cell lines. 12: 1641–1646.

Bagheri-Yarmand R, Kourbali Y, Mabilat C, Morere JF, Martin A, Lu LH, Soria C, Jozefonvicz J & Crepin M (1998a). *Br J of Cancer.* The supression of fibroblast growth factor2/fibroblast growth factor 4- dependent tumor angiogenesis and growth by the anti-growth factor activity of dextran derivative (CMDB7).78: 111–118.

Bagheri-Yarmand R, Kourbali Y, Morere JF, Jozefonvicz J & Crepin M (1998b) *Cell Growth Differ* Inhibition of MCF-7ras tumor growth by benzylamide dextran: blockage of the paracrine effect and receptor binding of transforming growth factor β1 and platelet-derived growth factor-BB. 9: 497–504.

Bagheri-Yarmand R, Kourbali Y, Rath AM, Vassy R, Martin A, Jozefonvicz J, Soria C, He L &Crépin M (1999) *Cancer Res.* Carboxymethyl benzylamide dextran blocks

angiogenesis of MDA-MB 435 breast carcinoma xenografted in fad pad and its lung metastases in nude mice. 59: 507–510

Bagheri-Yarmand R, Liu JF, Ledoux D, Morere JF &Crépin M (1997) *Biochem Biophys Res Commun* Inhibition of human breast epithelial HBL 100 cell proliferation by a dextran derivative (CMDB7) with FGF2 autocrine loop. 239:424–428.

Benyettou F, Lalatonne Y, Chebbi I, Benedetto MD, Serfaty JM, Lecouvey M & MotteL. (2011) *Phys Chem Chem Phys.* A multimodal magnetic resonance imaging nanoplatform for cancer theranostics. In press

Bronzert K, Pantazis P, Antoniades HN, Kasid A, Davidson N, Dickson RB & Lippman ME (1987) *Proc. Natl. Acad. Sci.* USA.Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. 84: 5763–5767.

Caraglia M, Santini D, Marra M, Vincenzi B, Tonini G, & Budillon A. (2006) *Endocr Relat Cancer* Emerging anti-cancer molecular mechanisms of aminobisphosphonates. 13: 7–26.

Chang SM, Kuhn JG, Robin HI, Clifford Schold S, Spence AM, Berger MS, Metha MP, Bozik ME, Pollack I, Schiff D, Gilbert M, Rankin C & Prados MD (1999) *J Clin Oncol* Phase II study of phenylacetate in patients with recurrent malignant glioma: a north American brain tumor concortium. 17: 984–990.

Clezardin P, Fournier P, Boissier S, Peyruchaud O (2003) *Curr Med Chem.* In vitro and in vivo antitumor effects of bisphosphonates. 10: 173–180.

Clezardin P, Ebetino FH, Fournier P (2005) Bisphosphonates and cancer induced bone disease: beyond their antiresorptive activity. Cancer Res 65:4971–4974

Cunha GR, Alarid ET, Turner T, Donjacour AA, Boutin EL, Foster BA (1992). *J Androl.* Normal and abnormal development of the male urogenital tract. Role of androgens, mesenchymal-epithelial interactions, and growth factors. 13(6):465-75.

Di Benedetto M, Kourbali Y, Starzec A, Vassy R, Jozefonvicz J,Perret G, Crepin M & Kraemer M. *Br. J. Cancer.* Sodium phenylacetate enhances the inhibitory effect of dextran derivative on breast cancer cell growth in vitro and in nude mice. 85 (2001) 917–923

Di Benedetto M, Starzec A, Colombo BM, Briane D, Perret GY, Kraemer M & Crépin M. Aponecrotic, antiangiogenic and antiproliferative effects of a novel dextran derivative on breast cancer growth in vitro and in vivo. Br J Pharmacol. 135(2002) 1859-71.

Di Benedetto M, Starzec A, Vassy R, Perret GY, Crépin M & Kraemer M. Inhibition of epidermoid carcinoma A431 cell growth and angiogenesis in nude mice by early and late treatment with a novel dextran derivative. Br J Cancer. 16;88 (2003) 1987-94.

Dickson RB, Kasid A, Huff KK, Bates SE, Knabbe C, Bronzert D, Gelman EP and Lippman ME (1987). *Proc Natl Acad Sci USA* Activation of growth factor secretion in tumorigenic,states of breast cancer induced by 17β estradiol or V-Ha-ras oncogene. 84: 837–841.

Diel IJ, Fogelman I, Al-Nawas B, Hoffmeister B, Migliorati C, et al. Gligorov J, Väänänen K, Pylkkänen L, Pecherstorfer M & Aapro MS. (2007) *Crit Rev Oncol Hematol.* Pathophysiology, risk factors and management of bisphosphonate-associated osteonecrosis of the jaw: Is there a diverse relationship of amino- and nonaminobisphosphonates? 64: 198–207.

Ducan R, Vicent MJ, Greco F, Nicholson RI. (2005) *Endocrine-related Cancer* Polymer-drug conjugates: towards a novel approach for the treatment of endocrine-related cancer. 12:S189-S199.

Ezra A & Golomb G (2000) *Adv Drug Deliv Rev* Administration routes and delivery systems ofbisphosphonates for the treatment of bone resorption. 42:175–195.

Ferrandina G, Melichar B, Loercher A, Verschaegen CF, Kudelka AP, Edwards CL, Scambia G, Kavanagh JJ, Abbruzzese JL & Freedman RS (1997) *Cancer Res.* Growth inhibitory effects of sodium phenylacetate (NSC 3039) on ovarian carcinoma cells in vitro. 57: 4309–4315.

Fromigue O, Lagneaux L & Body JJ (2000) *J Bone Miner Res.* Bisphosphonates induce breast cancer cell death in vitro. 15(11): 2211–2221.

Guenin E, Ledoux D, Oudar O, Lecouvey M & Kraemer M (2005) *Anticancer Res.* Structureactivity relationships of a new class of aromatic bisphosphonates that inhibit tumor cell proliferation in vitro. 25: 1139–1145.

Green JR (2003) *Cancer* Antitumor effects of bisphosphonates. 97: 840–847.

Hamma-Kourbali Y, Di Benedetto M, Ledoux D, Oudar O, Leroux Y, Lecouvey M & Kraemer M.(2003) *Biochem Biophys Res Comm.* A novel non-containing-nitrogen bisphosphonate inhibits both in vitro and in vivo angiogenesis. 310: 816–882.

Hamma-Kourbali Y, Vassy R, , Starzec A, Le Meuth-Metzinger V, Oudar O,Bagheri-Yarmand R, Perret G & Crepin M (2001). *J.Biol.Chem.* VEGF165 activities are inhibited by carboxymethyl benzylamide dextran competing for heparin binding to VEGF165 and VEGF165 : KDR complexes. 276, 39748- 39754.

Haslam SZ &Woodward TL (2001) *Breast Cancer Res.* Reciprocal regulation of extracellular matrix proteins and ovarian steroid activity in the mammary gland. 3(6):365-72.

Hiraga T, Williams PJ, Mundy GR & Yoneda T. (2001) *Cancer Res.* The bisphosphonate ibandronate promotes apoptosis in MDA-MB-231 human breast cancer cells in bone metastases.1;61(11):4418-24.

Jenkins DE, Hornig YS, Oei Y, Dusich J & Purchio T (2005) *Breast Cancer Res* Bioluminescenthuman breast cancer cell lines that permit rapid and sensitive in vivo detection of mammary tumors and multiple metastases in immune deficient mice. 7(4): 444–454.

Journe F, Chaboteaux C, Dumon J-C, Leclercq, Laurent G & Body J-J (2004) *Brit J Cancer.* Steroid-free medium discloses oestrogenic effects of bisphosphonate clodronate on breast cancer cells. 91: 1703–1710.

Knabbe C, Wakefield L, Flanders K, Kasid A, Derynck R, Lippman ME andDickson RB (1987) *Cell* Evidence that TGF β is a hormonally regulated negative growth factor in human breast cancer cell lines. 48: 417–428

Ledoux D, Hamma-Kourbali Y, Di Benedetto M, Foucault-Bertaud A, Oudar O, Sainte-Catherine O, Lecouvey M &Kraemer M. (2006) *Anti-Cancer Drugs.* A new dimethyl ester bisphosphonate inhibits angiogenesis and growth of human epidermoid carcinoma xenograft in nude mice. 17: 479–485.

Luckman SP, Hughes DE, Coxon FP, Russell GG & Rogers MJ (1998) *J Bone MinerRes* Nitrogencontaining biophosphonates inhibit the mevalonate pathway and prevent posttranslational prenylation of GTP- binding proteins, including Ras. 7(4): 581–599.

Mao J, Mukherjee S, Zhang Y, Cao R, Sanders JM, Song Y, Zhang Y, Meints GA, GaoYG, Mukkamala D, Hudock MP & Oldfield E. (2006) *J Am Chem Soc.* Solid-state NMR, crystallographic, and computational investigation of bisphosphonates and farnesyl diphosphate synthase-bisphosphonate complexes. 128:14485–14497.

Matsumura Y & Maeda H. *Cancer Res* A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritopic accumulation of proteins and the antitumour agent SMANCS. 6:6387-6392, 1986.

Migianu E, Monteil M, Even P & Lecouvey M (2005) *Nucleosides Nucleotides Nucleic Acids.* Novel approach to nucleoside-59-(1-hydroxymethylene-1, 1-bisphosphonates): synthesis of new AZT analogues. 24: 121–133.

Monkkonen H, Kuokkanen J, Holen I, Evans A, Lefley DV, Jauhiainen M, Auriola S &Mönkkönen J. (2008). *Anticancer Drugs.* Bisphosphonate-induced ATP analog formation and its effect on inhibition of cancer cell growth. 19(4): 391–399.

Noguchi Y, Wu J, Ducan R, Strohalm J, Ulbrich K, Akaike T & Maeda H. (1998) *Japanese J. Cancer Res.* Early phase tumor accumulation of macromolecules: a great difference in clearance rate between tumor and normal tissues. 89: 307-314.

Perret GY & Crepin M (2008) *Fundamental & Clinical Pharmacology.* New pharmacological strategies against metastatic spread. 5: 465-492.

Plouet J, Schilling J, & Gospodarowicz D. (1989). *EMBO J.* Isolation and characterization of a newly identifed endothelial cellmitogen produced by AtT-20 cells. 8, 373 ± 378.

Prasanna P, Thilbault A, Liu L& Samid D (1996) *Clin Cancer Res.* Lipid metabolism as a target for brain cancer therapy: synergistic activity of lovastatin and sodium phenylacetate and phenylbutyrate. 2: 865– 872.

Roelofs AJ, Thompson K, Gordon S & Rogers MJ (2006) *Clin Cancer Res* Molecular mechanisms of action of bisphosphonates: current status. 12: 6222s–6230s.

Rogers MJ (2003) *Curr Pharm Des.* New insights into the molecular mechanisms of action of bisphosphonates. 9(32): 2643–2658.

Samid D, Shack S &Myers CE (1993) *J Clin Invest.* Selective growth arrest and phenotypic reversion of prostate cancer cells in vitro by non toxic pharmalogical concentration of phenylacetate. 91: 2288–2295.

Samid D, Ram Z, Hudgins WR, Shack S, Liu L, Walbindge S, Oldfild EH & Myers CE (1994) *Cancer Res.* Selective activity of phenylacetate against malignant gliomas: ressemblance to fetal brain damage in phenylketonuria. 54: 891–895.

Samid D, Hudgins WR, Shack S, Liu L, Prasanna P & Myers CE (1997) *Adv Exp Med Biol.* Phenylacetate a nd phenylbutyrate as novel, nontoxic differenciation inducers. 400A: 501–505.

Samid D, Wells M, Greene ME, Shen W, Palmer CN & Thibault A (2000). *Clin Cancer Res.* Peroxisome proliferator-activated receptor gamma as a novel target in cancer therapy: binding and activation by an aromatic fatty acid with clinical antitumor activity. 3: 933–941.

Sasaki A, Boyce BF, Story B, Wright KR, Chapman M, Boyce R, Mundy GR 1Yoneda T.(1995) *Cancer res.* Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone. 15;55(16):3551-7.

Sebbah-Louriki M, Colombo BM, el Manouni D, Martin A, Salzmann JL, Leroux Y, Perret GY & Crepin M. (2002) *Anticancer Res.* A new phenylacetate-bisphosphonate

inhibits breast cancer cell growth by proapoptotic and antiangiogenic effectsin nude mice.22(6C): 3925–3931.

Senaratne SG, Pirianov G, Mansi JL, Arnett TR & Colston KW (2000) *Br J Cancer.* Bisphosphonates induce apoptosis in human breast cancer cell lines. 82(8): 1459–1468.

Seymour LW, Miyamoto Y, Brereton M, Subr V, Strohalm J & Ducan R. *European J. cancer.* Influence of molecular size of passive tumor-accumulation of soluble macromolecular drug carriers. 5:766-770, 1995.

Shekhar MP, Pauley R & Heppner G (2003) Breast Cancer Res. Host microenvironment in breast cancer development: extracellular matrix-stromal cell contribution to neoplastic phenotype of epithelial cells in the breast. 5(3):130-5.

Skhiri, L, Pavon-Djavid, G, Helary, G, Bartegi, A, & Migonney, V. *Pathologie Biologie.* New antitumor agent: In vitro activity on breast carcinoma cells,, 2008, 57, 3, Pages 9-15.

Sommers CL, Papageorge A, Wilding G & Gelmann EP (1990) *Cancer Res* Growth properties and tumorigenesis of MCF-7 cells transfected with isogenic mutants of rasH. 50: 67–71.

Spandidos DA & Agnantis NJ (1984). *Anticancer Res.* Human malignant tumors of the breast as compared to their respective normal tissue have elevated expression of the Harvey ras oncogene: 4: 269– 272.

Steeg PS (2006). *Nature Med.* Tumor metastasis: mechanistic insights and clinical challenges.12(8): 895– 904.

Stresing V, Daubine F, Benzaid I, Monkkonen H &Clezardin P (2007) *Cancer Lett.* Bisphosphonates in cancer therapy. 257 (1): 16–35.

Thibault A, Cooper MR, Figureg WD, Venzan DJ, Sartor OA, Tompkins AC, Weinberg MS, Headlee DJ, Mc Coll Na, Samid D & Myers CE (1994) *Cancer Res*. A phase I and pharmacokinetic study of intravenous phenylacetate in patients with cancer. 54: 1690–1694

Thibout D, Di Benedetto M, Kraemer M, Sainte-Catherine O, Derbin C & Crepin M (1998) *Anticancer Res* Sodium phenylacetate modulates the synthesis of autocrine and paracrine growth factors secreted by breast cancer cell lines.18: 2657–2662.

Van den Wyngaert T, Huizing MT, Vermoken JB (2006) *Ann Oncol.* Bisphosphonates and osteonecrosis of the jaw: cause and effect of a post hoc fallacy? 17:1197–1204.

Wakchoure S, Merrell MA, Aldrich W, Millender-Swain T, Harris KW, Triozzi P & Selander KS. (2006) *Clin Cancer Res.* Bisphosphonates inhibit the growth of mesothelioma cells in vitro and in vivo. 1;12(9):2862-8.

Wang W, Wyckoff JB, Frohlich VC, Oleynikov Y, Hu¨ttelmaier S, Zavadil J, Cermak L & Bottinger EP, Singer RH, White JG, Segall JE & Condeelis JS. (2002) *Cancer Res.* Single cell behaviour in metastatic primary tumors correlated with gene expression patterns revealed by molecular profiling. 62: 6278–6288.

Winter M, Holen I & Coleman RZ (2008) *Cancer Treat rev.* Exploring the anti-tumor activity of bisphosphonates in early breast cancer. 34(5): 453–75.

20

Future Therapeutic Strategies: Implications for Brk Targeting

Amanda Harvey and Rajpal Burmi
Brunel University,
United Kingdom

1. Introduction

Over the last two decades the survival of patients with breast cancer has improved significantly. This is partly as a result of national screening programmes resulting in earlier detection, but also due to major advances in the range of therapies that are now available for patients. However, spread of the disease and resistance to therapy is still an issue for many patients. It is therefore vitally important that the current rate of treatment advances continues for the foreseeable future. In addition to understanding resistance, and therefore generating solutions to overcome it, there is a need for new drug targets to be identified. This chapter will review the published work that has lead to Brk being identified as a potential new target for breast cancer therapy, and discuss the practicalities and implications of a Brk-targeted therapy.

2. Brk discovery and identification

The intracellular protein kinase, Brk (known as **br**east tumour **k**inase, or protein tyrosine kinase 6, PTK6), has been implicated in the development and progression of a number of different tumor types. It was first identified in 3 separate studies in the early 1990s. Initially it was identified in a study to determine which tyrosine kinases were expressed in human melanocytes (S.-T. Lee et al., 1993). Publication of the full-length sequence followed in 1994 after it was cloned from metastatic breast cancer as part of a screen to identify novel kinases with therapeutic potential, i.e. those that were expressed in breast cancers but were not found in normal mammary tissue (Mitchell et al., 1994). Simultaneous identification of the murine orthologue, sik (Src-related intestinal kinase), in mouse intestinal cells was achieved through the generation of a library of kinase catalytic domains (Siyanova et al., 1994).

3. Structure of gene and protein

3.1 *PTK6* gene

The *ptk6* gene comprises 8 exons which span 10kb (Mitchell et al., 1997) and encodes a protein of 451 amino acids in size (Mitchell et al., 1994). Only the boundary between Exons 1 and 2 is conserved with members of the src family (H. Lee et al., 1998; Mitchell et al., 1997) whereas the gene structure of other src family members exhibits high levels of evolutionary conservation, suggesting that *ptk6* belongs to a related, but distinct family of tyrosine

kinases. As 6 out of 7 exon boundaries however, are conserved with *Dsrc41*, *ptk6* may share a common ancestoral evolution with *Dsrc41* (Mitchell et al., 1997).
Fluorescent in situ hybridization studies assigned the *ptk6* gene to chromosome 20q 13.3 and initial analysis of the promoter region identified a number of cis-acting elements including those for Sp1, SIE, AP2 and NFκB (Mitchell et al., 1997). Both NFκB and Sp1 have been shown to bind to cis-acting elements within the promoter region, suggesting that they can play a role in regulating *ptk6* gene transcription (Kang et al., 2002).
Crompton and colleagues reported that the *ptk6* gene sequences derived from normal and tumour tissue were identical, suggesting that the ability of Brk/PTK6 to regulate cancer cell growth was not associated with gene mutations (Mitchell et al., 1994). A search of the COSMIC database in March 2011 (Catalogue of Somatic Mutations in Cancer, hosted by the Wellcome Trust Sanger Institute) revealed that out of 359 tumour samples analysed, only 1 contained a mutation, which provides further corroboration that Brk's role in tumour development occurs as a result of aberrant expression and/or altered cellular localization (see section 5).
In addition, an alternatively spliced variant of Brk has been identified that codes for a 134 amino acid protein, termed λ5 (Mitchell et al., 1997) or ALT-PTK6 (Brauer et al., 2011).

3.2 Brk/PTK6 protein

The protein product of the *PTK6* gene is the non-receptor tyrosine kinase, Brk. It comprises SH3, SH2 and kinase domains and shares 45% amino acid sequence homology with DSRC41 and 44% with human Src (H. Lee et al., 1998; Mitchell et al., 1997). As a 451 amino acid protein, Brk has a predicted molecular weight of 52kDa but typically resolves to around 48kDa on an SDS-PAGE gel. The protein also comprises an SH2-kinase linker region and a C-terminal tyrosine residue (Y447), both of which are involved in regulation of catalytic activity (Figure 1).
The backbone dynamics and solution structure of the SH2 domain of Brk were proposed by Yonsei University in 2004 (Hong et al., 2004). The peptide used in these studies had a dissociation constant of around 60uM. This is a much weaker Kd than had previously been reported for Src family members; again highlighting that although similar to Src, Brk belongs to a distinct family. Differences in Kd values are indicative of different interactions between 'receptors' and their ligands. In support of this, BrkSH2-ligand interactions differ from those involving canonical SH2 domains suggesting that Brk's SH2 domain might have unique binding features that are required for its specific ligand interactions. In addition, NMR data have suggested that the SH3 domain of Brk undergoes severe conformational instability in response to a change in the pH of its environment (Koo et al., 2002). Changes in conformation as a result of subtle changes in cellular pH could therefore alter possible Brk-substrate interactions. In indentifying the intramolecular SH3 binding site, Qiu and Miller were also able to demonstrate the importance of the SH3 domain in regulating Brk-substrate interactions (Qiu and Miller, 2004).
In contrast to src family members, where the interaction between the SH2-kinase linker region and the kinase domain inhibits the enzyme's kinase activity, the linker-kinase interaction is fundamental for Brk's catalytic activity (Kim and S.-T. Lee, 2005). Conversely, linker-SH3 domain interactions negatively regulate the kinase. Proline residues 175, 177 and 179 in the N-terminal part of the linker are required for the linker-SH3 domain interaction, which, alongside the C-terminal phospho-tyrosine-SH2 interaction hold the protein in a negative conformation (Kim et al., 2007).

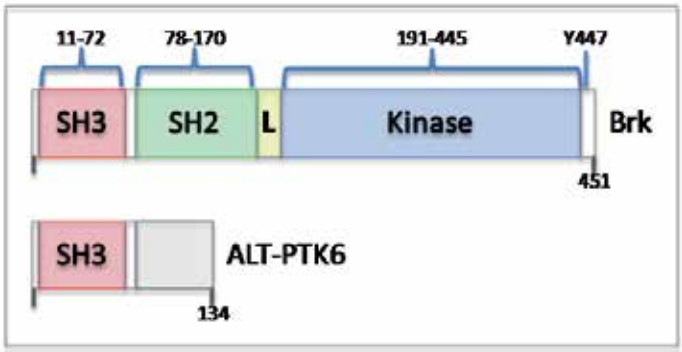


Fig. 1. Schematic representation of Brk and its alternative isoform ALT-PTK6. PTK6 consists of SH3 (red), SH2 (green) and kinase (blue) domains. The linker region (L) containing prolines 175, 177 and 179 is in yellow.

The alternatively spliced isoform, ALT-PTK6, is 15kDa in size and comprises the SH3 domain and a novel proline rich sequence but is truncated before the end of the SH2 domain so lacks functional SH2 and kinase domains (Mitchell et al., 1997). The biological role of this isoform of Brk is unknown, but it is possible that it competes with wild-type Brk for SH3 binding potentially acting as a competitive inhibitor (Brauer et al., 2011).

4. Brk expression profile

4.1 In normal cells

Physiological Brk expression is typically found within a number of normal epithelial tissue types where it is involved in differentiation and has a negative role in cell proliferation. In normal tissue Brk/sik expression is restricted to the cell layers immediately above the proliferative cell zone in these epithelia (Vasioukhin et al., 1995).

Brk/sik is highly expressed in the gastrointestinal (GI) tract where it is present within the non-dividing villus epithelium of the small intestine as well as detectable in the crypt cells post-irradiation (Llor et al., 1999; Vasioukhin et al., 1995; Haegebarth et al., 2009). In the colon Brk/sik was expressed at high levels in the upper crypts in cells undergoing terminal differentiation (Llor et al., 1999), and Brk expression has also been detected in the nuclei of normal luminal prostate epithelial cells (Derry et al., 2003) and oral epithelia (Petro et al., 2004).

Within the epidermis of the skin Brk expression was detected mainly in differentiating layers in the suprabasal keratinocytes (T. C. Wang et al., 2005). *In vitro* studies have supported a role for Brk/Sik in calcium-induced keratinocye differentiation, which was accompanied by the elevation of the epidermal differentiation markers such as Keratin10 or Filaggrin (Tupper et al., 2011; T.C. Wang et al., 2005; Vasioukhin and Tyner, 1997).

Perhaps most surprisingly, although Brk is over-expressed in breast tumours, it is not detected in normal mammary tissues or fibroadenomas (Barker et al., 1997), or at various stages of mouse mammary development (Llor et al., 1999).

4.2 In breast cancers

Brk is known to be low or undetectable in normal mammary tissue and benign lesions, but, in contrast, has been shown to be highly detectable in breast tumours. Typically, Brk was

detected in approximately two thirds of the breast tumours analysed, where approximately a third of these are overexpressed by levels ranging from five-fold to forty three-fold compared to normal tissue (Barker et al., 1997).
Brk is highly expressed in lobular and medullary carcinoma samples (Lukong et al., 2005), and its expression has been reported in up to 86% of breast cancers (Aubele et al., 2007; Ostrander et al., 2007; Harvey et al., 2009). In patient samples Brk mRNA expression correlated with an increase in histological tumour grade (Harvey et al., 2009) and immunostaining revealed an increased level of Brk protein in higher grade tumours (Chakraborty et al., 2008) and specimens with a higher percentage of carcinoma within the sample (Ostrander et al., 2007).

4.3 In other cancers

Brk expression has been detected in a number of cancer types from a variety of tissues. It was found to be highly expressed in 70% of high-grade, serous ovarian carcinomas, but absent in normal ovarian surface epithelia. Expression of Brk was also detected in approximately half of the ovarian cancer cell lines examined, but was again undetected in immortalized ovarian surface epithelium (Schmandt et al., 2006).
Although Brk expression has been detected in the normal human GI tract, it is highly expressed in colon tumour samples and cell lines (Derry et al., 2000; Llor et al., 1999). Indeed, Brk mRNA expression has also been evaluated and detected in tissue in origin from normal colon, polyps and tumours (Chen W et al., 1999).
Brk is expressed in secretory epithelial cells in prostate adenocarcinoma where localisation is believed to be important, as nuclear Brk has been correlated with lower tumour grade (Derry et al., 2003). There were detectable levels of Brk in human oral squamous cell carcinomas (Petro et al., 2004), head and neck squamous cell carcinoma (HNSCC) specimens (Lin et al., 2004), as well as in a large proportion of cutaneous T-cell lymphomas and other transformed T- and B-cell populations (Kasprzycka et al., 2006).

4.4 Regulation of expression

Although the *ptk6* gene promoter has been analysed, and Sp1 and NFkB are proposed to regulate gene transcription (Kang et al., 2002), little is known about the cellular events that bring about regulation of *ptk6* gene transcription in breast cancers. Brk expression is not cell cycle dependent (Barker et al., 1997), nor have there been any studies to suggest an increase in Brk in response to exogenous or autocrine factors known to be involved in breast cancer progression such as oestrogen, progesterone and ErbB receptor ligands. *ptk6* expression may occur as a result of Klf9 transcription factor activity, although it is likely that *ptk6* expression is an indirect effect of Klf9 action (Simmen et al., 2007).
ptk6 is co-expressed with *ErbB2* in breast cancers (Born et al., 2005) and amplification of the *ptk6* gene alongside E*rbB2* gene amplification has been reported (Xiang et al., 2008), however this latter observation does not appear to be consistent with studies on other tumour cohorts (Irie et al., 2010).
Regions of chromosome 20q are frequently amplified in breast cancer (Isola et al., 1995; Kallioniemi et al., 1994) so it is possible that over expression of the PTK6 gene occurs as a result of this amplification event. However, this is unlikely to be the complete picture. Aubele and colleagues reported that *ptk6* over expression in breast cancer is unlikely to be attributed solely to gene amplification (Aubele et al., 2009). In their study, the *ptk6* gene was

amplified in only 15% of 389 Brk-positive tumours and a further 30% of tumours had polysomy of chromsome 20. A normal gene copy number was detected in 55% of invasive breast cancers. These data are supported by reports showing that 85% breast cancers express Brk (Harvey et al., 2009) and between 60 and 86% of breast cancers have elevated Brk protein compared to normal breast tissue (Aubele et al., 2007; Barker et al., 1997; Ostrander et al., 2007).

Taken together these data show that, while in some breast cancers *ptk6* over-expression is likely to be related to amplification events, elevated Brk protein also arises as a result of alternative mechanisms. As Brk has not been detected in normal human mammary epithelial cells, or during development of the mouse mammary gland (Llor et al., 1999), the events triggering expression (irrespective of whether the gene is amplified or not) still remain elusive.

5. Brk localization

Brk has been reported to have different functions in different tissue types; for example, in normal tissues Brk's role appears to be related to regulating the differentiation process, whereas in tumour cells Brk promotes proliferation and cell survival. Variations in cellular localisation are thought to be one of the underlying factors contributing to Brk's opposing roles in differentiation and proliferation. Alterations in cellular localization will no doubt affect the variety of substrates and binding partners that are available for association with Brk, thereby contributing to the different functions and effects that have been ascribed to expression of the *ptk6* gene (reviewed in Brauer and Tyner 2010).

Myristoylation is a post- or co-translational protein modification, whereby a fatty acid-derived group is attached to an N-terminal amino acid. Such modifications are important as they allow proteins to associate directly with membrane structures rather than relying on interactions with additional membrane-associated proteins for membrane localisation (reviewed in Sorek et al., 2011). Although Brk is structurally related to Src, it lacks the amino-terminal myristoylation site. Without this myristoylation site, Brk is not able to interact directly with the plasma membrane, and as it lacks a nuclear localization sequence (NLS), Brk was originally thought to be solely a cytoplasmic kinase (Mitchell et al., 1994).

Without cellular targeting via an NLS or myristoylation, Brk's cellular localization is not tightly regulated; as a result, we now know that it can be found localized in different cellular compartments based on its protein-protein interactions. It has been reported at the membrane via association with ErbB growth factor receptors (Aubele et al., 2010) and the adamalysin ADAM15 (Zhong et al., 2008), in the cytoplasm interacting with paxillin and mitogen activated protein (MAP) kinase (Aubele et al., 2008; Chen et al., 2004) as well as in the nucleus through interactions with RNA binding proteins such as Src-associated in mitosis-68 (Sam68) and the Sam68-like mammalian proteins, SLM1 and SLM2 (Derry et al., 2000; Haegebarth et al., 2004). In normal human prostate epithelial cells and well-differentiated prostate carcinomas, Brk was localized in the nucleus whereas poorly differentiated prostate tumours had cytoplasmic Brk (Derry et al., 2003). In oral epithelia Brk was localized in the nucleus and cytoplasm, but within the perinuclear regions in the oral squamous carcinoma cells (Petro et al., 2004). These data suggest that Brk's cellular localisation may affect its role in oncogenesis as much, if not more, than the level of over-expression. Recent cell culture experiments sustain this hypothesis. Association of proteins to the plasma membrane can be mimicked by experimental inclusion of a myristoylation

site. Adding a myristoylation site to the N-terminus of Brk, enhanced its oncogenic role by promoting the proliferation, survival and migration of the human embryonic kidney cell line, HEK293. Trapping Brk in the nucleus with a synthetic NLS abrogated these effects (Kim and S.-T. Lee, 2009), indicating that Brk's oncogenic role may be dependent on its cellular localization. Brk's effects on β-catenin-mediated transcriptional activity were also found to be dependent on the cellular localization of Brk itself (Palka-Hamblin et al., 2010).
Without either a myristoylation site or a nuclear localization signal it is unclear how Brk delocalizes from one sub-cellular compartment to another and which cellular signals are responsible for controlling this transition. One plausible hypothesis is that it is the Brk-substrate interactions that regulate localization. We would therefore predict that Brk can 'travel' into the nucleus by 'piggy-backing' on a binding partner. The same hypothesis could be applied to cytoplasmic localization and indeed, supporting this theory, unpublished work from Angela Tyner's laboratory suggests that Brk is held in the cytoplasm by an as yet unidentified protein (Brauer and Tyner 2010). Although a less intriguing possibility from a research perspective, we should also not rule out that Brk may simply diffuse from one cellular location to another.

6. Brk interactions, substrates and activation

6.1 Substrates and interacting proteins

Brk is capable of phosphorylating a number of target molecules and a wealth of information on possible substrates and interacting proteins has been compiled. So far, at least 30 proteins have been shown to interact with Brk (summarised in Table 1), however, not all of these associations result in phosphorylation, neither do all of these proteins bind directly to Brk.
Many interactions are likely to be mediated via a 'third-party' that may be known, such as the signal transducing adapter protein-2 (STAP-2) which is also known as Brk kinase substrate (BKS), in the case of both signal transducing and activators of transcription STAT3 and STAT5 (Sekine et al., 2005; Ikeda et al., 2009; Ikeda et al., 2011) or by as yet unidentified interactions. Given that Brk is reported to have a kinase-independent function (Harvey and Crompton 2003), it is highly likely that not all the interacting proteins will be substrates of Brk's kinase activity. Brk may also function as an adaptor molecule; therefore one of Brk's functions could be to stabilize signalling complexes to allow phosphorylation of some of its interacting proteins (and/or additional molecules within the complex) by other kinases. Association of Brk in large signalling complexes, as an adaptor or scaffolding molecule, may also contribute to its cellular localization. The unidentified protein holding Brk in the cytoplasm that has been reported by Brauer and Tyner (Brauer and Tyner 2010) may shed further light on Brk's role as an adaptor protein.
Some of the proteins that interact with Brk have yet to be fully characterized. The protein which approximates to 100kDa and interacts with both Brk and BKS-STAP2 (Mitchell et al., 2000) has yet to be fully identified despite a number of known Brk substrates such as STAT5b, β-catenin and KAP3A being around 100kDa. Proteins such as β-catenin are not thought to be potential candidates (Mitchell et al., 2000) making it likely that the 100kDa protein will be identified as a *de novo* Brk-interacting protein.
The variety of binding partners identified indicates that Brk can interact and potentially regulate a number of significantly important pathways that are known to be involved in breast cancer cell growth and proliferation. For example, the importance of ErbB signalling in breast tumour progression has been well-documented (reviewed in Navolanic et al., 2003) and

clinical inhibition of this pathway with therapies such as trastuzumab and lapatinib is now routine for relevant sub-types of breast cancer (CRUK website). Brk associates with all 4 members of the ErbB receptor family (Aubele et al., 2008; 2010; Kamalati et al., 1996; 2000) and therefore is capable of promoting downstream signalling in response to ErbB ligand binding. Brk also attenuates EGFR signalling through interaction with and phosphorylation of ARAP1 (also known as Arf-GAP, Rho-GAP, ankyrin repeat, and pleckstrin homology (PH) domain-containing protein 1) (Kang et al., 2010), as well as regulating possibly regulating signalling via reported interactions with PTEN and Akt (Aubele et al., 2008; Zhang et al., 2005).

Brk Substrates and Interacting Proteins	
Interacting Protein	**Localisation**
EGFR	Membrane
HER2	Membrane
ErbB3	Membrane
ErbB4	Membrane
IGF-1R	Membrane
*ARAP1	Membrane-associated
*Akt	Cytoplasmic/Membrane-associated
ADAM-15A	Membrane
ADAM-15B	Membrane
*β-Catenin	Membrane/Cytoplasmic/Nuclear
*KAP3A	Cytoplasmic/Nuclear
*STAT3	Cytoplasmic/Nuclear
*STAT5a/b	Cytoplasmic/Nuclear
IRS-1	Cytoplasmic/Membrane-associated
*IRS-4	Cytoplasmic/Membrane-associated
Erk5	Cytoplasmic
Erk	Cytoplasmic
MAPK	Cytoplasmic
PTEN	Cytoplasmic
*Paxillin	Cytoplasmic
*BKS-STAP-2	Cytoplasmic
*GNAS	Cytoplasmic
*FL139441	Cytoplasmic
GapA-p65	Cytoplasmic/Membrane-associated
*Sam68	Nuclear
*SLM-1	Nuclear
*SLM-2	Nuclear
PSF	Nuclear
*β-Tubulin	Cytoplasmic
*p190 Rho GAP	Cytoplasmic
23KDa	Cytoplasmic
100KDa	Cytoplasmic ?

Table 1. Brk Substrates and Interacting Proteins. Proteins known to interact with Brk are listed along with their usual cellular location. Proteins marked with * are confirmed as *bona fide* Brk substrates.

Research into insulin-like growth factor (IGF) signalling is also gaining momentum, especially given that IGF receptor (IGFR) expression is linked to poor outcome in ER-negative breast cancer patients (Railo et al., 1994) and IGF-1R expression and signalling are believed to mediate resistance to trastuzumab (Lu et al., 2001; Nahta et al., 2005). Immunoprecipitation and mass spectrometry experiments have identified insulin receptor substrate (IRS)-4 (IRS-4) as a Brk binding partner (Qiu et al., 2005). In HEK 293 cells, exogenous Brk and IRS-4 were demonstrated to associate in both resting and IGF1-1 stimulated cells. IGF-1 increased the phosphorylation of Brk in MCF-7 breast cancer cells, and this effect was enhanced in the presence of IRS-4 (Qui et al., 2005). Brk also co-precipitates with IRS-1 and IGF-1R in MCF10A-IGF1R cells (Irie et al., 2010).

Brk's association with ADAM-15 variants is of particular interest. Brk showed strong binding to ADAM-15A and ADAM-15B, but not ADAM-15C. Of the 4 alternatively spliced isoforms that were differentially expressed in human breast carcinoma tissue, high expression of ADAM-15A and ADAM-15B were associated with poor relapse-free survival in node-negative breast cancer patients, whereas higher levels of ADAM-15C appeared to predict a more favourable outcome (Zhong et al., 2008).

In addition to phosphorylating Sam68 and polypyrimidine tract-binding (PTB) protein-associated splicing factor (PSF), Brk can also bind to and phosphorylate the SLM-1 and SLM-2 RNA binding proteins (Haegebarth et al., 2004), as well as the transcription factors NFκB (Chakraborty et al., 2008), STAT3 (Liu et al., 2006) and STAT5b (Weaver and Silva 2007). Both Brk and its alternatively spliced isoform ALT-PTK6 have been reported to bind to β-Catenin (Palka-Hamblin et al., 2010; Brauer et al., 2011). Nuclear-targeted Brk negatively regulated β-Catenin/TCF transcription, whereas membrane associated Brk enhanced transcription (Palka-Hamblin et al., 2010); expression of ALT-PTK6, downregulated PTK6 activity and enhanced the inhibition of β-Catenin/TCF transcription that was mediated by Brk (Brauer et al., 2011).

Therefore Brk is capable of regulating both gene transcription and post-transcriptional RNA processing, although the outcome of this regulation will be dependent of Brk's cellular localisation.

6.2 Brk activation

As might be expected from the spectra of protein-protein interactions, Brk is activated by a number of different ligands (Figure 2), as well as exhibiting a small amount of basal autophosphorylation in *in vitro* kinase assays (Castro and Lange, 2010).

Unsurprisingly the signalling via ErbB and IGF-1R receptor ligands, EGF and IGF, activates Brk (Kamalati et al., 1996; Ostrander et al., 2007; Qiu et al., 2005). Activation of the MET receptor by hepatocyte growth factor (HGF) also activated Brk in both breast cancer and keratinocyte cell lines (Castro and Lange 2010). As all these growth factors have roles in cell proliferation, Brk activation by these ligands could increase the proliferative index of tumours. Activation of Brk by osteopontin (OPN) (Chakraborty et al., 2008), a chemokine-like protein that is known to enhance metastasis (Denhardt et al., 2003), has consequences for tumour progression if downstream signalling from Brk promotes the effects of OPN.

Keratinocyte differentiation is also important in terms of Brk-mediated biology. Brk can be activated in response to calcium or ionomycin, although in this context Sik/Brk activation results in the initiation of differentiation (Vasioukhin and Tyner 1997; T.C. Wang et al., 2005). Combined with the fact that Sik promotes differentiation of murine intestinal cells

(Haegebarth et al., 2006), the activation of Brk clearly has the potential for cell-type specific consequences that could be exploited for therapeutic purposes (see section 9).

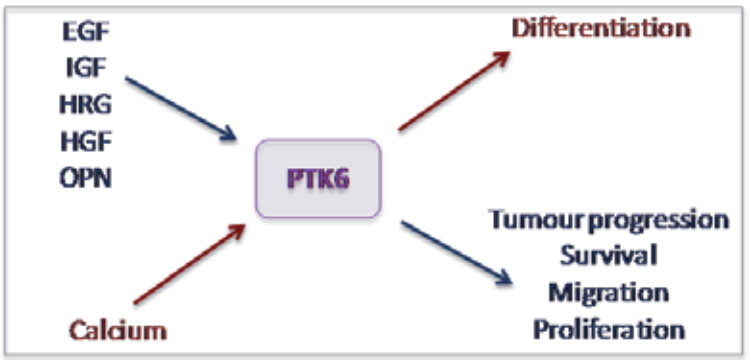


Fig. 2. Brk is activated by a number of ligands. Activation pathways that result in effects considered to be tumour promoting are shown in blue, and those resulting in differentiation are in brown.

6.3 Negative regulation of Brk

Little is known about the negative regulation of Brk activity, as much emphasis has been placed on trying to understand how Brk is activated and the biological effects of this activation. However, studies have demonstrated that the cytokine signalling suppressor, SOCS3, is able to inhibit Brk activity and the subsequent phosphorylation and transcriptional activity of STAT3 (Liu et al., 2006).

Sam68 has anti-proliferative properties and it has been suggested that these are neutralised, possibly by Brk, in breast cancer cells (Lukong et al., 2005). Over-expression of Sam68 in rat astrocytes has been shown to inhibit Brk-induced cell cycle progression (Lukong et al., 2005), indicating that the balance of expression, as well as localisation of both Brk and Sam68 could be important in mediating breast cancer cell proliferation.

7. Tumour-related effects of Brk expression

The elevated levels of Brk expression in tumour samples relative to the restricted levels in normal or differentiating tissues suggest that Brk may have a role in the processes underlying tumourigenesis, such as promotion of cancer cell proliferation and migration and evasion of cell death (Reviewed in Hanahan and Weinberg 2000; 2011).

7.1 Proliferation and cell cycle progression

There is a mounting body of evidence highlighting Brk's role in promoting proliferation and cell cycle progression in breast tumour cells. Brk can increase breast cancer cell proliferation as well as anchorage independent growth in normal mammary epithelial cells (Harvey and Crompton, 2003; Kamalati et al., 1996; Ostrander et al., 2007) and suppression of Brk levels by RNA interference has been shown to result in decreased proliferation in breast cancer cells (Chan and Nimnual, 2010; Harvey and Crompton, 2003; Ostrander et al., 2007).

Brk's role in promoting proliferation in response to EGF remains the best characterised. Brk has been shown to promote proliferation of the mammary epithelial cell line, Hb4a by

potentiating the effects of EGF, associating with EGFR and also recruiting PI3-K to ErbB3 receptors resulting in increased Akt activation (Kamalati et al., 1996; 2000). Furthermore, co-expression of HER2 and Brk in the non-tumourigenic breast cell line, MCF10A, was shown to increase the levels of Cyclin E and decrease p27 to induce cell cycle progression as well as increase Akt phosphorylation (Xiang et al., 2008). As Brk can directly activate Akt by phosphorylation of tyrosine residues 315 and 326 (Zheng et al., 2010), Brk could directly contribute to downstream signalling and presumably increased proliferation through interactions with Akt, as well as enhancing proliferation in response to EGF via activation of p190RhoGAP (Shen et al., 2008). EGF-mediated activation of Brk induced phosphorylation of Sam68 and promoted cell cycle progression, suggesting that Brk's oncogenic functions are, in part, mediated by inhibiting the tumour-suppressive functions of molecules such as Sam68 (Lukong et al., 2005; reviewed in Brauer and Tyner, 2010). The combined EGFR/HER2 inhibitor lapatinib inhibits HER2 mediated proliferation, however over-expression of Brk in MCF10A-HER2 cells reduced the effectiveness of lapatinib in inhibiting proliferation (Xiang et al., 2008).

Other ErbB receptor ligands are also capable of mediating receptor-Brk interactions and altering its activity. For example, heregulin has been shown to activate Brk's kinase activity in T-47D breast cancer cells (Ostrander et al., 2007). Increased proliferation as a result of activated Brk in response to stimulation with either EGF or heregulin resulted in activation of Rac GTPase, extracellular signal regulated kinase (ERK) 5, and p38 mitogen-activated protein kinase (MAPK), as well as an increase in Cyclin D1 expression (Ostrander et al., 2007).

Additional downstream targets of Brk that are involved in augmenting Brk's effects in promoting proliferation include STAT3 (Liu et al., 2006) and STAT5b (Weaver and Silva, 2007). Phosphorylation of both these proteins resulted in increased proliferation and transcriptional activity.

Brk's role in promoting proliferation is one way in which it can contribute to breast cancer development and progression. Interestingly however, Lukong and colleagues showed that Brk could phosphorylate the nuclear protein PSF causing it to delocalise to the cytoplasm resulting in growth arrest (Lukong et al., 2009). Their data provide mechanistic evidence supporting previous studies that cytoplasmic Brk is oncogenic, whereas nuclear Brk could play a role in negatively regulating cell cycle progression and/or proliferation (Derry et al., 2003; Kim and S.-T. Lee, 2009).

There is also some evidence to suggest that Brk does not always promote proliferation when transfected into non-transformed cells. Brk over-expression in rat fibroblasts did not promote either anchorage independent growth or cell cycle progression, but did affect cellular responses to DNA-damage and stress (Haegebarth et al., 2005). Intestinal epithelial cells from PTK null mice showed an increase in basal levels of proliferation compared to cells from wild-type mice, but in response to γ-irradiation both proliferation and BrdU labelling were increased in wild-type cells compared to PTK6 null cells (Haegebarth et al., 2009).

7.2 Cell death

Brk has been shown to participate in a number of signalling pathways that could ultimately regulate cell death depending on the cellular context. The disruption of the cell-cell matrix interactions acts a stimulus for apoptosis (Frisch and Francis, 1994), and given that Brk has been shown to transform mammary epithelial cells such that they proliferate in anchorage

independent conditions (Kamalati et al., 1996), it is likely that Brk also promotes anchorage-independent cell survival by protecting cells from cell death. Recent data has shown that Brk, via IGF-1 signalling, protected breast cancer cells from classical apoptosis/anoikis (Irie et al., 2010). In a separate study, Brk expression reduced breast cancer cell death via an autophagic pathway (Harvey et al., 2009). These studies indicate that PTK6 can protect cells from different types of programmed cell death.

The Bcl-x alternatively spliced variants, Bcl-x_L and Bcl-x_S, were shown to be differentially expressed in response to Brk targeting (Harvey et al., 2009); Brk suppression resulted in a concurrent reduction in the anti-apoptotic Bcl-x_L and an induction in Bcl-x_S, suggesting that targeting Brk could modulate a tumour cell's capacity for cell death. Investigations as to whether Brk mediates these effects in breast cancer cells through regulation of alternative splicing or through protein stability are on-going, however the Brk substrate Sam68 (Lukong et al., 2005) is able to regulate the alternative splicing of Bcl-x in HEK293 cells (Paranetto et al., 2007).

Contrary to the data outlined above, Brk sensitizes non-transformed rat fibroblasts to inducers of apoptosis such as serum starvation and UV irradiation/serum starvation (Haegebarth et al., 2005). As previously discussed (Harvey et al., 2009) parallels can be drawn with data on the c-*myc* oncogene, as Myc is also capable of sensitizing fibroblasts to induction of apoptosis by serum deprivation (Evan et al., 1992; Harrington et al 1994). In intestinal crypt epithelial cells PTK6 is induced by stress and promotes apoptosis through inhibition of Akt and Erk1/2 (Haegebarth et al., 2009). This further underlines the fact that that the pro-survival functions of Brk are likely to be dependent on cellular context.

7.3 Migration

Brk has been shown to promote the migration of breast cancer cell lines towards Heregulin, HGF and EGF (Castro and Lange 2010; H.-Y. Chen et al., 2004; Ostrander et al., 2007). Through its interaction with paxillin, Brk mediated EGF-induced migration and invasion of breast tumour cells has also been demonstrated to occur via a mechanism involving CrkII and Rac (Chen et al., 2004). As well as promoting proliferation, Brk phosphorylation of p190RhoGAP promoted migration and invasion (Shen et al., 2008), and KAP3A has been identified as physiological substrate of Brk during migration of BT20 breast cancer cells (Lukong and Richard 2008).

Studies to date therefore suggest that Brk can promote the migration of breast cancer cells through more than one mechanism, and in response to a number of different ligands.

7.4 Tumour formation

The *in vitro* data in a number of tumour types, but especially breast cancer, strongly support a role for Brk in augmenting some of the processes underlying breast cancer progression and dissemination.

Brk promoted ErbB2 induced tumourigensis in orthoptic transplantation-based models. Cells co-expressing Brk and ErbB2 formed tumours 2-3 weeks earlier, on average, than cells with ErbB2 alone. The ErbB2/Brk positive tumours also showed increased proliferation compared with ErbB2-only tumours (Xiang et al., 2008). OPN enhanced VEGF-dependent tumour progression in xenograft models, and in vitro experiments suggest that this is through activation of a PTK6/NFκB/ATF-4 signalling cascade (Chakraborty et al., 2008). There exists, in this scenario, potential for a feedback loop whereby Brk activation of NFκB

results in increased Brk expression, given that NFκB binding sites have been identified in the *ptk6* gene promoter (Kang et al., 2002; Mitchell et al., 1997).

7.5 Role of Brk's kinase domain in Brk-mediated biology

Brk has been reported to have kinase-independent function and it is proposed that it may act as an adaptor protein in signal transduction (Harvey and Crompton, 2003). Certainly the kinase inactive PTK6 K219M mutant is capable of promoting proliferation in over-expression studies in the PTK6 positive cell line T-47D, (Harvey and Crompton 2003) and Brk's association with, and regulation of, β-catenin is also independent of kinase activity (Palka-Hamblin et al., 2010).

However, certain aspects of Brk function including anchorage independent growth, and regulation of cell death phenotypes appear to require functional kinase activity (Harvey et al., 2009; Irie et al., 2010; Kamalati et al., 1996).

The reliance on functional kinase activity in migration is more clearly defined. Migration of breast cancer cells towards EGF and foetal bovine serum appeared to require Brk's kinase activity (H.-Y. Chen et al., 2004; Lukong and Richard 2008), however kinase inactive Brk was found to be able to promote migration towards HGF (Castro and Lange 2010).

Kinase inactive Brk did not appear to bind to ARAP-1 as well as wild-type Brk, indicating that the catalytic activity of Brk is required for the interaction with ARAP-1 and the maintenance of EGFR protein and the subsequent prolonging of EGFR signalling (Kang et al., 2010).

8. Brk and breast cancer patient prognosis

The literature relating to Brk expression and breast cancer prognosis is conflicted. Much of the *in vitro* cell culture data, support an oncogenic role for Brk as it has been shown to augment breast cancer cell proliferation and migration, ErbB receptor signalling, as well as inhibit cell death via different mechanisms. Gene expression data (Harvey et al., 2009) and immunohistochemistry staining (Aubele et al., 2007; Ostrander et al., 2007) from different cohorts of patient tumour samples indicated that Brk expression is correlated with higher-grade tumours initially suggesting that expression is likely to be linked with poorer prognosis for breast cancer patients as these tumours are more likely to disseminate (Porter et al., 2004). Indeed a recent study indicates that high Brk expression is associated with adverse patient outcomes (Irie et al., 2010).

Conversely, Aubele and colleagues also showed that whilst initially the probability of disease-free-survival was lower for patients with higher levels of Brk expression, beyond 50-100 months high Brk expression was linked with an improved probability of distant recurrence-free survival (P=0.001 at 240 months) (Aubele et al., 2007). As we have previously discussed one explanation for this discrepancy is that PTK6 expression may be correlated with expression of the oestrogen receptor, a known positive prognostic indicator (discussed in Harvey et al., 2009). The underlying cause of Brk overexpression may also contribute to patient outcome, especially in tumours where the *ptk6* gene is amplified as 20q and 20q13 amplifications have been associated with poor prognosis and more aggressive tumour phenotypes (Isola et al., 1995; Tanner et al., 1995).

In addition, it is also possible that driving tumour cells to proliferate may make them more susceptible to the effects of conventional chemotherapy agents. These are known to target actively-dividing rather than 'resting' cells, so increasing susceptibility to these agents

would enhance the benefits of such therapy thereby aiding patient survival. It would be particularly beneficial to examine patient survival in relation to both Brk expression and combination of therapy received before a definitive conclusion on the role of Brk expression in patient prognosis can be reached.

9. Brk-targeted therapies: Opportunities and implications

Brk has been considered as a potential therapeutic target for breast cancer for a number of years (reviewed in Harvey and Crompton 2004) and the wealth of more recently published *in vitro* and *in vivo* data, supports this hypothesis. Inhibition of Brk would be predicted to reduce breast cancer cell proliferation, migration and survival, as well as down-regulate some of the processes underlying tumour development and possibly dissemination. Given the high percentage of breast cancers that express Brk (up to 86%) (Harvey et al., 2009; Ostrander et al., 2007), a high proportion of patients could benefit from such a therapy.

The fact that the *ptk6*-null mouse survives into adulthood, is fertile, and there are minimal effects on health apart from the developmental issues such as changes in crypt length (Haegebarth et al., 2006), suggests that a Brk targeted therapy could be tolerable to patients. The biggest risk factor for developing breast cancer is increased age (BCC website), meaning that patients are diagnosed with breast cancer at a time in their life when development has already been completed, indicating that a Brk-targeted therapy would not cause any development-related adverse effects. In colonic crypt cells, Brk is induced in response to external stresses (Haegebarth et al., 2009) so any potential effects of Brk inhibitors on colonic cells may need to be monitored. PTK6 has been shown to sensitise cell to inducers of cell death in fibroblasts (Haegebarth et al., 2005), however this is unlikely to have any clinical impact as fibroblasts have yet to be shown as sites of PTK6 expression.

As well as inhibiting proliferation, survival and migration, Brk inhibition should reduce the signalling mediated via ErbB and IGF receptors. As inhibitors for both these receptor families are currently in clinical use or in clinical trials as combination therapies, it could be hoped that therapeutic inhibition of Brk could produce similar anti-tumour effects. Co-targeting Brk alongside EGFR or Her-2, may produce significant clinical benefit especially in tumours where both ptk6 and ErbB2 are over-expressed or co-amplified. Brk has already been implicated in mediating resistance in *in vitro* studies to the dual EGFR/Her-2 inhibitor, lapatinib (Xiang et al., 2008), suggesting that co-targeting of ErbB receptors and Brk is of therapeutic value.

Brk-targeted therapies may well have consequences for expression of cell death related proteins, as Brk suppression has been shown to modulate Bcl-x_L:Bcl-x_S ratios in favour of Bcl-x_S (Harvey et al., 2009). The induction of Bcl-x_L has been linked with resistance to both traditional chemotherapy agents as well anti-hormonal treatments in breast cancer (Kumar et al., 1996; Mercatante et al., 2002; Minn et al., 1995). As reducing Bcl-x_L or over-expressing Bcl-x_S increases the sensitivity of response to chemotherapeutic agents in breast cancer cell lines (Simões-Wüst et al., 2002; Sumantran et al., 1995), targeting Brk may also modulate chemotherapeutic responses to existing treatments. This possibility is of particular importance in triple negative breast cancers that are intrinsically less sensitive to chemotherapeutic agents and where, due to their negative-receptor status, targeted therapies are not suitable.

If combinations of already existing therapies, such as radiation, are to be used alongside Brk inhibitors, treatment protocols/rationales will need to be strictly determined. Brk is induced

with DNA-damage in response to γ-irradiation (Haegebarth et al., 2009), meaning that the order of treatment for patients will need careful consideration.

Kinase inhibitors for a number of cellular targets such as the kinase domains of EGFR and HER2, and bcr-abl are now widely used, and the side effects appear to be manageable and the drugs are tolerated reasonably well by patients. A kinase inhibition strategy is often the preferred option by pharmaceutical companies (reviewed in Keri et al., 2006) and one that could be considered for Brk. This strategy is not without issues as Brk is reported to have kinase independent role in both proliferation (Harvey and Crompton, 2003) and the regulation of β-catenin (Palka-Hamblin et al., 2010) suggesting that perhaps targeting of the SH2 or SH3 domains maybe more beneficial (Harvey and Crompton, 2004). However, there is an increasing body of knowledge showing that anchorage independent growth and cell death (Harvey et al., 2009; Irie et al., 2010; Kamalati et al., 1996), as well as migration (H.-Y. Chen et al., 2004; Lukong and Richard, 2008) and regulation of EGFR signalling (Kang et al., 2010) do require kinase function suggesting, that while caution is required, inhibition of kinase activity would offer some clinical benefit via inhibition of these processes. Data presented in 2010 at the American Association for Cancer Research annual conference suggest that kinase inhibitors are being considered by the pharmaceutical industry as Brk-targeted therapies (Y. Wang et al., 2011).

We have previously discussed the implication for kinase inhibition and the potential effects on differentiation (Harvey and Crompton 2004), however given that EGF signalling is heavily implicated in keratinocyte differentiation (Nanney et al., 1990; Peus et al., 1997) and that EGFR and Brk are proposed to be co-regulated in differentiation (Tupper et al., 2011), it is possible that the adverse effects would be no greater than those observed with EGFR inhibitors.

10. Future perspectives

Inhibiting Brk remains an attractive option for the treatment of breast cancer patients. However, there are a number of 'knowledge gaps' that need to be addressed.

The role of ALT-PTK6 in regulating PTK6 function could be vital to furthering our understanding of how Brk is negatively regulated. If SH3 inhibition proves to be an appropriate therapeutic strategy, understanding ALT-PTK6 function, as an SH3-only protein, will be important.

There are a number of groups working within the PTK6 research community and some of our knowledge of PTK6 function is gained from studies on other tumour types such as prostate and oral squamous carcinomas (Derry et al., 2003; Petro et al., 2004). There is now a need to assess whether the same conclusions can be made for breast cancer.

PTK6 expression is increased with increasing tumour grade in breast cancer, however the clinical implications of Brk expression need further clarification. Is PTK6 a negative prognostic indicator? Or does patient outcome depend on treatment regime in the context of Brk expression?

One of the major 'knowledge gaps' is our lack of understanding as to how or why *ptk6* gene expression is triggered in breast cancer cells, when expression is absent in normal mammary development. What are the underlying events that 'switch-on' expression?

Many Brk-protein associations and interactions have been shown a wide range of cell types, some by over expression studies of both Brk and its proposed substrate/binding partner. Further studies are now warranted to determine which of these reported interactions are relevant from a pathological perspective. These investigations, combined with fully understanding Brk's cellular localisation and how it translocates from one compartment to

another, will be particularly helpful especially in the context of designing SH3 or SH2 inhibitors to disrupt specific disease-related protein-protein interactions.

11. Conclusion

The relatively limited physiological expression profile of Brk, and its high level of *de novo* expression in breast tumours make it an attractive therapeutic target. Much progress has been made in the last decade in our understanding of Brk's role in the processes underlying tumour development (summarised in Figure 3). More recent studies indicate that a kinase-inhibitor approach to 'anti-Brk' drug development may warrant further investigation. The next decade will undoubtedly be crucial for providing further knowledge that will consolidate Brk as a viable therapeutic target for breast cancer.

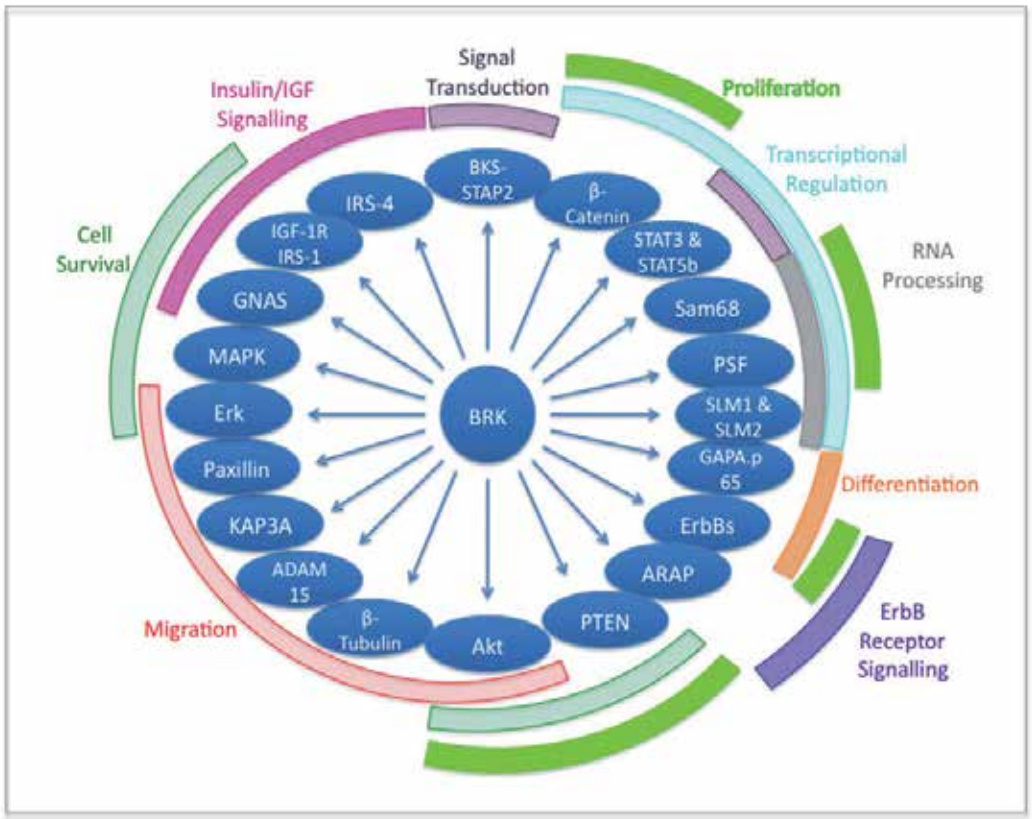


Fig. 3. Summary of the known interactions of Brk and the proposed biological effects that are regulated by these interactions.

12. Acknowledgements

The authors would like to thank Dr Ester Hammond, University of Oxford, UK for her comments and helpful discussions on this chapter. Rajpal Burmi was supported by a Breast Cancer Campaign project grant.

13. References

Aubele, M., Auer, G., Walch, AK., Munro, A., Atkinson, MJ., Braselmann, H., Fornander, T. and Bartlett, JM. (2007). PTK (protein tyrosine kinase)-6 and HER2 and 4, but not HER1 and 3 predict long-term survival in breast carcinomas. *British Journal of Cancer*, Vol.96, No.5, (February 2007), pp. 801-807.

Aubele, M., Walch, AK., Ludyga, N., Braselmann, H., Atkinson, MJ., Luber, B., Auer, G., Tapio, S., Cooke, T., and Bartlett, JMS. (2008). Prognostic value of protein tyrosine kinase 6 (PTK6) for long-term survival of breast cancer patients. *British Journal of Cancer*, Vol.99, No.7, (October 2008), pp. 1089 – 1095.

Aubele, M., Vidojkovic, S., Braselmann, H., Ritterswürden, D., Auer, G., Atkinson, MJ., Tapio, S., Höfler, H., Rauser, S. and Bartlett, JM. (2009). Overexpression of PTK6 (breast tumor kinase) protein-a prognostic factor for long-term breast cancer survival-is not due to gene amplification. *Virchows Archive,* Vol.455, No.2, (July 2009), pp. 117-123.

Aubele, M., Spears, M., Ludyga, N, Braselmann, H., Feuchtinger, A., Taylor, KF, Lindner, K., G Auer, Stering, K., Höfler, H., Schmitt, M. and Bartlett, JMS. (2010). In situ quantification of HER2–protein tyrosine kinase 6 (PTK6) protein–protein complexes in paraffin sections from breast cancer tissues. *British Journal of Cancer*, Vol.103, No.5, (August 2010), pp. 663-337.

Barker, KT., Jackson, LE. and Crompton, MR. (1997). BRK tyrosine kinase expression in a high proportion of human breast carcinomas. *Oncogene,* Vol.15, No.7, (August 1997), pp. 799-805.Brauer and Tyner 2010.

BCC website: http://www.breastcancercampaign.org/breastcancer/6/ accessed May 2011.

Born, M., Quintanilla-Fend, L., Braselmann, H., Reich, U., Richter, M., Hutzler, P. and Aubele, M. (2005). Simultaneous over-expression of the Her2/neu and PTK6 tyrosine kinases in archival invasive ductal breast carcinomas. *Journal of Pathology*, Vol.205, No.5, (April 2005), pp. 592-596.

Brauer, PM., Zheng, Y., Evans, MD., Dominguez-Brauer, C., Peehl, DM. and Tyner, AL. (2011). The alternative splice variant of protein tyrosine kinase 6 negatively regulates growth and enhances PTK6-mediated inhibition of β-catenin. *PLoS One*, Vol.6, No.3, (March 2011), pp. e14789.

Brauer, PM. And Tyner, AL. (2010). Building a better understanding of the intracellular tyrosine kinase PTK6 - BRK by BRK. *Biochimica et Biophysica Acta*, Vol.1806, No.1, (August 2010), pp. 66-73.

Castro, NE. and Lange, CA. (2010). Breast tumor kinase and extracellular signal regulated kinase 5 mediate Met receptor signaling to cell migration in breast cancer cells. *Breast Cancer Research,* Vol.12, No.4, (August 2010), pp. R60.

Chan, E. and Nimnual, AS. (2010). Deregulation of the cell cycle by breast tumor kinase (Brk). *International Journal of Cancer*, Vol.127, No.11, (December 2010), pp. 2723-2731.

Chakraborty, G., Jain, S., and Kundu, GC. (2008). Osteopontin promotes vascular endothelial growth factor–dependent breast tumor growth and angiogenesis via autocrine and paracrine mechanisms. *Cancer Research,* Vol.68, No.1, (January 2008), pp. 152-161.

Chen, HY., Shen, CH., Tsai, YT., Lin, FC., Huang, YP. and Chen, RH. (2004). Brk activates rac1 and promotes cell migration and invasion by phosphorylating paxillin. *Molecular and Cellular Biology*, Vol. 24, No.24, (December 2004), pp. 10558-10572.

Chen, WS., Kung, HJ., Yang, WK. and Lin, W. (1999). Comparative tyrosine-kinase profiles in colorectal cancers: enhanced arg expression in carcinoma as compared with adenoma and normal mucosa. *International Journal of Cancer*, Vol.83, No.5, (November 1999), pp. 579-584.

COSMIC: http://www.sanger.ac.uk/genetics/CGP/cosmic/ accessed (March 2011).

CRUK website: http://www.cancerhelp.org.uk/type/breast-cancer/treatment/biological-therapy-for-breast-cancer accessed May 2011.

Denhardt, DT., Mistretta, D., Chambers, AF., Krishna S., Porter, JF., Raghuram, S. and Rittling, SR. (2003). Transcriptional regulation of osteopontin and the metastatic phenotype: evidence for a Ras-activated enhancer in the human OPN promoter. *Clinical and Experimental Metastasis*, Vol.20, No.1, (2003), pp. 77–84.

Derry, JJ., Richard, S., Valderrama Carvajal, H., Ye, X., Vasioukhin, V., Cochrane, AW., Chen, T. and Tyner, AL. (2000). Sik (BRK) phosphorylates Sam68 in the nucleus and negatively regulates its RNA binding ability. *Molecular and Cellular Biology,* Vol.20, No.16, (August 2000), pp. 6114-6126.

Derry, JJ., Prins, GS., Ray, V. and Tyner, AL. (2003). Altered localization and activity of the intracellular tyrosine kinase BRK/Sik in prostate tumor cells. *Oncogene.* Vol.22, No.27, (July 2003), pp. 4212-4220.

Evan, G., Wyllie, A., Gilbert, C., Littlewood, T., Land, H., Brooks, M., Waters, C., Penn, L. and Hancock, D. (1992). Induction of apoptosis in fibro-blasts by c-myc protein. *Cell*, Vol.69, (Apr 1992), pp. 119–128.

Frisch, SM. and Francis, H. (1994). Disruption of epithelial cell-matrix interactions induces apoptosis. *The Journal of Cell Biology*, Vol.124, No.4, (February 1994), pp. 619-626.

Haegebarth, A., Heap, D., Bie, W., Derry, JJ., Richard, S. and Tyner, AL. (2004). The nuclear tyrosine kinase BRK/Sik phosphorylates and inhibits the RNA-binding activities of the Sam68-like mammalian proteins SLM-1 and SLM-2. *Journal of Biological Chemistry*, Vol.279, No.52, (December 2004), pp. 54398-54404.

Haegebarth, A., Nunez, R. and Tyner, AL. (2005). The intracellular tyrosine kinase Brk sensitizes non-transformed cells to inducers of apoptosis. *Cell Cycle*, Vol.5, No.2, (January 2005), pp. 1239-1246.

Haegebarth, A., Bie, W., Yang, R., Crawford, SE., Vasioukhin, V., Fuchs, E. and Tyner, AL. (2006). Protein tyrosine kinase 6 negatively regulates growth and promotes enterocyte differentiation in the small intestine. *Molecular and Cellular Biology,* Vol.26, No.13, (July 2006), pp. 4949-4957.

Haegebarth, A., Perekatt, AO., Bie, W., Gierut, JJ. and Tyner, AL. (2009). Induction of protein tyrosine kinase 6 in mouse intenstinal crypt epithelial cells promotes DNA damage-induced apoptosis. *Gastroenterology*, Vol.137, No.3, (September 2009), pp. 945-954.

Hanahan, D. and Weinberg, RA. (2000). Hallmarks of cancer. *Cell*, Vol. 100, No.1, (January 2000), pp. 57-70.

Hanahan, D. and Weinberg, RA. (2011). Hallmarks of cancer: the next generation. *Cell*, Vol.144 No.5, (March 2011), pp. 646-674.

Harrington, E., Bennett, M., Fanidi, A. and Evan, G. (1994). c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO Journal*, Vol.13, No.14, (July 1994), pp. 3286 –3295.

Harvey, AJ. and Crompton, MR. (2003). Use of RNA interference to validate Brk as a novel therapeutic target in breast cancer: Brk promotes breast carcinoma cell proliferation. *Oncogene*, Vol.22, No.32, (August 2003), pp. 5006–5010.

Harvey, AJ. and Crompton, MR. (2004). The Brk protein tyrosine kinase as a therapeutic target in cancer: opportunities and challenges. *Anticancer Drugs*, Vol.15, No.2 (February 2004), pp. 107-111.

Harvey, AJ., Pennington, CJ., Porter, S., Burmi, RS., Edwards, DR., Court, W., Eccles, SA., and Crompton, MR. (2009). Brk protects breast cancer cells from autophagic cell death induced by loss of anchorage. *The American Journal of Pathology*, Vol.175, No.3, (September 2009), pp. 1226-1234.

Hong, E., Shin, J., Kim, HI., Lee, ST. and Lee, W. (2004). Solution structure and backbone dynamics of the non-receptor protein-tyrosine kinase-6 Src homology 2 domain. *Journal of Biological Chemistry*, Vol.279, No.28, (July 2004), pp. 29700-29708.

Ikeda, O., Miyasaka, Y., Sekine, Y., Mizushima, A., Muromoto, R., Nanbo, A., Yoshimura, A. and Matsuda, T. (2009). STAP-2 is phosphorylated at tyrosine-250 by Brk and modulates Brk-mediated STAT3 activation. Biochem Biophys Res Commun. Vol. 384, No.1, (June 2009) pp. 71-75.

Ikeda, O., Mizushima, A., Sekine, Y., Yamamoto, C., Muromoto, R., Nanbo, A., Oritani, K., Yoshimura, A. and Matsuda, T. (2011). Involvement of STAP-2 in Brk-mediated phosphorylation and activation of STAT5 in breast cancer cells. *Cancer Science*, Vol.102, No.4, (April 2011), pp. 756-761.

Irie, HY., Shrestha, Y., Selfors, LM., Frye, F., Iida, N., Wang, N., Zou, L, Yao, J, Lu, Y, Epstein, CB., Natesan, S., Richardson, AL. Polyak, K., Mills, GB., Hahn, WC., and Brugge, JS. (2010). PTK6 regulates IGF-1-induced anchorage-independent survival. *PLoS ONE,* Vol.5, No.7, (July 2010), pp. e11729.

Isola, J., Kallioniemi, O., Chu, L., Fuqua, S., Hilsenbeck, S., Osborne, C. and Waldman, F. (1995). Genetic aberrations detected by comparative genomic hybridization predict outcome in node-negative breast cancer. *Am J Pathol*, Vol.147, No.4, (October 1995), pp. 905-911.

Kallioniemi, A., Kallioniemi, O., Piper, J., Tanner, M., Stokke, T., Chen, L., Smith, H., Pinkel, D., Gray, J. and Waldman, F. (1994). Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *The Proceedings of the National Academy of Sciences of the United States of America*, Vol.91, No.6, (March 1994), pp. 2156-2160.

Kamalati, T., Jolin, HE., Mitchell, PJ., Barker, KT., Jackson, LE., Dean, CJ., Pagei, MJ., Gusterson, BA., and Crompton, MR. (1996). Brk, a breast tumor-derived non-receptor protein-tyrosine kinase, sensitizes mammary epithelial cells to epidermal growth factor. *The Journal of Biological Chemistry*, Vol.271, No.48, (November 29), pp. 30956–30963.

Kamalati, T.,Jolin, HE., Fry, MJ. and Crompton, MR. (2000). Expression of the BRK tyrosine kinase in mammary epithelial cells enhances the coupling of EGF signalling to PI 3-kinase and Akt, via erbB3 phosphorylation. *Oncogene*, Vol.19, No.8, (Novermber 2000), pp. 5471 – 5476.

Kang, K., Kim, M.,Pae, K., and Lee, S. (2002). Characterization of the 5′-fanking region of the human PTK6 gene. *Biochimica et Biophysica Acta*, Vol.1574, No.3, (April 2002), pp. 365-369.

Kang, SA., Lee, ES., Yoon, HY., Randazzo, PA. and Lee, S.-T. (2010) PTK6 inhibits down-regulation of EGF receptor through phosphorylation of ARAP1. *Journal of Biological Chemistry*, Vol.285, No.34, (August 2010), pp. 26013-26021.

Kasprzycka, M., Majewski, M., Wang, ZJ., Ptasznik, A., Wysocka, M., Zhang, Q., Marzec, M., Gimotty, P., Crompton, MR. and Wasik, MA. (2006). Expression and oncogenic role of Brk (PTK6/Sik) protein tyrosine kinase in lymphocytes. *The American Journal of Pathology,* Vol.168, No.5, (May 2006), pp. 1631-1641.

Kéri,G., Orfi, L., Eros, D., Hegymegi-Barakonyi, B., Szántai-Kis C., Horváth, Z., Wáczek, F., Marosfalvi, J., Szabadkai1, I., Pató, J., Greff, Z., Hafenbrad, D., Daub, H., Müller, G., Kleb, B. and Ullrich, A. (2006). Signal transduction therapy with rationally designed kinase inhibitors. *Current Signal Transduction Therapy*, Vol.1 (2006) pp. 67-95.

Kim, HI. and Lee, S.-T. (2005). An intramolecular interaction between SH2-kinase linker and kinase domain is essential for the catalytic activity of protein-tyrosine kinase-6. *Journal of Biological Chemistry*, Vol. 280, No. 32, (August 2005), pp. 28973-28980.

Kim, HI., Jung, J., Lee, ES., Kim, YC., Lee, W. and Lee, S.-T. (2007). Molecular dissection of the interaction between the SH3 domain and the SH2-Kinase Linker region in PTK6. *Biochemical and Biophysical Research Communications*, Vol.362, No.4, (November 2007) pp. 829-834.

Kim, HI. and Lee, S-T. (2009). Oncogenic functions of PTK6 are enhanced by its targeting to plasma membrane but abolished by its targeting to nucleus. *The Journal of Biochemistry*, Vol.146, No.1, (March 2009), pp. 133–139.

Koo, BK., Kim, MH., Lee, S.-T. and Lee, W. (2002). Purification and spectroscopic characterization of the human protein tyrosine kinase-6 SH3 domain. *Journal of Biochemistry and Molecular Biology*, Vol.35, No.3, (May 2002), pp. 343-347.

Kumar, R., Mandal, M., Lipton, A., Harvey, H. and Thompson, CB. (1996). Overexpression of HER2 modulates bcl-2, bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells. Clinical Cancer Research, Vol.2, No.7, (July 1996), pp. 1215-1219.

Lee, H., Kim, M., Lee, KH., Kang, KN. and Lee, S.-T. (1998). Exon-intron structure of the human PTK6 gene demonstrates that PTK6 constitutes a distinct family of non-receptor tyrosine kinase. *Molecules and Cells*, Vol.8, No.4, (August 1998), pp. 401-407.

Lee, S.-T., Strunk, KM. and Spritz, RA. (1993). A survey of protein tyrosine kinase mRNAs expressed in normal human melanocytes. *Oncogene*, Vol.8, No.12, (December 1993), pp. 3403-3410.

Lin, HS., Berry, GJ., Fee, WE., Jr, Terris, DJ. and Sun, Z. (2004). Identification of tyrosine kinases overexpressed in head and neck cancer. *Archives of Otolaryngology-Head & Neck Surgery,* Vol.130, No.3, (March 2004), pp. 311-316.

Liu, L., Gao, Y., Qiu, H, Miller, WT., Poli, V and Reich, NC. (2006). Identification of STAT3 as a specific substrate of breast tumor kinase. *Oncogene*, Vol.25, No.35, (August 2006), pp. 4904–4912.

Llor, X., Serfas, MS., Bie, W., Vasioukhin, V., Polonskaia, M., Derry, J., Abbott, CM. and Tyner, AL. (1999). BRK/Sik expression in the gastrointestinal tract and in colon tumors. *Clinical Cancer Research,* Vol.5, No.7, (July 1999), pp. 1767-1777.

Lu, Y., Zi, X., Zhao, Y., Mascarenhas, D. and Pollak, M.(2001). Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). *Journal of the National Cancer Institute*, Vol.93, No.24, (December 2001), pp. 1852-1857.

Lukong, KE., Larocque, D., Tyner, AL. and Richard, S. (2005). Tyrosine phosphorylation of sam68 by breast tumor kinase regulates intranuclear localization and cell cycle progression. *The Journal of Biological Chemistry*, Vol. 280, No.46, (November 2005), pp. 38639-38647.

Lukong, KE. and Richard, S. (2008). Breast tumor kinase BRK requires kinesin-2 subunit KAP3A in modulation of cell migration. *Cell Signalling*, Vol.20, No.2, (February 2008), pp. 432-442.

Lukong, KE., Huot, ME. and Richard, S. (2009). BRK phosphorylates PSF promoting its cytoplasmic localization and cell cycle arrest. *Cell Signalling*, Vol.21, No.9, (September 2009), pp. 1415-1422.

Mercatante, DR., Mohler, JL. and Kole, R. (2002). Cellular response to an antisense-mediated shift of Bcl-x pre-mRNA splicing and antineoplastic agents. *Journal of Biological Chemsitry*, Vol.277, No.51, (December 2002), pp. 49374-82.

Minn, AJ., Rudin, CM., Boise, LH. and Thompson, CB. (1995). Expression of bcl-xL can confer a multidrug resistance phenotype. *Blood*, Vol.86, No.5, (Spetember 1995), pp. 1903-1910.

Mitchell, PJ., Barker, KT., Martindale, JE., Kamalati, T., Lowe, PN., Page, MJ., Gusterson, BA. and Crompton, MR. (1994). Cloning and characterisation of cDNAs encoding a novel non-receptor tyrosine kinase, brk, expressed in human breast tumours. *Oncogene*. Vol.9, No.8, (August 1994), pp. 2383-2390.

Mitchell, PJ., Barker, KT., Martindale, JE., Kamalati, T., Lowe, PN., Page, MJ., Gusterson, BA. and Crompton, MR. (1997). Characterisation and chromosome mapping of the human non receptor tyrosine kinase gene, brk. *Oncogene*. Vol.15, (May 1997), pp. 1497-1502.

Mitchell, PJ., Sara, EA. and Crompton, MR. (2000). A novel adaptor-like protein which is a substrate for the non-receptor tyrosine kinase, BRK. *Oncogene*, Vol.19, No.37, (August 2000), pp. 4273-4283.

Nahta, R., Yuan, LX., Zhang, B., Kobayashi, R. and Esteva, FJ. (2005). Insulin-like growth factor-I receptor/human epidermal growth factor receptor 2 heterodimerization contributes to trastuzumab resistance of breast cancer cells. *Cancer Research*, Vol.65, No.23 (December 2005), pp. 11118-11128.

Nanney, LB., Stoscheck, CM., King Jr, LE. Underwood, RA. and Holbrook, KA. (1990). Immunolocalization of epidermal growth factor receptors in normal developing human skin. *Journal of Investigative Dermatology*, Vol.94, No.6, (June 1990), pp. 742–748.

Ostrander, JH., Daniel, AR., Lofgren, K., Kleer, CG. and Lange, CA. (2007). Breast tumor kinase (Protein Tyrosine Kinase 6) regulates heregulin-induced activation of ERK5 and p38 MAP kinases in breast cancer cells. *Cancer Research*, Vol.67, No.9, (May 2007), pp. 4199-4209.

Palka-Hamblin, HL., Gierut, JJ., Bie, W., Brauer, PM., Zheng, Y., Asara, JM. And Tyner, AL. (2010). Identififcation of β-Catenin as a tartget of the intracellular tyrosine kinase PTK6. *Journal of Cell Science*, Vol.123, No.2 (January 2010), pp236-245.

Paronetto, MP., Achsel, T., Massiello, A., Chalfant, CE. and Sette, C. (2007). The RNA-binding protein Sam68 modulates the alternative splicing of Bcl-x. *The Journal of Cell Biology,* Vol.176, No.7, (March 2007), pp. 929-939.

Petro, BJ., Tan, RC., Tyner, AL., Lingen, MW. and Watanabe, K. (2004). Differential expression of the non-receptor tyrosine kinase BRK in oral squamous cell carcinoma and normal oral epithelium. *Oral Oncology.* Vol.40, No.10, (November 2004), pp. 1040-1047.

Peus, D., Hamacher, L. and Pittelkow, MR. (1997). EGF-receptor tyrosine kinase inhibition induces keratinocyte growth arrest and terminal differentiation. *Journal of Investigative Dermatology*, Vol. 109 No.6, (December 1997), pp. 751–756.

Porter, G., Evans, A., Pinder, S., James, J., Cornford, E., Burrell, H., Chan, S., Cheung, K. and Robertson, J. (2004). Patterns of metastatic breast cancer: influence of tumor histological grade. *Clinical Radiology*, Vol.59, (Dec 2004), pp. 1094–1098.

Qiu, H. and Miller, WT. (2004). Role of the Brk SH3 domain in substrate recognition. *Oncogene*, Vol.23, No.12, (March 2004), pp. 2216-2223.

Qiu, H., Zappacosta, F., Su, W., Annan, RS. and Miller, WT. (2005). Interaction between Brk kinase and insulin receptor substrate-4. *Oncogene,* Vol.24, No.36, (August 2005), pp. 5656-5664.

Railo, MJ., von Smitten, K. and Pekonen, F. (1994). The prognostic value of insulin-like growth factor-I in breast cancer patients. Results of a follow-up study on 126 patients. *European Journal of Cancer*, Vol.30A No.3 (1994) pp. 307-11.

Schmandt, RE., Bennett, M., Clifford, S,. Thornton, A., Jiang, F., Broaddus, RR., Sun, CC., Lu, KH., Sood, AK. and Gershenson, DM. (2006). The BRK tyrosine kinase is expressed in high-grade serous carcinoma of the ovary. *Cancer Biology & Therapy,* Vol.5, No.9, (September 2006), pp. 1136-1141.

Sekine, Y., Yamamoto, T., Yumioka, T., Sugiyama, K., Tsuji, S., Oritani, K., Shimoda, K., Minoguchi, M., Yoshimura, A. and Matsuda, T. (2005). Physical and functional interactions between STAP-2/BKS and STAT5. *Journal of Biological Chemistry*, Vol. 280, No.9, (Mar 2005), pp. 8188-8196.

Shen, CH., Chen, HY., Lin, MS., Li, FY., Chang, CC., Kuo, ML., Settleman, J. and Chen, RH. (2008). Breast tumor kinase phosphorylates p190RhoGAP to regulate rho and ras and promote breast carcinoma growth, migration, and invasion. *Cancer Research,* Vol.68, No.19, (October 2008), pp. 7779-7787.

Simmen, FA., Xiao, R., Velarde, MC., Nicholson, RD., Bowman, MT., Fujii-Kuriyama, Y., Oh, SP. and Simmen, RC. (2007). Dysregulation of intestinal crypt cell proliferation and villus cell migration in mice lacking Kruppel-like factor 9. *Am J Physiol Gastrointest Liver Physiol*, Vol.292, No.6, (June 2007), pp. 1757-1769.

Simões-Wüst, AP., Schürpf, T., Hall, J., Stahel, RA. and Zangemeister-Wittke, U. (2002). Bcl-2/bcl-xL bispecific antisense treatment sensitizes breast carcinoma cells to doxorubicin, paclitaxel and cyclophosphamide. *Breast Cancer Research and Treatment,* Vol.76, No.2, (November 2002), pp. 157-166.

Siyanova, EY., Serfas, MS., Mazo, IA. and Tyner, AL. (1994). Tyrosine kinase gene expression in the mouse small intestine. Oncogene, Vol.9, No.7, (July 1994), pp. 2053-2057.

Sorek, N., Bloch, D. and Yalovsky S. (2009). Protein lipid modifications in signaling and subcellualr targeting. *Current Opinion in Plant Biology*, Vol.12, No.6, (December 2009), pp. 714-720.

Sumantran, VN., Ealovega, MW., Nuñez, G., Clarke, MF. and Wicha, MS. (1995). Overexpression of Bcl-XS sensitizes MCF-7 cells to chemotherapy-induced apoptosis. *Cancer Research*, Vol.55, No.12, (June 1995), pp. 2507-2510.

Tanner, M., Tirkkonen, M., Kallioniemi, A., Holli, K., Collins, C., Kowbel, D., Gray, J., Kallioniemi, O. and Isola, J. (1995). Amplification of chromosomal region 20q13 in invasive breast cancer: prognostic implications. *Clinical Cancer Research*, Vol.1, No.12, (December 1995), pp. 1455–1461.

Tupper, J., Crompton, MR. and Harvey, AJ. (2011). Breast tumor kinase (Brk/PTK6) plays a role in the differentiation of primary keratinocytes. *Archives of Dermatological Research,* Vol.303, No.4, (May 2011), pp. 293-297.

Vasioukhin, V., Serfas, MS., Siyanova, EY., Polonskaia, M., Costigan, VJ., Liu, B., Thomason, A. and Tyner, AL. (1995). A novel intracellular epithelial cell tyrosine kinase is expressed in the skin and gastrointestinal tract. *Oncogene.* Vol.10, No.2, (January 1995), pp. 349-357.

Vasioukhin, V. and Tyner, AL. (1997). A role for the epithelial-cell-specific tyrosine kinase Sik during keratinocyte differentiation. *The Proceedings of the National Academy of Sciences of the United States of America*, Vol.94, No.26, (December 1997), pp. 14477-14482.

Wang, TC., Jee, SH., Tsai, TF., Huang, YL., Tsai, WL. and Chen, RH. (2005). Role of breast tumour kinase in the in vitro differentiation of HaCaT cells. *British Journal of Dermatology,* Vol.153, No.2, (August 2005), pp. 282-289.

Wang, Y., Malkowski, M., Jin, W., Belanger, D., Zeng, H., Curran, PJ., Siddiqui, MA., Maio, H., Shipps, GW., Hailey, H., Maxwell, E., Carr, D. and Seidel-Dugan, C. (2011). Inhibition of PTK6 kinase activity reduces proliferation and migration of tumour cells. In*: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; 2011. Abstract nr 1945.*

Weaver, AM. and Silva, CM. (2007). Signal transducer and activator of transcription 5b: a new target of breast tumor kinase/protein tyrosine kinase 6. *Breast Cancer Research.* Vol.9, No.6, (November 2007), pp. R79

Xiang, B., Chatti, K., Qiu, H., Lakshmi, B., Krasnitz, A., Hicks, J., Yu, M., Miller, WT. and Muthuswamy, SK. (2008). Brk is coamplified with ErbB2 to promote proliferation in breast cancer. *The Proceedings of the National Academy of Sciences of the United States of America*, Vol.105, No.34, (August 2008), pp. 12463-12468.

Zhang, P., Ostrander, JH., Faivre, EJ., Olsen, A., Fitzsimmons, D. and Lange CA. (2005). Regulated association of protein kinase B/Akt with breast tumor kinase. *The Journal of Biological Chemistry*, Vol.280, No.3, (January 2005), pp. 1982-1991.

Zheng, Y., Peng, M., Wang, Z., Asara, JM. and Tyner, AL. (2010). Protein tyrosine kinase 6 directly phosphorylates AKT and promotes AKT activation in response to epidermal growth factor. *Molecular and Cellular Biology.* Vol.30, No.17, (September 2010), pp. 4280-4292.

Zhong, JL., Poghosyan, Z., Pennington, CJ., Scott, X., Handsley, MM., Warn, A., Gavrilovic, J., Honert, K., Krüger, A., Span, PN., Sweep, FC. and Edwards, DR. (2008). Distinct functions of natural ADAM-15 cytoplasmic domain variants in human mammary carcinoma. *Molecular Cancer Research*, Vol.6, No.3, (March 2008), pp. 383-394.

21

Immunoliposomes: A Multipurpose Strategy in Breast Cancer Targeted Therapy

Enrique Barrajón-Catalán, María P. Menéndez-Gutiérrez, Alberto Falcó, Miguel Saceda, Angela Catania and Vicente Micol
Institute of Molecular and Cell Biology (IBMC), Miguel Hernández University, Spain

1. Introduction

One of the main challenges in chemotherapy is the delivery of effective doses of cytotoxic agents to the tumor site and simultaneously to minimize the side effects on normal cells. The use of drug delivery systems (DDS) can improve the pharmacological properties of many agents, modifying their pharmacokinetics and biodistribution.

Among the DDS available, liposomes are one of the most used and promising. Cytotoxic drug incorporation into liposomes has been reported since 1970. Thanks to their special structure, they are able to encapsulate both hydrophilic and hydrophobic compounds. Today several drugs with various chemical properties (hydrophilic drugs such as N-(phosphoacetyl)-L-aspartate or hydrophobic drugs such as doxorubicin) have been succesfully encapsulated into liposomes. Compared to conventional chemotherapeutic drugs (non-encapsulated), the encapsulation of these drugs into liposomes shows several advantages, such as a considerable reduction of side effects associated to conventional chemotherapy, increase of solubility, improvement of therapeutic index and prolonged duration of exposure (Papahadjopoulos, Allen et al., 1991; Drummond, Meyer et al., 1999). Moreover, tumours are one of the primary sites for accumulation of liposomes, where they concentrate due to the higher permeability of the vascular endothelium surrounding tumours. However, despite their unquestionable advantages, unfortunately, liposomes are non site-specific drug delivery systems.

Antibody based targeted therapies are the most promising strategies to improve the selectivity and potency of current cancer treatments. The increasing number of new anticancer drugs based on antibodies demonstrates this assumption (Sznol & Holmlund, 1997; Reichert, Rosensweig et al., 2005). However, selectivity provided by the use of antibodies can also be used, not only for direct treatment but to target other anticancer drugs. In this sense, the conjugation of complete or fragmented antibodies to liposomes has resulted in the next generation of drug delivery systems, i.e. immunoliposomes (Park, Hong et al., 1997; Noble, Kirpotin et al., 2004). In this strategy, the liposome acts as a drug carrier, and the antibody allows bringing the drug system specifically to its target.

Tumour cells have few specific molecules for recognition as they share many common features with normal cells. Then, the main issue when designing selective immunoliposomes is to find a proper target. Since complete selectivity is hard to achieve, the target must be as selective as possible, i.e. by using tumor-overexpressed proteins as

targets. These proteins increase their expression levels up to 100 times compared to normal cells, and are suitable to be used as best target proteins (Barrajón-Catalán, Menéndez-Gutiérrez et al., 2010).

In this chapter, two promising targets are presented as examples of the immunoliposomes versatility and their usefulness in breast cancer models. The first one is the epithelial cell adhesion molecule (EpCAM), which is overexpressed in carcinomas of several origins including breast carcinomas (Armstrong & Eck, 2003; Went, Lugli et al., 2004). The second one is the human epidermal growth factor receptor 2 (HER2), which is overexpressed in 20–30% of breast and ovarian cancers carrying a bad prognosis (Barrajón-Catalán, Menéndez-Gutiérrez et al., 2010). Currently, several antibody-derived drugs based on these targets are already in use in cancer clinical therapies. For instance, Edrecolomab and Trastuzumab are antibodies against these proteins which are used in cancer therapies (Punt, Nagy et al., 2002; Reichert, Rosensweig et al., 2005).

The diversity of chemotherapeutic drugs vehiculized into immunoliposomes is continuously growing, as new drugs or natural compounds are incorporated to the anticancer therapeutic arsenal (Mamot, Drummond et al., 2003; Yang, Choi et al., 2007; Yuji Yamamoto, 2011 Jan). Alternatively, other new strategies based on the use of immunoliposomes, but including lytic peptides, are focused on specifically promoting the lysis of cancer cells (Barrajón-Catalán, Menéndez-Gutiérrez et al., 2010). In this regard, melittin represents one of the most studied lytic peptides, and it has shown anti-inflammatory and anticancer activities and low toxicity, and it has been used in several therapeutical approaches (Son, Lee et al., 2007; Soman, Baldwin et al., 2009).

This chapter is focused on the latest mentioned type of immunoliposomes, the so-called lytic immunoliposomes (LILs), which might become a fast and specific new treatment for breast cancer. The feasibility of LILs carrying melittin and containing antibodies against the two above mentioned targets, EpCAM and HER2, is shown. These immunoliposomes have been used to treat a panel of human breast cancer cells bearing different EpCAM or HER2 expression levels. In both cases, the morphological changes observed in treated cancer cells suggested a cytolytic process, as it could be expected given melittin lytic properties.

The approach presented here may suppose a specific, versatile and effective strategy for the treatment of tumors overexpressing a surface antigen. The incorporation of new antibodies to this model will allow targeting additional molecular sites in cancer cells, which will surely provide treatments for cancer therapeutics with increased selectivity and lower side effects and, it will probably contribute to decrease the appearance of resistances.

2. Preliminary aspects to be considered

2.1 The molecular target

As stated above, the most difficult task when designing immunoliposomes is choosing the right molecular target. Since complete selectivity is hard to achieve, the therapy must be as specific as possible in order to reduce side effects and to increase the potency of the model.

To obtain optimal results, the target must fulfil the following conditions:

- It must be a surface antigen and has to be located in an accessible domain of the cytoplasmic membrane.
- It must be as much antigenic as possible in order to produce the corresponding antibody.

- Proteins are preferred rather than other antigens such as carbohydrates.
- Tumor specific antigens are preferred, but these are rare. Then, tumor overexpressed antigens are a more accessible alternative. The higher the overexpression level is, the more selective the system gets.

Finding the perfect target is unlikely, but there are several promising candidates. The most used and characterized antigens belong to the HER family. Moreover, other targets have been used and well characterized: EpCAM in breast and gastric cancers (Cimino, Halushka et al., 2009; Wenqi, Li et al., 2009), PSMA in prostate cancer (Ikegami, Tadakuma et al., 2005; Ikegami, Yamakami et al., 2006) or MUC1 in colon and prostate cancer (McDermott, Crocker et al., 2001; Papadopoulos, Sivridis et al., 2001).

The continuous discovery of new tumor specific and/or tumor overexpressed antigens by many research groups working in this field will enormously contribute to the development of these treatments, by generating a constant increase in the number of potential antibodies. Moreover, new advances in proteomic investigation will also help to establish successful therapies (Conrotto, Roesli et al., 2008; Madoz-Gúrpide, Kuick et al., 2008).

2.2 The immunoliposome design

The linking of antibodies to liposomes has been intensely studied from different perspectives (see review (Hansen, Kao et al., 1995)). Most of these techniques have been used in different cellular and animal models. Since every case may have specific requirements, the linking technology is normally chosen depending on the availability of the chemical groups involved, such as lipid polar head groups, linker's nature and antibody characteristics.

Once the technique is selected, the right linker will be chosen according to the procedure. The most common linkers are modified lipids containing the maleimide moiety. On the other hand, the antibody must be derivatized in order to be linked to the lipid moieties. One of the most common derivatization techniques uses Traut's reagent, which yields thiol groups in the antibody, what allows the subsequent reaction with the maleimide group of the modified lipids through a covalent bonding (Kirpotin, Park et al., 1997).

Regardless the selected protocol, there are some aspects to take into account when immunoliposomes are designed:

- Immunogenicity of the final product must be as low as possible, and the drug must remain in blood circulation as long as possible. Pegylation solves both aspects in a smart way. First, the use of pegylated lipids into liposomes at 1-5% molar percentage increases blood permanence time because it reduces uptake rate by the reticuloendothelial system. Second, it decreases the immunogenicity of the preparation as the liposomes become "invisible" to the immune system due to the polyethylene glycol (PEG) tails (Petrak, 2005; Elbayoumi & Torchilin, 2006). In addition, pegylated liposomes show a superior tumor delivery capacity compared with conventional liposomes composed of natural phospholipids (Allen & Cullis, 2004). Although there are different PEG lengths, PEG-2000 is the most used in pharmacological preparations (Sadzuka, Kishi et al., 2003).
- Lipid composition is also very important because it determines the fluidity of the liposomal membrane, and therefore, its ability to encapsulate or retain compounds. For example, the presence of cholesterol increases the rigidity of the liposome membrane, contributing to the encapsulation improvement of hydrophilic compounds into the inner aqueous cavity. When hydrophobic molecules are used, the lipid composition is

also an important issue, because it must provide an adequate environment, capable to retain hydrophobic molecules in the required active conformation and the ability to transfer them to target membranes (Maruyama, Kennel et al., 1990).

- Finally, the antibody attached to the liposome can be used either as a whole protein or fragmented. Several approaches have been developed using the single-chain antibody variable region (scFv) (Mamot, Drummond et al., 2003; Hu, Chen et al., 2006), the complete variable region (Fab) (Kirpotin, Drummond et al., 2006) or the whole antibody (Barrajón-Catalán, Menéndez-Gutiérrez et al., 2010). It must be considered that, scFv and Fab (Figure 1) are less immunogenic than the whole antibody, however these fragments are not easy to obtain. The production of Fab requires a large amount of the original antibody and the protocol includes its digestion by pepsin or other enzymes. In contrast, the use of scFv fragments requires cloning designs and recombinant production systems.

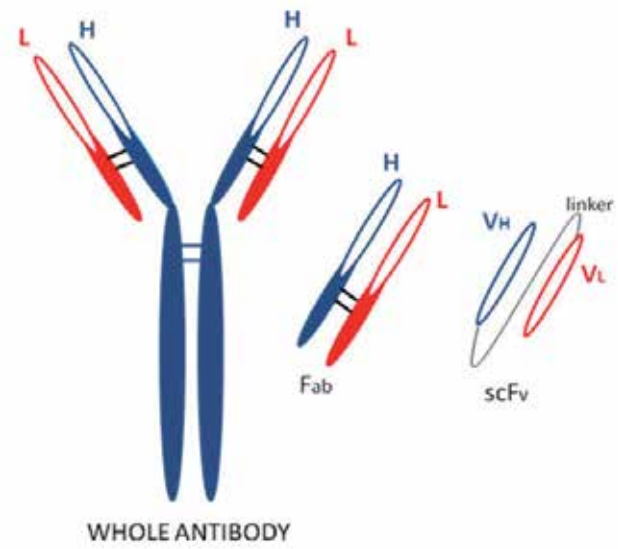


Fig. 1. Different classes of antibody derivatives used for immunoliposome synthesis. Heavy chain (H) is shown in blue and light chain (L) in red, variable region appears as empty ellipses. Whole antibody (on the left) can be used to obtain the Fab fragment with constant and variable region linked by disulfide bonds. scFV fragment contains only the variable regions of both heavy and light chains bound by a linker.

2.3 The lytic peptides

Antimicrobial peptides are gene encoded natural peptide antibiotics being part of the innate defences of many organisms (Patrzykat & Douglas, 2005). Among them, lytic peptides (LPs) are those showing highest cytolytic activity. Several studies have shown that LPs have cationic and amphipathic properties, which allow them to interact with lipid cell membranes of most pathogens, often negatively-charged (Lohner & Blondelle, 2005), by forming pores or ion channels (Bechinger, 1997). Several models have been proposed to explain this mechanism, i.e. barrel-stave, carpet-like, toroidal pore formation or detergent-type micellization models (Allende, Simon et al., 2005). Regardless the molecular mechanism, LPs promote increased

membrane permeabilization, leakage of cell content and osmotic instability, which leads to the unavoidable cell death. In some cases, peptide diffusion to intracellular targets has also been described. The capacity of LPs to promote cell lysis has been extensively studied in order to develop new anticancer approaches (Hoskin & Ramamoorthy, 2008).

LPs can be included in LILs as active molecules that promote cytotoxic effects. LPs have already been used covalently linked to hormone segments in order to increase their efficacy and selectivity on several types of cancer cells (Gawronska, Leuschner et al., 2002; Hansel, Enright et al., 2006). Nevertheless, a therapy based on this approach might be valid only for those tumors expressing hormone receptors, such as prostate or breast cancers. However, LILs may be applied to any kind of tumors. For instance, previous reports have shown the activity of LILs against hepatoma cancer cells (Hu, Chen et al., 2006).

Among the LPs derived from insects and amphibians, melittin, from bee venom, has recently shown anticancer properties but its precise mechanism of action is still uncertain (Son, Lee et al., 2007). Melittin is a 26 amino acid linear peptide (GIGAVLKVLTTGLPALISWIKRKRQQ) with alpha-helix conformation. The distribution of its amino acid sequence is the main responsible of the amphipathic nature of this peptide: the amino-terminal region (residues 1–20) is predominantly formed by hydrophobic residues, whereas the carboxy-terminal region (residues 21–26) is hydrophilic due to the presence of a tandem of basic amino acids (Raghuraman & Chattopadhyay, 2007). Melittin has been the LP choice in several studies using LILs. Among the reasons for this are: its particular amphipatic nature, which allows it to bind to lipid membranes with high affinity, its activity against tumor cells, its relatively low market value and its availability. It is also water soluble, which facilitates its manipulation.

Although melittin molecular mechanism seems to be controversial, the most accepted model proposes that melittin destroys target membranes by pore formation and destabilization through the barrel-stave mechanism (Sui, Wu et al., 1994; Lin & Baumgaertner, 2000). In this sense, once LIL binds to targeted cell, a lytic process occurs in a few hours with no signal of apoptosis (Barrajón-Catalán, Menéndez-Gutiérrez et al., 2010).

To fully characterize LILs model, the selected LP must be adequately quantified. For this task, HPLC coupled to fluorescent, diode array or mass detection is the most commonly used method (Perez-Paya, Braco et al., 1991; Barrajón-Catalán, Menéndez-Gutiérrez et al., 2010) but also capillary chromatography (Pacakova & Stulik, 2000) can be utilised.

2.4 The cellular model

The choice of a suitable cellular model is as important as the target to test the efficacy of the LIL system. Several cell lines showing gradual differences in the expression levels of the targeted protein may be required for these studies in order to make sure that cytotoxic effects are dependent on the antigen expression. Thus, one of the preliminary tasks is the quantification of the expression levels of the selected cellular lines. Nevertheless, it must be checked that all the quantified protein is located on the cell membrane. In some cases, a significant percentage of the overexpressed protein shows an intracellular location, and then, this protein is obviously not suitable to be used as target for LILs. Fortunately, many breast cancer cellular lines are readily available and well characterized, facilitating this task.

When selecting cell lines, to assess their *in vivo* tumorogenic capacity becomes also an important issue. One of the critical steps in preclinical investigations with LILs and other pharmaceutical formulations, is to prove their *in vivo* activity against xenotransplanted

cancer cells in athymic mice. Not all cells are able to establish and grow in these mice on their own. For example, some breast cancer cells present strogen-dependent growth (Saceda, Knabbe et al., 1991) and estradiol must be used as coadjuvants in order to obtain successful *in vivo* tumourogenic activity. On the other hand, other cells need the use of polymeric coadjuvants, such as matrigel, to be implanted in mice and the tumor cells must be coinjected with this polymer.

3. Lytic immunoliposome models

3.1 EpCAM immunoliposomes

EpCAM is a transmembrane protein (Armstrong & Eck, 2003; Went, Lugli et al., 2004; Trzpis, McLaughlin et al., 2007) which mediates epithelium-specific Ca^{2+} independent cell-cell adhesions. EpCAM is overexpressed in carcinomas of several origins such as breast, gastric, colorectal, prostate and liver (Went, Lugli et al., 2004; Wenqi, Li et al., 2009). EpCAM antigen has been used in immunotherapy studies (Braun, Hepp et al., 1999; Winkler, Martin-Killias et al., 2009) against breast cancer cells with good results. There is a commercial anti-EpCAM antibody called Edrecolomab (Panorex ®), which is currently being used in the treatment of colorectal, breast and gastric cancers (Braun, Hepp et al., 1999; Haller, 2001). Due to these reasons, EpCAM supposes an attractive and promising target to be challenged through the use of lytic immunoliposomes therapy.

In this first study, anti-EpCAM coupled immunoliposomes were used to target human cancer cells *in vitro*. Large unilamellar vesicles were sterically stabilized by using a pegylated phospholipid (Kirpotin, Park et al., 1997) and then, covalently coupled to a commercial anti-EpCAM antibody using a phospholipid linker. Subsequently, pure melittin was incorporated into the immunoliposome mixture. Unbonded peptide was separated by gel filtration chromatography. For the complete protocol, see reference (Barrajón-Catalán, Menéndez-Gutiérrez et al., 2010).

Once immunoliposomes were obtained, the expression levels of EpCAM were analyzed in the cell lines. In this case, the selected cell lines were MCF7 and MDA-MB231, both derived from a pleural effusion of a human breast adenocarcinoma; MCF7 is documented to have high expression levels of EpCAM. On the contrary, MDA-MB231 shows a very low expression (Prang, Preithner et al., 2005). To ensure this point, EpCAM expression was analyzed by Western blot using an anti-EpCAM antibody (Figure 2) in both cell lines, confirming the previously published results.

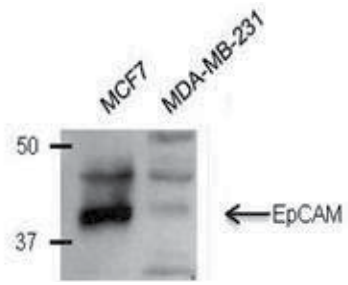


Fig. 2. Anti-EpCAM Western blot showing the expression levels of the protein in MCF7 and MDA-MB231 cells. Molecular weight references (in kDa) are shown in the left side of the picture.

To ensure that targeted antigen is mainly expressed as a surface protein instead of being located in endosomes or vacuoles, and that it can be reached by the antibodies linked to the LILs, fluorescence-activated cell sorting (FACS), a type of flow cytometry, can be used. Membrane expression levels of surface antigens can be measured and compared using this sensitive technique. Other alternative is to perform Western blot analysis of membrane protein extracts obtained after subcellular fractionation. One example of the results obtained by FACS is shown in figure 3, where EpCAM membrane expression levels in MCF7 and MDA-MB231 cell lines are compared.

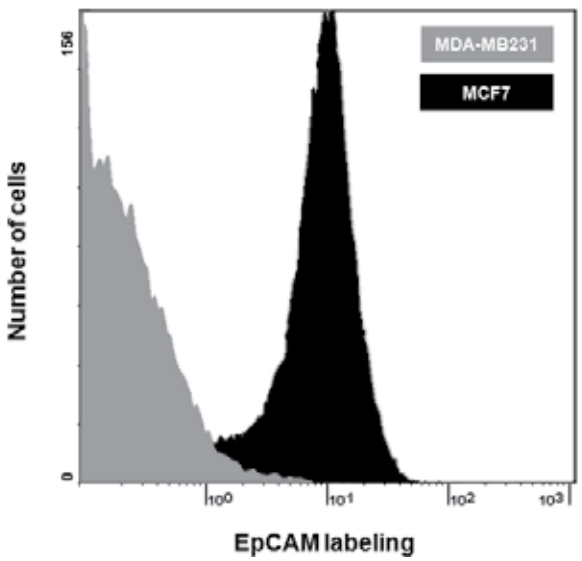


Fig. 3. Analysis of EpCAM expression on cell membranes by FACS in MDA-MB231 (grey) and MCF7 (black).

Once the expression levels of the chosen antigen have been determined in both cell lines, binding of immunoliposomes to the cells can be assessed by fluorescence microscopy. For this purpose, the lipid mixture can be labeled using a fluorescent phospholipid conjugated with rhodamine. Alternatively, fluorescein isothiocyanate (FTIC) conjugated antibodies can also be used. In the present example, images of two cell lines were obtained after treatment with fluorescent immunoliposomes, which were labeled both with rhodamine conjugated phosphatidylethanolamine (PE) and anti-EpCAM FTIC-conjugated (Figures 4A and 4B). MCF7 cells, which overexpress EpCAM antigen, present a double labeling due to the binding of complete immunoliposomes (red of rhodamine-PE and green of FITC-labeled antibody). However, MDA-MB231 cells showing low EpCAM expression levels present only a weak red fluorescence due to the background signal deriving from rhodamine-labeled liposomes lacking the antibody. The cytotoxic activity of the LILs can be also analyzed using fluorescence microscopy or flow cytometry. Some probes, such as DAPI are able to penetrate only in permeabilized cells or cells with damaged membranes, then these probes are specific for cell death. The efficacy of the cytotoxic effect of LILs may be also monitorized using DAPI fluorescence because of the effect of lytic peptides on cell membrane (Figure 4C). MCF7 cells show a triple-labeling (red from lipids, green from antibody and blue from DAPI), indicating that the binding of the LILs to the targeted cells is followed by cell death.

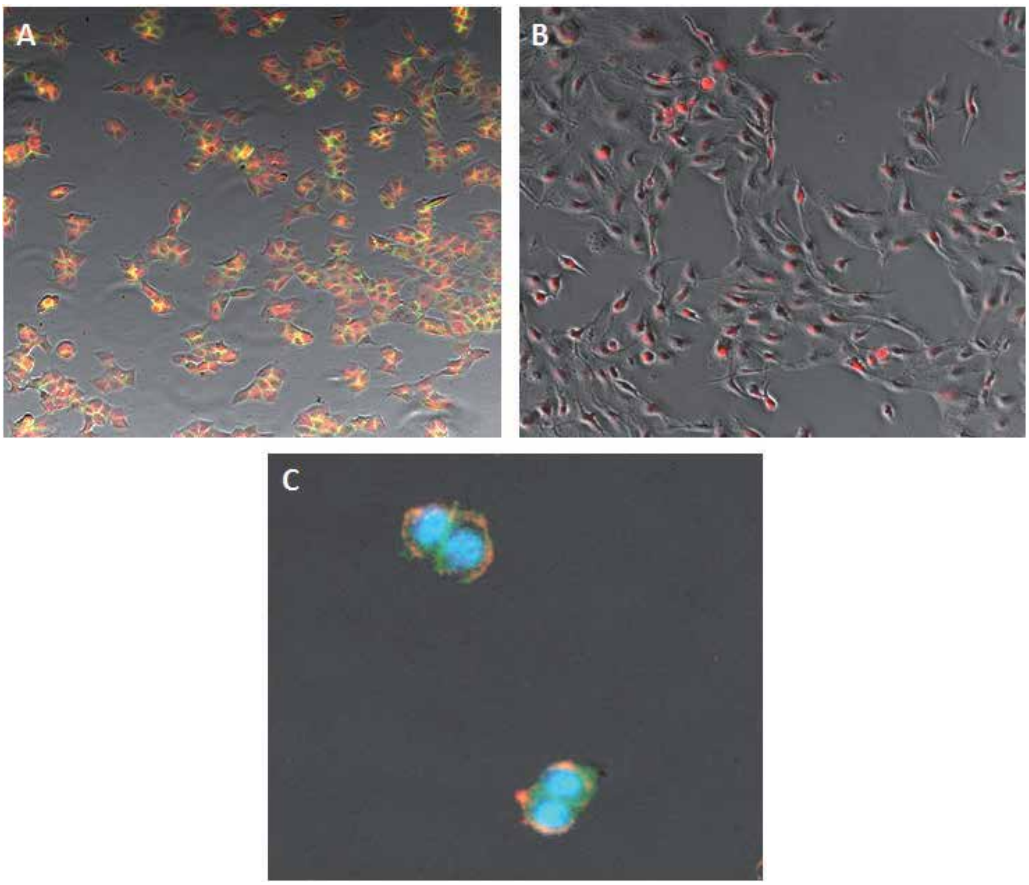


Fig. 4. Fluorescence microscopy imaging of the treatment of breast cancer cells with LILs containing EpCAM antibody. MCF7 (A) and MDA-MB231 (B) cells treated with LILs carrying rhodamine conjugated PE and anti-EpCAM FTIC conjugated antibody. (C) MCF7 cells treated with the same LILs but in the presence of DAPI (blue fluorescence).

Fluorescent microscopy imaging is a useful technique for obtaining qualitative results, nevertheless quantification of the binding and cytotoxic effects of LILs are key aspects to consider in order to test LILs efficacy. The use of flow cytometry is a great approach to quantify these two aspects simultaneously. To achieve this goal, LILs carrying FITC-conjugated EpCAM antibody were used to treat both cell lines and then propidium iodine, which only penetrates permeabilized cells, was added to monitorize cell death. Figure 5 shows the treatment of MDA-MB231 and MCF-7 cells using labeled LILs and propidium iodine. The whole bar indicates the percentage of EpCAM FITC labeled cells and stripped bar shows dead cells (propidium iodine labeled), which are EpCAM positive, i.e. cells showing double labeling. EpCAM overexpressing MCF7 cells treated with LILs showed ten times more fluorescence, due to antibody labeling, than MDA-MB231 cells, which showed a low EpCAM expression level (Figure 5). This indicates that MCF7 cells were succesfully reached by LILs. Furthermore, only MCF7 cells were labeled with propidium iodine, indicating that the binding of LILs to these cells was followed by permeabilization and cell death. This confirms previous fluorescence microscopy results.

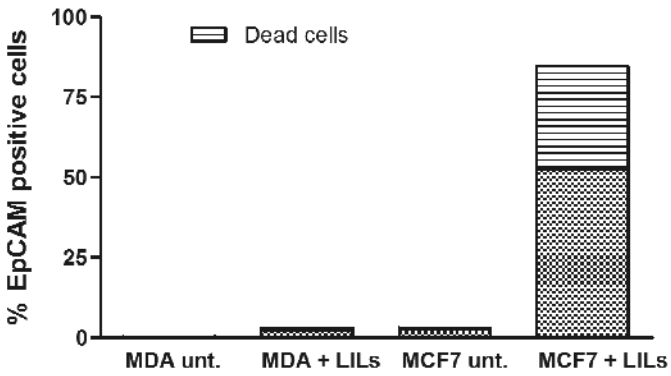


Fig. 5. Flow cytometry results of MDA-MB231 and MCF-7 cells treated with LILs carrying EpCAM antibody and propidium iodine. Total bars indicate the percentage of EpCAM positive-expressing cells while stripped fraction of the bars indicates the percentage of these cells reached by propidium iodine.

Other techniques to quantify the effects of LILs on cancer cells are those focused to determine cell viability. MTT (Barrajon-Catalan, Fernandez-Arroyo et al., 2010) and violet crystal are among the techniques most commonly used for detecting cell survival. Adequate control experiments must also be established in cell survival assays in order to obtain conclusions about LILs specificity. Target cells must be treated comparatively, using identical concentrations of either complete LILs (LILs carrying labeled antibody and lytic peptide), with immunoliposomes lacking the lytic peptide (LIP), or with liposomes bearing the lytic peptide but lacking the antibody (LIP + LP) (Figure 6). Although some level of unspecific cytotoxicity was found in the cells showing low antigen expression levels (MDA-MB231), LILs exerted a strong cytotoxic effect on cells expressing high levels of EpCAM, which demonstrates LILs selectivity.

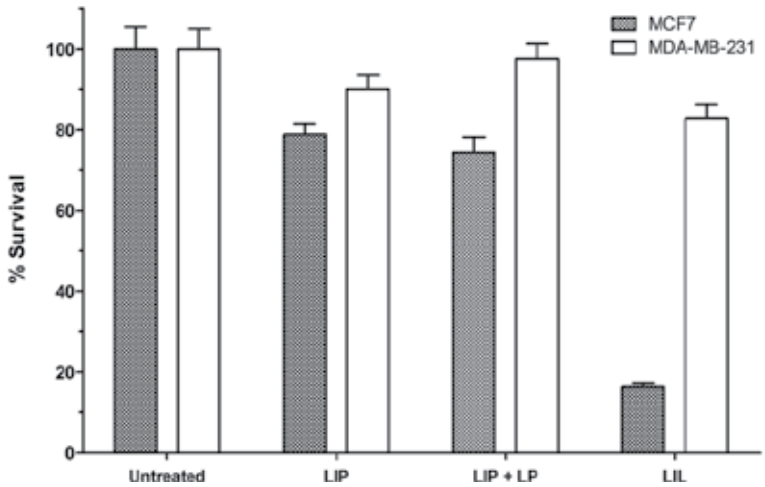


Fig. 6. Survival plots for two breast cancer cell lines having different EpCAM expression levels treated with various liposomal systems. Percentage of surviving cells was measured by MTT assay. MCF7 (dotted bars) and MDA-MB231 cells (white bars) were challenged with different constructs of liposomes and LILs.

3.2 HER2 immunoliposomes

HER2 molecule is probably the most studied and clinically used target in anticancer studies involving immunotherapies. HER2 (or ErbB2) is a protooncogene belonging to the epidermal growth factor receptor family (EGFR or ErbB) of receptor tyrosine kinases (RTK) (Olayioye, Neve et al., 2000). HER2 is overexpressed in 20–30% of breast and ovarian cancers (Slamon, Clark et al., 1987). Moreover, in normal adult tissues, HER2 is present only at low levels in certain epithelial cell types (Press, Cordon-Cardo et al., 1990). Several anticancer therapies targeting ErbB receptors have been developed, and the humanized monoclonal antibody that binds to HER2 (Herceptin® or trastuzumab) is currently in clinical use as an effective treatment for HER2 positive breast cancer (Slamon, Leyland-Jones et al., 2001). Trastuzumab has been used in several therapeutical approaches coupled to liposomes in order to increase anticancer therapy selectivity. Anti-HER2 immunoliposomes have shown binding and internalization capacities in HER2-overexpressing cells (Kirpotin, Park et al., 1997), which leads to intracellular drug delivery. Moreover, the use of doxorubicin-loaded anti-HER2 immunoliposomes exhibited significantly better therapeutic results, in HER2-overexpressing xenograft models, compared to other treatments (Park, Hong et al., 2002).

The cellular model presented above for EpCAM LILs was composed of two cell lines, but more complex models can be used when several cell lines with different antigen expression levels are available. Figure 7 shows the level of HER2 expression of the total cellular extracts

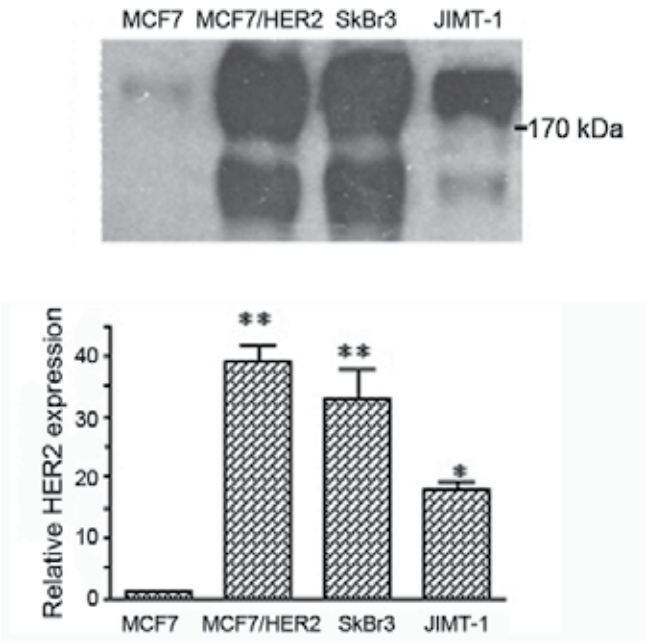


Fig. 7. HER expression level of four breast cancer cells by Western blot analysis. Top image shows the expression level in total cellular extracts from the different cell lines (MCF7, MCF7/HER2, SKBr3 and JIMT-1). Bottom image shows the results of the quantification of the bands using densitometric analysis. MCF7 and SKBr3 derive from a pleural effusion of a human breast adenocarcinoma, MCF7/HER2 stably overexpress HER2 after transfection and JIMT-1 derives from a human breast cancer clinically resistant to trastuzumab (Barrajón-Catalán, Menéndez-Gutiérrez et al., 2010).

deriving from four different breast cancer cell lines (Barrajón-Catalán, Menéndez-Gutiérrez et al., 2010). Western blot analysis and quantification of the bands by densitometric analysis showed that MCF7/HER2 and SkBr3 exhibited the highest HER2 expression levels followed by JIMT-1 and MCF7 cells.

Nevertheless, to confirm the extracellular location of the antigen detected by Western blot, flow cytometry analysis using an antiboby able to detect only the antigen on the cell membrane was also performed. In this case, the different expression level of each cell line correlates to a displacement of the peak population to higher fluorescent levels, as shown in Figure 8.

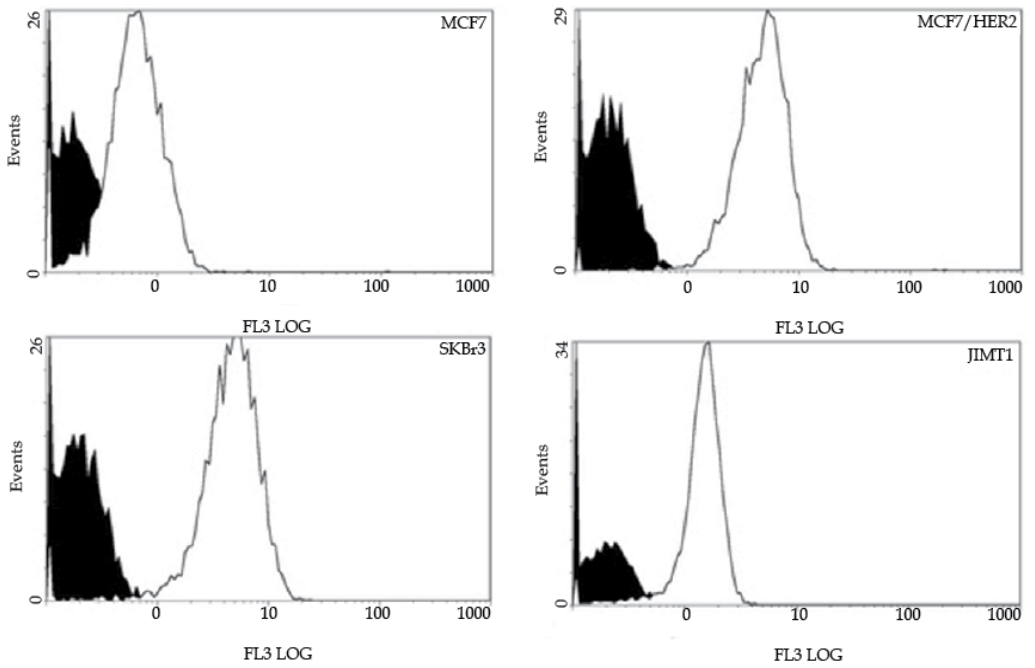


Fig. 8. FACS analysis of membrane HER2 expression in different breast cancer lines, named in the histograms.

The efficacy and selectivity of HER2-containing LILs on the different cell lines were tested in dose/response experiments using increasing concentrations of the LILs. Viability of MCF7 cells (with low HER2 expression) was the least affected by LILs. JIMT-1 cells viability was decreased in a higher degree than that one observed for MCF7 cells. Besides, MCF7/HER2 and SKBr3 cells, showing the highest levels of HER2 expression, underwent a sharp decrease of their viability at almost all the concentrations of the immunoliposomes preparation studied. Therefore, the higher the expression of HER2 was, the more active LILs system was (Figure 9A). Nevertheless, high concentrations of immunoliposomes would make the cytotoxic activity to be less cell-specific. Therefore, the biggest differences in cell viability were found at 0.3 mM total lipid concentration (Figure 9B).

Flow cytometry experiments using specific probes for cell death are good complementary information to cell viability assays. However, new technical approaches as ImageStream analysis allow, not only the quantification of the fluorescence probes, but also obtaining *in situ* fluorescent images of the treated cells, when lipid or antibodies are labeled with

fluorescent dyes. ImageStream-based analysis employs flow cytometry combined with microscopy and allows for statistical analysis of a variety of cellular parameters, as well as the visualization of cells in suspension during flow analysis via high-resolution bright-field, dark-field and fluorescence images (Zuba-Surma, Kucia et al., 2007).

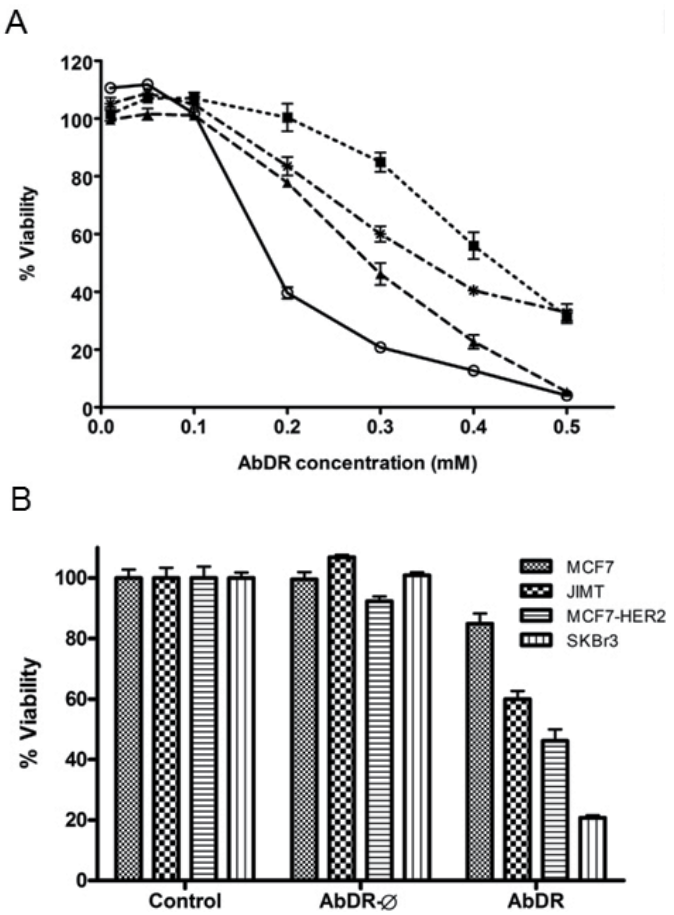


Fig. 9. Survival assays for HER2 LILs. (A) Plots showing the percentage of viable cells in each breast cancer cell line as determined by MTT assay after treatment with HER2 labeled LILs (--✹-- JIMT-1, -▲- MCF7/HER2, -○- SKBr3, - ■- MCF7). (B) Percentage of viable cells determined by MTT assay for the four breast cancer cell lines after the treatment with 0.3 mM of LILs (AbDR) or immunoliposomes lacking the lytic peptide (AbDR-ø).

Figure 10 shows the ImageStream assay results of the treatment of SkBr3 breast cancer cells with liposomes containing a rhodamine labeled lipid, but lacking antibody (Rhod-L, panel A) in comparison to labeled liposomes bearing HER antibody (Rhod-LILs, panel B) (Barrajón-Catalán, Menéndez-Gutiérrez et al., 2010). For quantification purposes, cell populations were separated in three different groups (subpopulations number 1, 2, and 3) attending to increasing fluorescence intensity signal. Figure 10 shows that Rhod-L (panel A) and Rhod-LILs (panel B) treated cells evidenced similar subpopulation 1 (those cells having lowest intensity and Rhod labeling). In contrast, Rhod-LILs treated cells showed a greater

cell subpopulation 2 than Rhod-L treated cells (around double, i.e. 28% vs. 14%) (see panels A and B and quantification in panel C). Moreover, Rhod-LILs treated cells showed another cell subpopulation, which supposed 5% of total cells (subpopulation 3, panel B), bearing a very strong Rhod labeling intensity, which was negligible in Rhod-L treated cells. The images corresponding to examples of sorted cells deriving from both treatments (Figure 10) clearly showed that subpopulations 2 and 3 exhibited a strong rhodamine labeling signal, which demonstrates the higher specificity of Rhod-LILs for SKBr3 cells. The total fluorescence in Rhod-LILs treated cells was approximately three times higher than that one of Rhod-L treated cells.

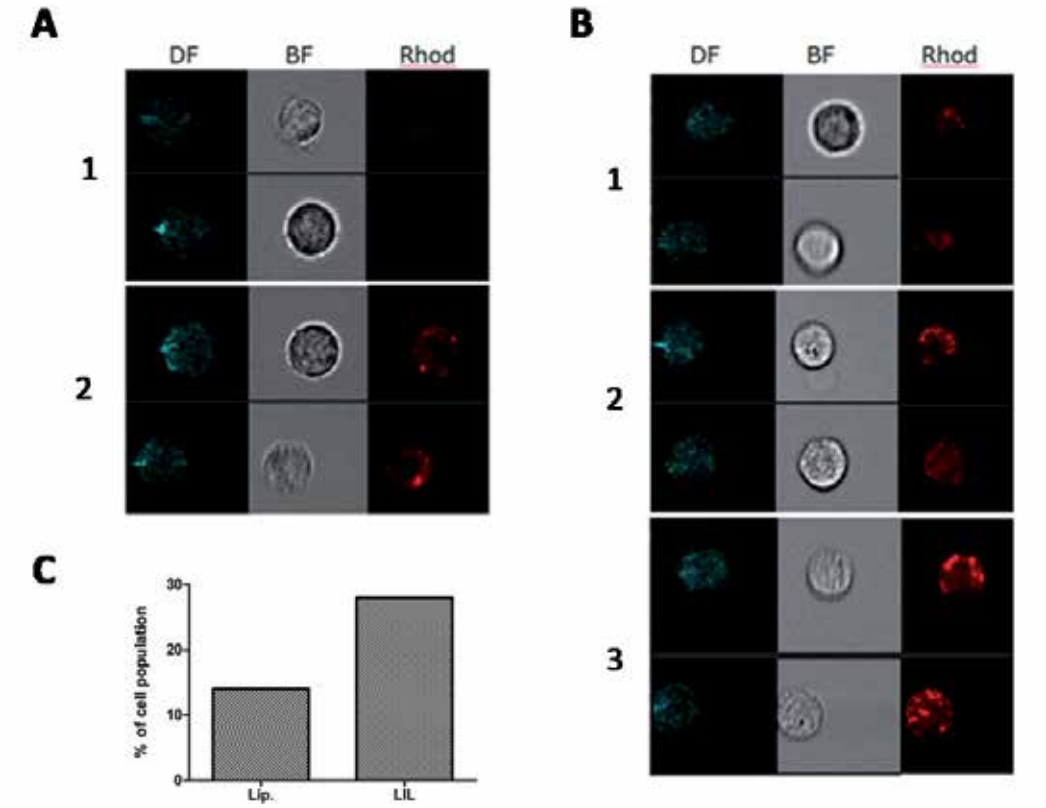


Fig. 10. (A and B) ImageStream results. SkBr3 breast cancer cells treated with liposomes containing a rhodamine labeled lipid, but lacking antibody (Rhod-L, panel A) in comparison to treatment with labeled liposomes bearing HER antibody (Rhod-LILs, panel B). 1, 2 and 3 are mean cell subpopulations having increasing fluorescence intensity signal derived from Rhod labeling. Composite images of dark-field (DF), bright-field (BF) and rhodamine fluorescence (Rhod) images are shown for both conditions. (C) Gated subpopulation 2 in Rhod-L and Rhod-LILs treated cells.

3.3 LILs potential mechanism

The cytotoxic effect of LILs on the tested breast cancer cells shown in these studies is quite fast. The main responsible molecule for this effect is the lytic peptide melittin, since immunoliposomes lacking melittin were devoid of such effect. Then it must be hypothesized

that the cytotoxic action of LILs is mediated by a cytolytic process in which cells lysis is present. This event occurs within a few hours. Some descriptive images were taken to show this action (Figure 11). The figure clearly shows that cells died in a few hours period suffering a presumably lytic process, showing membrane cell disruption and leading to the release of the intracellular content followed by cell membrane retraction.

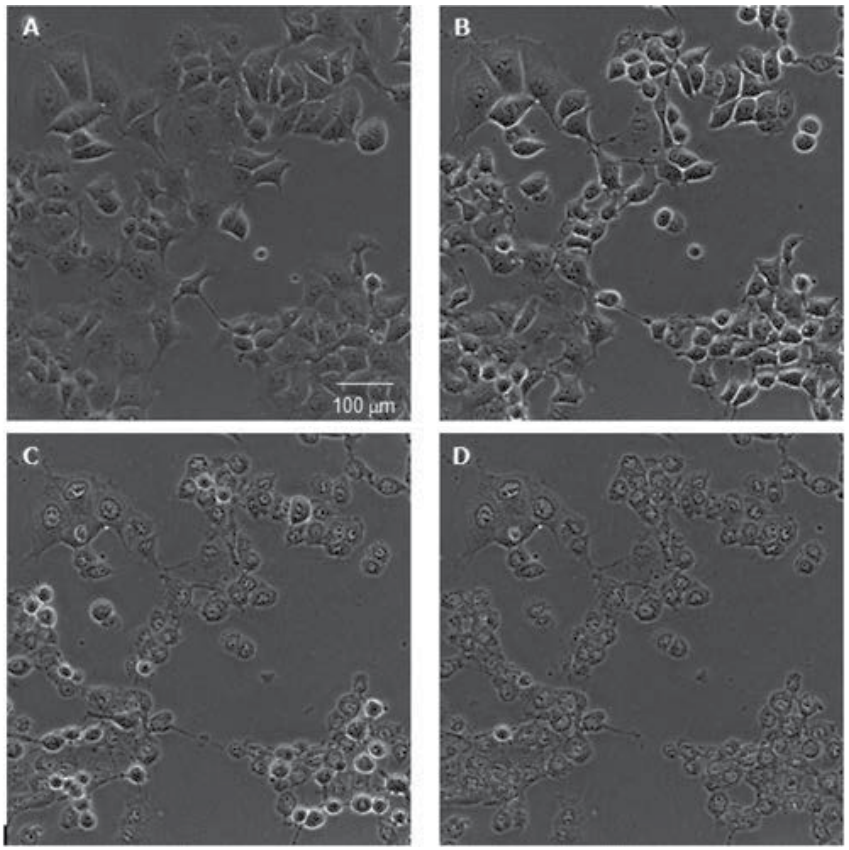


Fig. 11. Images from a LILs treatment. Images extracted from a time-course follow up experiment of the treatment of MCF7/HER2 cells for 4 h with LILs in a Nikon Eclipse TE2000U fluorescence microscope. The images were taken a 0 (A), 1 (B), 2 (C), and 4 h (D) using a 20x lens.

Apparently, apoptosis is not directly implicated in the LILs mechanism as the time-lapse is too short (only a few hours) and no signals of DNA fragmentation or apoptotic bodies were observed after DAPI labeling (Figure 12).

LILs must be also structurally characterized by several techniques in order to test size and stability of the delivery system. Size, polydispersity and Z-potential can be obtained by Dinamic Light Scattering (Villari & Micali, 2008), a potent and simple technique that allows to obtain these parameters without disrupting the LILs. The quantification of the amount of antibody present in LILs by Western blot is also useful in order to confirm the number of antibody molecules per liposome, which can give information about the efficacy of the derivatization of liposomes (Barrajón-Catalán, Menéndez-Gutiérrez et al., 2010).

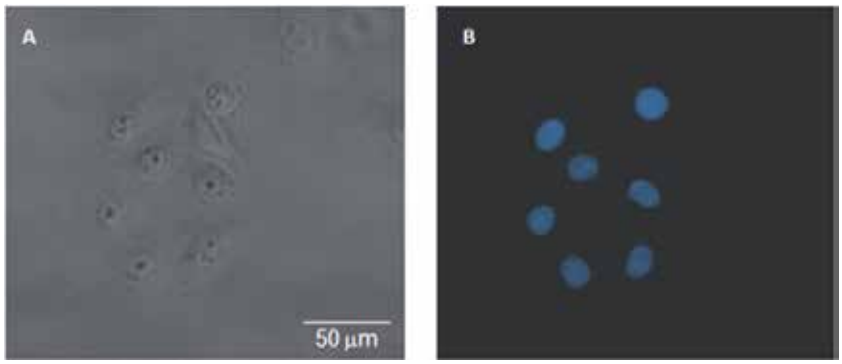


Fig. 12. DAPI labeling after LILs treatment. Contrast phase (A) and fluorescence images (B) of DAPI staining of MCF7–HER2 cells treated with LILs, images were taken using 40x lens.

4. Conclusions

LILs are a suitable pharmacological tool for the selective treatment of cancer cells. For their synthesis, liposomes containing an adequate lipid composition, and a choice of lytic peptides and antibody are required. LILs exhibit specific cytotoxicity against antigen overexpressing breast cancer cells. Moreover, this capacity correlates to the antigen expression level at the cell membranes. The morphological changes observed through microscopy, when cancer cells are treated, suggest a very fast mechanism of cell death, which might take place primarily through membrane pore formation involving a cytolytic process.

The approach presented here may suppose an specific and effective strategy for the treatment of tumors that show overexpression of a surface antigen, by using a low toxicity peptide such as melittin. The examples presented in this chapter describe the elements needed to synthesize lytic immunoliposomes and the techniques to assay their activity on cancer cells. Although these studies have been focused on cancer cells, if an overexpressed surface antigen exists, this approach may be extended to other cell types. Finally, immunoliposomes could be also used as delivery systems for other cytotoxic molecules, either from synthetic or natural origin, which could improve the selectivity or efficacy of these molecules on their cellular targets.

5. Acknowledgments

This investigation has been supported by Grant AGL2007-60778 and private funds provided by CGB-EDSON. We thank personnel of Elche University Hospital for their advice and help in the ImageStream analysis and for providing us with the commercial antibody. We also thank Dr. José A. Ferragut and Dr. Alfredo Carrato from IBMC, and Dra. Inmaculada Jiménez from Elche University Hospital and Dr. Jose Solla from Alicante University for their invaluable help. Figures 7-12 are reprinted from *Cancer letters*, 290, Barrajón-Catalán et al., Selective death of human breast cancer cells by lytic immunoliposomes: Correlation with their HER2 expression level, 192-203, with permission from Elsevier.

6. References

Allen, T. M. and P. R. Cullis (2004). "Drug delivery systems: entering the mainstream." Science 303: 1818-1822.

Allende, D., S. A. Simon, et al. (2005). "Melittin-induced bilayer leakage depends on lipid material properties: evidence for toroidal pores." Biophys.J. 88(3): 1828-1837.

Armstrong, A. and S. L. Eck (2003). "EpCAM: A new therapeutic target for an old cancer antigen." Cancer Biol.Ther. 2(4):320-326.

Barrajon-Catalan, E., S. Fernandez-Arroyo, et al. (2010). "Cistaceae aqueous extracts containing ellagitannins show antioxidant and antimicrobial capacity, and cytotoxic activity against human cancer cells." Food and Chemical Toxicology 48: 2273–2282.

Barrajón-Catalán, E., M. P. Menéndez-Gutiérrez, et al. (2010). "Selective death of human breast cancer cells by lytic immunoliposomes: Correlation with their HER2 expression level." Cancer Letters 290: 192-203.

Bechinger, B. (1997). "Structure and functions of channel-forming peptides: magainins, cecropins, melittin and alamethicin." J.Membr.Biol. 156(3): 197-211.

Braun, S., F. Hepp, et al. (1999). "Monoclonal antibody therapy with Edrecolomab in breast cancer patients: Monitoring of elimination of disseminated cytokeratin-positive tumor cells in bone marrow." Clinical Cancer Research 5(12): 3999-4004.

Cimino, A., M. Halushka, et al. (2009). "Epithelial cell adhesion molecule (EpCAM) is overexpressed in breast cancer metastases." Breast Cancer Research and Treatment: 1-8.

Conrotto, P., C. Roesli, et al. (2008). "Identification of new accessible tumor antigens in human colon cancer by ex vivo protein biotinylation and comparative mass spectrometry analysis." International Journal of Cancer 123(12): 2856-2864.

Drummond, D. C., O. Meyer, et al. (1999). "Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors." Pharmacol.Rev. 51: 691-743.

Elbayoumi, T. A. and V. P. Torchilin (2006). "Enhanced accumulation of long-circulating liposomes modified with the nucleosome-specific monoclonal antibody 2C5 in various tumours in mice: gamma-imaging studies." Eur. J. Nucl. Med. Mol. Imaging.

Gawronska, B., C. Leuschner, et al. (2002). "Effects of a lytic peptide conjugated to beta HCG on ovarian cancer: studies in vitro and in vivo." Gynecol.Oncol. 85(1): 45-52.

Haller, D. G. (2001). "Update of clinical trials with edrecolomab: A monoclonal antibody therapy for colorectal cancer." Seminars in Oncology 28(1 SUPPL. 1): 25-30.

Hansel, W., F. Enright, et al. (2006). "Destruction of breast cancers and their metastases by lytic peptide conjugates in vitro and in vivo." Mol.Cell Endocrinol. 260-262:183-189.

Hansen, C. B., G. Y. Kao, et al. (1995). "Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures." Biochim.Biophys.Acta. 1239(2): 133-144.

Hoskin, D. W. and A. Ramamoorthy (2008). "Studies on anticancer activities of antimicrobial peptides." Biochim.Biophys. Acta 1778(2): 357-75.

Hu, H., D. Chen, et al. (2006). "Target ability and therapy efficacy of immunoliposomes using a humanized antihepatoma disulfide-stabilized Fv fragment on tumor cells." J.Pharm.Sci. 95(1):192-199.

Ikegami, S., T. Tadakuma, et al. (2005). "Selective gene therapy for prostate cancer cells using liposomes conjugated with IgM type monoclonal antibody against prostate-specific membrane antigen." Human cell : official journal of Human Cell Research Society 18(1): 17-23.

Ikegami, S., K. Yamakami, et al. (2006). "Targeting gene therapy for prostate cancer cells by liposomes complexed with anti-prostate-specific membrane antigen monoclonal antibody." Human Gene Therapy 17(10): 997-1005.

Kirpotin, D., J. W. Park, et al. (1997). "Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro." Biochemistry 36(1): 66-75.

Kirpotin, D. B., D. C. Drummond, et al. (2006). "Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models." Cancer Res. 66(13): 6732-6740.

Lin, J. H. and A. Baumgaertner (2000). "Stability of a melittin pore in a lipid bilayer: a molecular dynamics study." Biophys.J. 78(4): 1714-1724.

Lohner, K. and S. E. Blondelle (2005). "Molecular mechanisms of membrane perturbation by antimicrobial peptides and the use of biophysical studies in the design of novel peptide antibiotics." Comb.Chem. High Throughput Screen. 8(3): 241-56.

Madoz-Gúrpide, J., R. Kuick, et al. (2008). "Integral protein microarrays for the identification of lung cancer antigens in Sera that induce a humoral immune response." Molecular and Cellular Proteomics 7(2): 268-281.

Mamot, C., D. C. Drummond, et al. (2003). "Epidermal growth factor receptor (EGFR)-targeted immunoliposomes mediate specific and efficient drug delivery to EGFR- and EGFRvIII-overexpressing tumor cells." Cancer Res. 63(12): 3154-3161.

Maruyama, K., S. J. Kennel, et al. (1990). "Lipid composition is important for highly efficient target binding and retention of immunoliposomes." Proc.Natl.Acad.Sci.U.S.A. 87(15): 5744-5748.

McDermott, K. M., P. R. Crocker, et al. (2001). "Overexpression of MUC1 reconfigures the binding properties of tumor cells." International Journal of Cancer 94(6): 783-791.

Noble, C. O., D. B. Kirpotin, et al. (2004). "Development of ligand-targeted liposomes for cancer therapy." Expert Opinion on Therapeuty Targets 8(4): 335-353.

Olayioye, M. A., R. M. Neve, et al. (2000). "The ErbB signaling network: receptor heterodimerization in development and cancer." EMBO J. 19(13): 3159-67.

Pacakova, V. and K. Stulik (2000). "Validation of a method for determination of phospholipase A2 and melittin in bee venom preparations by capillary electrophoresis." J.AOAC Int. 83(3): 549-554.

Papadopoulos, I., E. Sivridis, et al. (2001). "Tumor angiogenesis is associated with MUC1 overexpression and loss of prostate-specific antigen expression in prostate cancer." Clinical Cancer Research 7(6): 1533-1538.

Papahadjopoulos, D., T. M. Allen, et al. (1991). "Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy." Proc.Natl.Acad Sci.U.S.A. 88: 11460-11464.

Park, J. W., K. Hong, et al. (2002). "Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery." Clin.Cancer Res. 8: 1172-1181.

Park, J. W., K. Hong, et al. (1997). "Immunoliposomes for cancer treatment." Adv.Pharmacol. 40: 399-435.

Patrzykat, A. and S. E. Douglas (2005). "Antimicrobial peptides: cooperative approaches to protection." Protein Pep. Lett 12(19):19-25.

Perez-Paya, E., L. Braco, et al. (1991). "High-performance liquid chromatographic separation of modified and native melittin following transglutaminase-mediated derivatization with a dansyl fluorescent probe." J.Chromatogr. 548(1-2): 351-359.

Petrak, K. (2005). "Essential properties of drug-targeting delivery systems." Drug Discovery Today 10(23-24): 1667-1673.

Prang, N., S. Preithner, et al. (2005). "Cellular and complement-dependent cytotoxicity of Ep-CAM-specific monoclonal antibody MT201 against breast cancer cell lines." Br.J.Cancer. 92(2): 342-349.

Press, M. F., C. Cordon-Cardo, et al. (1990). "Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues." Oncogene 5: 953-962.

Punt, C. J. A., A. Nagy, et al. (2002). "Edrecolomab alone or in combination with fluorouracil and folinic acid in the adjuvant treatment of stage III colon cancer: A randomised study." Lancet 360(9334): 671-677.

Raghuraman, H. and A. Chattopadhyay (2007). "Melittin: a membrane-active peptide with diverse functions." Biosci.Rep. 27(4-5): 189-223.

Reichert, J. M., C. J. Rosensweig, et al. (2005). "Monoclonal antibody successes in the clinic." Nature Biotechnology 23(9): 1073-1078.

Saceda, M., C. Knabbe, et al. (1991). "Post-transcriptional destabilization of estrogen receptor mRNA in MCF-7 cells by 12-O-tetradecanoylphorbol-13-acetate." J.Biol.Chem. 266: 17809-17814.

Sadzuka, Y., K. Kishi, et al. (2003). "Effect of polyethyleneglycol (PEG) chain on cell uptake of PEG-modified liposomes." Journal of Liposome Research 13(2): 157-172.

Slamon, D. J., G. M. Clark, et al. (1987). "Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene." Science 235: 177-182.

Slamon, D. J., B. Leyland-Jones, et al. (2001). "Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2." N. Engl. J. Med. 344(11):783-792.

Soman, N. R., S. L. Baldwin, et al. (2009). "Molecularly targeted nanocarriers deliver the cytolytic peptide melittin specifically to tumor cells in mice, reducing tumor growth." Journal of Clinical Investigation 119(9): 2830-2842.

Son, D. J., J. W. Lee, et al. (2007). "Therapeutic application of anti-arthritis, pain-releasing, and anti-cancer effects of bee venom and its constituent compounds." Pharmacol.Ther. 115(2): 246-70.

Sui, S. F., H. Wu, et al. (1994). "Conformational changes of melittin upon insertion into phospholipid monolayer and vesicle." J.Biochem. 116: 482-487.

Sznol, M. and J. Holmlund (1997). "Antigen-specific agents in development." Seminars in Oncology 24(2): 173-86.

Trzpis, M., P. M. McLaughlin, et al. (2007). "Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule." Am. J. Pathol. 171:386-395

Villari, V. and N. Micali (2008). "Light scattering as spectroscopic tool for the study of disperse systems useful in pharmaceutical sciences." Journal of Pharmaceutical Sciences 97(5): 1703-1730.

Wenqi, D., W. Li, et al. (2009). "EpCAM is overexpressed in gastric cancer and its downregulation suppresses proliferation of gastric cancer." Journal of Cancer Research and Clinical Oncology 135(9): 1277-1285.

Went, P. T., A. Lugli, et al. (2004). "Frequent EpCam protein expression in human carcinomas." Hum.Pathol. 35(1): 122-128.

Winkler, J., P. Martin-Killias, et al. (2009). "EpCAM-targeted delivery of nanocomplexed siRNA to tumor cells with designed ankyrin repeat proteins." Molecular Cancer Therapeutics 8(9): 2674-2683.

Yang, T., M. K. Choi, et al. (2007). "Antitumor effect of paclitaxel-loaded PEGylated immunoliposomes against human breast cancer cells." Pharm.Res. 24(12): 2402-11.

Yuji Yamamoto, M. Y., Mitsunori Sato, Koichi Sato, Satoshi Kikuchi, Hiroki Sugishita, Jun Kuwabara, Yusuke Matsuno, You Kojima, Masamitsu Morimoto, Atsushi Horiuchi, Yuji Watanabe (2011 Jan). "Feasibility of tailored, selective and effective anticancer chemotherapy by direct injection of docetaxel-loaded immunoliposomes into Her2/neu positive gastric tumor xenografts." Int. J. Oncology 38(1): 33-39.

Zuba-Surma, E. K., M. Kucia, et al. (2007). "The ImageStream System: a key step to a new era in imaging." Folia Histochem. Cytobiol. 45(4):279-290.

22

Treatment of Breast Cancer Lytic Skeletal Metastasis Using a Model in Nude Rats

Michael Zepp et al.*
Unit of Chemotherapy & Toxicology, German Cancer Research Center, Heidelberg, Germany

1. Introduction

Cancer is a life-threatening disease, not as a result of the primary tumor that can be removed surgically in the vast majority of cases but from its metastatic spread to distant parts of the body. These metastases are often seen as a hopeless end-stage of the cancer disease and at this time only palliative treatments are applied. Some of the most prevalent solid tumors, such as breast-, lung- and prostate cancers, metastasize into the skeleton and cause either osteolytic (destructive) or osteoblastic lesions. Both types are often accompanied by bone pain and increased bone fragility and thus are reason for extended suffering. In breast cancer, bone is the site of first distant relapse and the clinical course of these women is relatively long, with a median survival of 2-3 years (1, 2). Lytic skeletal metastases are present in over 90% of patients who die from breast cancer (3).
Many factors are involved in the pathogenesis of lytic skeletal lesions among which the proteins osteopontin (OPN) and bone sialoprotein II (BSPII) are considered to play an important role. In patients with primary breast cancer, elevated serum BSPII was recognized as prognostic marker of subsequent bone metastasis and was associated with poor survival (4-8). BSPII is a noncollagenous protein of the extracellular bone matrix and a member of the SIBLING (**S**mall **I**ntegrin-**B**inding **Li**gand, **N**-linked **G**lycoprotein) family. The SIBLINGs are mainly clustered on human chromosome 4, and include bone sialoprotein II, osteopontin, dentin matrix protein 1 (DMP1), matrix extracellular phosphoglycoprotein (MEPE) and dentin sialophosphoprotein (DSPP) (12). These proteins are normally expressed in mineralizing tissues of bone and teeth but are also found in different cancers (13). In normal bone, BSPII is expressed by osteoblasts, osteoclasts and other skeleton-associated cell types, especially at sites of new mineral formation (12, 14-16). In this case, BSPII is a potential nucleator of hydroxyapatite formation and a specific marker of osteoblast differentiation (14). The sialoprotein is involved in hydroxyapatite and collagen binding, as well as in the attachment of bone cells including fibroblasts, osteoblasts and osteoclasts to solid surfaces,

* Tobias J. Bäuerle[1], Victoria Elazar[2], Jenny Peterschmitt[1], Rinat Lifshitz-Shovali[2], Hassan Adwan[1], Franz P. Armbruster[3], Gershon Golomb[2] and Martin R. Berger[1]
[1]*Unit of Chemotherapy & Toxicology, German Cancer Research Center, Heidelberg*
[2]*School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem*
[3]*Immundiagnostik AG, Bensheim*
[1,3]*Germany*
[2]*Israel*

but they are also secreted by breast cancer cells and this presumably is related to their specific homing into osseous tissue (12, 14, 16-19). This fact as well as the interaction with molecules as factor H, integrins, and the ensuing stimulation of signaling cascades promoting migration led to consider BSPII as an important player in the pathogenesis of lytic skeletal lesions (18, 20).

One of the other sibling proteins is osteopontin which is a secreted, adhesive non-collagenous phosphorylated glycoprotein. This molecule was found to be a metastasis-associated protein in human breast cancer in that over-expression of OPN in breast tumours as well as in blood of patients was highly correlated with tumour progression and bad prognosis (21, 22). OPN was initially described as a protein, the secretion of which was elevated in many transformed cells in culture (23). In addition, OPN can bind to a number of receptors such as integrins as well as to certain variant forms of CD44, and it can act as a cytokine (24-26).

For further investigation of the functions from BSPII and OPN in the course of bone metastasis formation, we set up an animal model that would allow following processes such as tumor cell extravasation, adhesion to the target tissue (bone) and formation of osteolytic lesions. For this complex scenario, animal models are indispensable tools to investigate the pathogenesis of bone metastasis *in vivo* and to examine the effects of a therapeutic intervention. For inducing breast cancer bone metastasis in rodents, most experimental models require the injection of human cancer xenografts into immunodeficient mice. This is commonly achieved by intracardial injection or intraosseous administration of breast cancer cells (27). Disadvantageously, the former mode of tumor cell administration into the left ventricle of the heart is associated with dissemination of tumor cells into all skeletal and visceral peripheries of the organism. Local intraosseous administration, however, causes bone damage and lacks processes such as tumor cell extravasation and invasion. Finally, methods based on the injection of bone specific tumor cell subclones are more likely to specifically induce and mimic the process of bone metastasis, but metastatic dissemination is still observed at multiple skeletal sites.

Here we describe a new experimental animal model for inducing site-specific osteolytic lesions in the hind leg of nude rats. This method is characterized by intra-arterial injection of human breast cancer cells into an anastomosing vessel between the femoral and the iliac arteries. This model can be applied to explore the effects of a drug against bone related tumors, which should result in a reduced growth of osteolytic lesions in the treated animals.

2. *In vivo* metastasis model

Nude rats (RNU strain) were obtained from Harlan Winkelmann (Borchen, Germany) or Charles River (Sulzfeld, Germany) at an age of 4–6 weeks. They were housed 4 per cage at specific pathogen-free conditions in a minibarrier system of the central animal facility. Autoclaved feed and water was given ad libitum to the animals that were maintained under controlled conditions (21 ± 2°C room temperature, 60% humidity, and 12 hour light-dark rhythm).

Subconfluent human breast cancer cells (MDA-MB-231^{GFP+}) were harvested using 2 mM EDTA in PBS- (phosphate-buffered saline without Ca^{2+} and Mg^{2+}) and 0.25% trypsin. The cells were washed twice with PBS (with Ca^{2+} and Mg^{2+}), subsequently counted and re-suspended in PBS to a concentration of $5x10^5$ cells per 1 ml. For tumor cell implantation, rats were anaesthetized with a mixture of laughing gas (nitrous oxide; 1 l/min), oxygen (0.5 l/min) and isoflurane (1–1.5 vol. %).

The respective area of a rat's hind leg was shaved and disinfected. A clear cut of 2–3 cm length was performed in the inguinal region (Fig. 1).

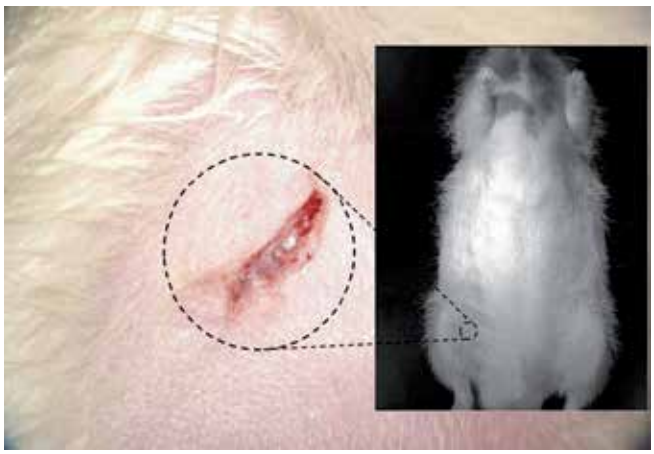


Fig. 1. Shaved and desinfected rat thigh, prepared for tumor cell implantation - showing the first incision.

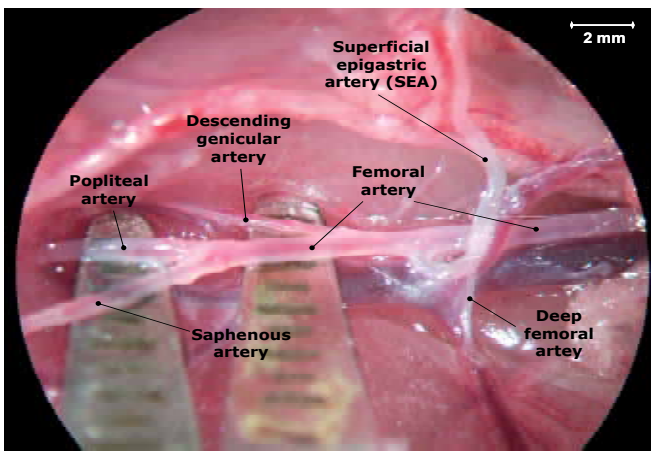


Fig. 2. The branching pattern of the femoral artery, photographed through an operation microscope (magnification 16-fold).

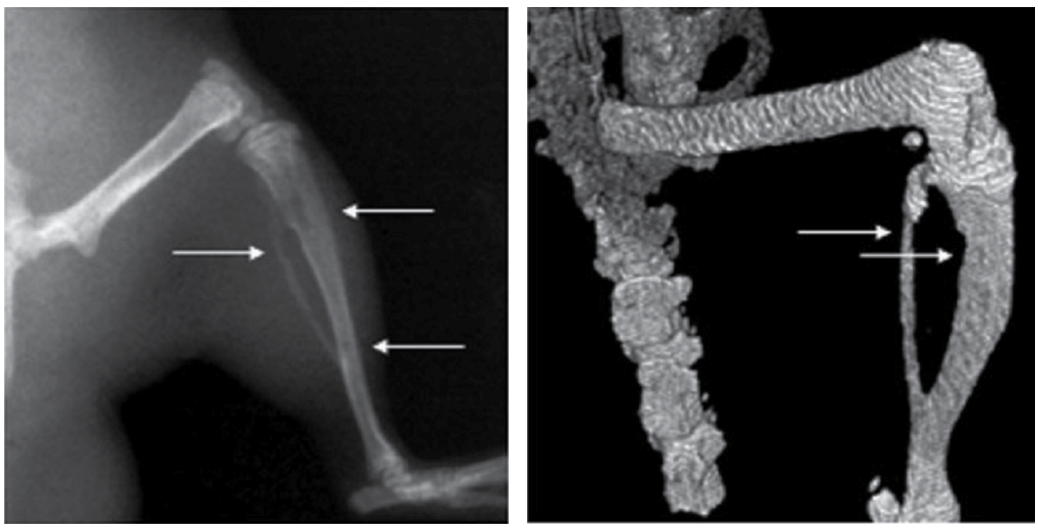
After preparation of all arterial branches as seen in Figure 2, the flow of the femoral artery (FA; Fig. 2) was temporarily occluded by clips that were placed proximal and distal of the superficial epigastric artery's (SEA; Fig. 2) origin. The deep femoral artery (Fig. 2), which normally branches off the FA thus supplying the medial and caudal muscles of the thigh, was also clipped in cases of an anatomical variant as seen in Figure 2. In addition, the SEA was ligated distally, which allowed the opening of this vessel without bleeding. In this context, it is interesting to note that a ligation of the distal SEA is possible because it anastomoses with the caudal epigastric artery that arises from the pudendoepigastric trunk, which is a branch of the iliac artery. After making an incision proximal of the ligation, a 1% papaverin solution was administered onto the SEA to facilitate the subsequent insertion of a

needle (0.3 mm diameter and 42 mm length). After insertion, the needle was fixed in an external support, which reduces irregular movements that would result in perforation of the arterial wall and allows connection with a syringe. Then the distal clip was removed from the FA and placed onto the saphenous artery, which runs superficially and supplies the dorsal and plantar aspects of the foot. MDA-MB-231^{GFP+} cells (10^5 cells suspended in 0.2 ml PBS) were slowly injected into the SEA and by virtue of the clips directed to the descending genicular and popliteal arteries (Fig. 2), both supplying the knee joint and muscles of the right hind leg.

The growth of these tumor cells as well as the formation of lytic lesions was followed subsequently by radiographic examinations. The imaging of the rats was performed every 7 – 14 days under general anesthesia. The animals were fixed in a.p. and p.a. position and exposed to X-rays. The X-ray films were processed by an automatic developing machine and the resulting images were scanned using a digital imaging program with a resolution of 300 dpi. After inverting the scanned radiographs (turning positive into negative), analyses were done with a computer based imaging program. From the beginning to the end of this study, tumor growth and lytic lesions occurred exclusively in the femur, tibia and fibula of the respective hind leg (Fig. 3a). For a 3-dimensional volume rendering reconstructions of the skeleton we performed a high resolution computed tomography (HRCT) using a Multidetector Somatom Plus 4 CT-scanner (based on CT slices with 0.5 mm thickness). Whole animals were investigated with a native spiral scan (Fig. 3b).

2.1 Pilot study

In a pilot study on the optimum take rate, the parameters tumor cell number and sex were varied. For this purpose, male and female animals were observed for a period of 90 days after administering increasing tumor cell numbers. Two male and 2 female rats, respectively, received an injection into the superficial epigastric artery of the right hind leg containing $2.5x10^4$, $7.5x10^4$, $2.5x10^5$ and $7.5x10^5$ MDA-MB-231 or MDA-MB-231^{GFP+} cells.



a1) b1)

Fig. 3. (Continued)

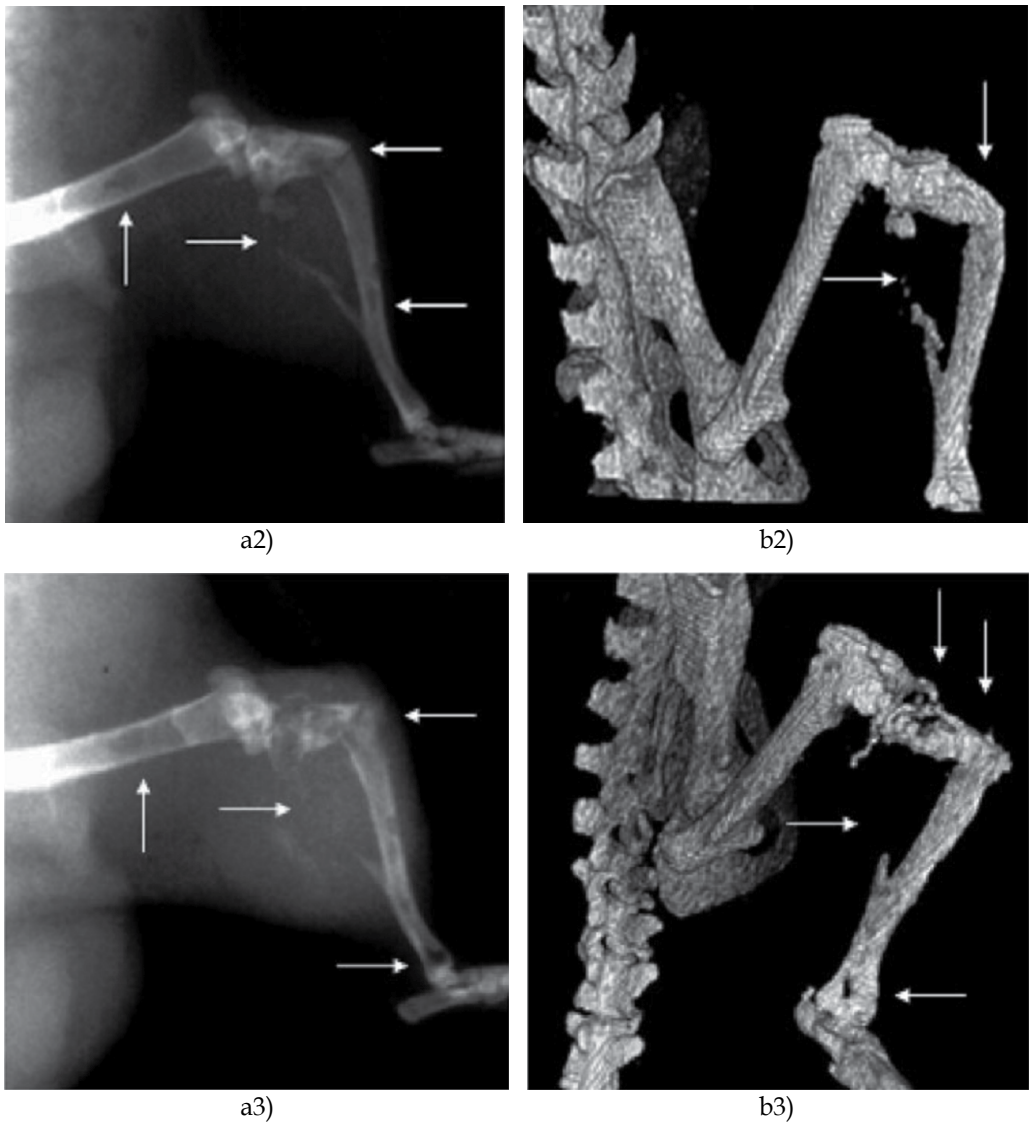


Fig. 3. (a, b) Comparison of the lytic lesions of an untreated control rat detected by 2 radiographic imaging techniques (lesions are indicated by arrows).
(a) X-ray in a.p. position
a1) at day 30 after tumor cell inoculation,
a2) at day 66 after tumor cell inoculation,
a3) at day 104 after tumor cell inoculation.
(b) Computed tomography scan reconstruction
b1) at day 30 after tumor cell inoculation,
b2) at day 66 after tumor cell inoculation,
b3) at day 104 after tumor cell inoculation.

As a result, 50% of all animals developed discernible lytic lesions within an observation period of 90 days. In 5 of 8 males and in 3 of 8 females, overt lytic lesions were observed by

X-rays. The tumor take rate did not differ between MDA-MB-231 and MDA-MB-231^{GFP+} cells (4 of 8 animals, respectively). Cell numbers between 7.5x10^4 and 2.5x10^5 were found appropriate, as these animals developed more and bigger lesions in comparison to rats receiving higher or lower numbers of cells. In an additional group of 7 female nude rats, only 3 developed discernible lytic lesions after inoculation of 1x10^5 MDA-MB-231^{GFP+} cells. Overt lytic lesions were detected by X-rays in some animals as early as 3 weeks after tumor cell implantation. The inoculation of 1x10^5 MDA-MB-231^{GFP+} cells was well tolerated as animals recovered quickly from general anesthesia and did not show weight loss or any signs of walking with a limb.
As a consequence, 1x10^5 MDA-MB-231^{GFP+} cells were inoculated into male rats as basis for all further animal studies.

2.2 Evaluation of the animal metastasis model

By using an amount of 1x10^5 tumor cells the corresponding tumor take rate was 92.7%. In the subgroup of control rats, 2 of 27 (7.4%) showed a spontaneous complete remission of an established lytic lesion within the observation period and 1 rat showed a delayed appearance of its lytic lesion at day 50 after tumor cell inoculation.
The appearance of lesions was usually first detected in the distal femur and the proximal tibia of the hind leg inoculated with tumor cells. Thereafter, small single lytic lesions started to increase in extend and to become confluent with adjacent lesions. Advanced lytic tumor growth was associated with development of surrounding soft tissue metastasis in 8 of 25 rats (32%), and with circular defects of cortical bone in 9 of 25 rats (36%). On average, soft tissue metastasis was detected earlier than circular cortical defects (60 days vs. 80 days after tumor cell inoculation, respectively), and was observed mainly in animals with fast growing lesions of the skeleton. In this rat model, the development of osteolytic lesions can be monitored up to at least 110 days after tumor cell inoculation.

3. *In vitro* experiments for characterization of an IgY antibody

The polyclonal antibodies used were developed in chicken against human bone sialoprotein II and were obtained from the company **Immundiagnostik** (Bensheim, Germany). Appropriate dilutions were made using PBS without Ca^{2+} and Mg^{2+}.
In this part of the study, MDA-MB-231^{GFP+} human breast cancer cells were exposed to an IgY antibody against bone sialoprotein II in order to determine the effect on proliferation, colony formation and migration (Table 1).
Proliferation assay. A volume of 100 µl RPMI medium per well containing 5x10^3 MDA-MB-231^{GFP+} cells was plated onto 96-well plates. After 24h 100 µl medium was added containing the anti-BSPII IgY antibody at final concentrations of 1-400 µg/ml anti-BSPII antibody. The plates were kept under standard cell culture conditions for 1-7 days of incubation. Thereafter, 10 µl/well of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; 10 mg/ml) was added to determine the number of surviving cells. The supernatant was removed after 3h of incubation, and formazan crystals that had been developed were dissolved by adding of 100 µl acidified 2-propanol/well (0.04 N HCl). Extinction was measured by an automated microtiter plate reader at 540 nm, reference filter 690 nm.
It was found that a concentration below 1 mg/ml medium was ineffective. Higher concentrations gradually decreased cell proliferation with the highest concentration (400 mg/ml) precluding any proliferative activity of MDA-MB-231^{GFP+} cells. The IC50

corresponded to 140 mg/ml at 7 days after start of treatment. Incubation of MDA-MB-231^{GFP+} cells for up to 7 days with concentrations from 1 to 400 µg/ml anti-BSPII antibody decreased the proliferation in a dose- and time-dependent manner as examined by MTT assay. The T/C% value of exposed cells decreased gradually from 101 (1 µg/ml) to 5 (400 µg/ml) after 7 days of incubation (Table 1).

Colony formation assay. For determining the response of MDA-MB-231^{GFP+} cells after exposure to the anti-BSPII IgY, 5x10^5 cells were pre-incubated for 48h in 2 ml RPMI medium containing anti-BSPII at concentrations of 1-400 µg/ml. Thereafter, MDA-MB-231^{GFP+} cells were harvested, counted and transferred into semi-solid medium with 0.8% RPMI-methylcellulose and 30% FBS. Finally, 1 ml of the semi-solid medium containing 5x10^3 MDA-MB-231^{GFP+} cells was plated onto 3.5 cm Petri-dishes. Triplicate Petri-dishes per treatment protocol were cultivated for 5-7 days at standard cell culture conditions. Colony formation (clusters of ≥30 cells) was visualized by staining with MTT and scored by an inverted microscope.

The same range of concentrations as in the MTT assay was chosen to study the colony formation of MDA-MB-231^{GFP+} cells after exposure to the anti-BSPII immunoglobulin. Following 2 days of pre-incubation in medium, colony formation on methyl cellulose was dose-dependently inhibited, with T/C% values ranging from 107 (1 µg/ml) to 17 (400 µg/ml; see Table 1).

Migration assay. In a model for cell migration, 1x10^3 MDA-MB-231^{GFP+} cells were incubated with final anti-BSPII IgY concentrations ranging from 1 to 200 µg/ml. They had been incubated for 48h before being transferred into a transwell migration system. The breast cancer cells were plated on a polycarbonate filter membrane with a pore size of 8 µm (upper layer). The bottom layer was set up by 0.5 ml RPMI medium containing 1x10^4 SaOs-2 cells (osteosarcoma), which were grown in 24-well plates. After 24h the medium was removed and a semi-liquid RPMI medium containing 0.2% methylcellulose and 20% FBS was transferred on top of the SaOs-2 cells (0.5 ml/well) in order to maintain a gradient between the two compartments and to provide an additional barrier for cell migration. The polycarbonate filter was removed from the bottom layer after 24h of co-cultivation and transferred onto a fresh well containing bottom layer (see above). Cells migrating through the pores were counted daily for 4 days by fluorescence microscopy. The mean growth rate (MGR) of cells after migration through the polycarbonate filter was determined by the equation:

$$MGR = \log_2 N_t - \log_2 N_0/t,$$

with N_0 as initial cell number, N_t as final cell number and t as time period of cell incubation in days.

Following pre-incubation for 2 days, MDA-MB-231^{GFP+} cells showed enhanced migration in response to 1 µg/ml anti-BSPII (146 T/C%, day 1). Cells pre-incubated with higher concentrations of anti-BSPII (25-200 µg/ml) showed decreased T/C% values ranging from 92 to 11 (25-200 µg/ml, day 1). This inhibition of migration decreased gradually with time. At day 4 after exposure, only cells pre-incubated with 200 µg/ml anti-BSPII showed significantly reduced migration (59 T/C %) compared to untreated control cells. Cells that had migrated towards the bottom layer were allowed to proliferate for up to three days.

Their mean growth rates (MGR; Table 1) ranged from 0.64 (25 µg/ml) to 0.25 (200 µg/ml), as compared to the MGR of untreated controls (0.80). A control IgY antibody did not show any significant effect (data not shown).

Assay	1 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml
Proliferation assay[a]	100.9	86.9	77.1	62.7	26.9	5.3
Colony formation assay[b]	106.6	94.2	86.4	54.2	43.2	17.3
Migration assay[c] (MGR)[d]	145.5 (0.72)	91.5 (0.64)	86.6 (0.69)	31.03 (0.32)	10.7 (0.25)	---

[a] Determined by MTT assay; T/C% values after 7 days of incubation with anti-BSPII IgY.
[b] T/C% values of colony counts at day 7 after plating; for treatment the cells were pre-incubated with anti-BSPII IgY for 2 days.
[c] Number of migrating cells in % of untreated control; T/C% values at day 1 after pre-incubation of MDA-MB-231^{GFP+} cells with anti-BSPII IgY.
[d] Mean growth rate (per day): mean of growth rates (day 1/day 2 and day 2/day 3) determined in cells migrating through pores with a diameter of 8 µm; MGR of control: 0.80.

Table 1. Overview of *in vitro* results.

4. Treatment of lytic skeletal metastasis with an anti-BSPII immunoglobulin

The whole *in vivo* study comprised seven groups of nude rats with a total number of 81 animals (Table 2). Untreated control rats (group 1) were observed for a period of 90 days. The effect of the antibody was assessed by either exposing MDA-MB-231^{GFP+} cells prior to their implantation (groups 2 and 3) or by treating rats bearing MDA-MB-231^{GFP+} cells early (group 4) or late (groups 5-7) after tumor inoculation (Fig. 4). Early treatment started at the day of tumor inoculation and additional s.c. injections were given 2 and 4 days later. Late treatment was administered to rats after the appearance of lytic lesions (Fig. 4). Treatment with erucylphospho-NNN-trimethylpropanolamine ($ErPC_3$), used as control, was given i.v. after the onset of lytic lesions (31-34 days following tumor inoculation; group 5, Table 2) for a period of 8 weeks. Treatment with anti-BSPII IgY and the combination consisting of the alkylphosphocholine $ErPC_3$ and anti-BSPII IgY was maintained over the same time period (groups 6 and 7, Table 2). The antibody and $ErPC_3$ were tolerated well without any side effects.

Group no.	Animal no. (% total study)	Mode of treatment	Agent	Concentration/ dosage	Time period of exposure/treatment schedule
1	25 (30.9)[a]	Untreated control rats	---	---	---
2	9 (11.1)	Pre-treatment of cells (HD[b])	Anti-BSPII IgY	600 µg/ml	2h[f]
3	11 (13.6)	Pre-treatment of cells (LD[c])	Anti-BSPII IgY	25 - 100 µg/ml	48h[f]
4	12 (14.8)	Early treatment	Anti-BSPII IgY	20 mg/kg s.c.	Day 0, 2, 4[g]
5	8 (9.9)	Late treatment (APC[d])	$ErPC_3$	60 µmol/kg i.v.	Twice weekly for 8 weeks[h]

Group no.	Animal no. (% total study)	Mode of treatment	Agent	Concentration/ dosage	Time period of exposure/treatment schedule
6	8 (9.9)	Late treatment (AB[e])	Anti-BSPII IgY	10 mg/kg s.c.	Once weekly for 8 weeks[h]
7	8 (9.9)	Late treatment (APC + AB)	$ErPC_3$ + Anti-BSPII IgY	60 µmol/kg i.v. $ErPC_3$ + 10 mg/kg s.c. anti-BSPII	Twice weekly ($ErPC_3$) + once weekly (anti-BSPII) for 8 weeks[h]

[a] Two rats with spontaneous regression were excluded; the initial number was 27.
[b] High dose. [c] Low dose. [d] Alkylphosphocholine. [e] Antibody.
[f] Pre-incubation of MDA-MB-231^{GFP+} cells before tumor implantation.
[g] Three injections of 20 mg/kg were given subcutaneously on days 0, 2 and 4 after tumor implantation. [h] Treatment of rats after the occurrence of lytic lesions.

Table 2. Design of *in vivo* experiments.

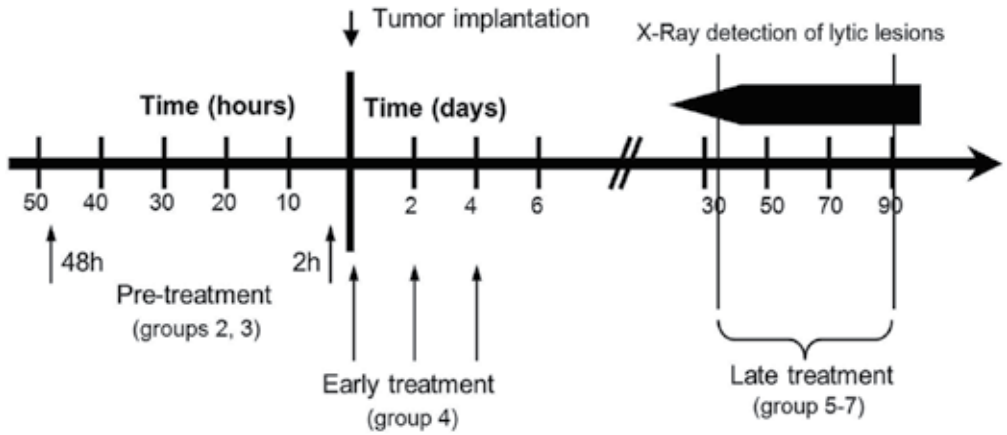


Fig. 4. Time axis of *in vivo* experiment.

In vivo experiments were performed with pre-treated MDA-MB-231^{GFP+} cells, which were incubated with anti-BSPII IgY for 2 days (group 3) or 2 h (group 2) before implantation into nude rats (pre-treatment). Animals of group 4 received the anti-BSPII IgY at the day of tumor implantation, as well as 2 and 4 days later (early treatment). In groups 5-7, treatment was administered to rats with lytic lesions for 8 weeks (late treatment).

4.1 Pre-treatment

The mean lytic lesion size of animals implanted with pre-treated MDA-MB-231^{GFP+} cells increased more slowly and was significantly smaller at days 70-90 than the corresponding mean lesion size of untreated controls (Fig. 5a). One of 11 rats (9%) did not develop any visible metastasis during the observation time of 90 days.

Rats receiving pre-treated cells (groups 2 and 3) showed no circular defects of cortical bone or any soft tissue metastasis surrounding the lytic lesions during the observation time of up to 90 days (Table 3). Untreated control rats developed a circular defect of cortical bone in 9 of 25 rats (36%) and soft tissue metastasis in 8 of 25 cases (32%; Table 3). In comparison to

untreated control rats, the average daily growth rate was distinctly lower in animals receiving pre-treated cells. For animals of group 2, the average daily growth rate was 0.07 mm^2 and for those of group 3 it was negative (-0.01 mm^2). In contrast, lesions of untreated control rats increased by 0.55 mm^2 per day on average.

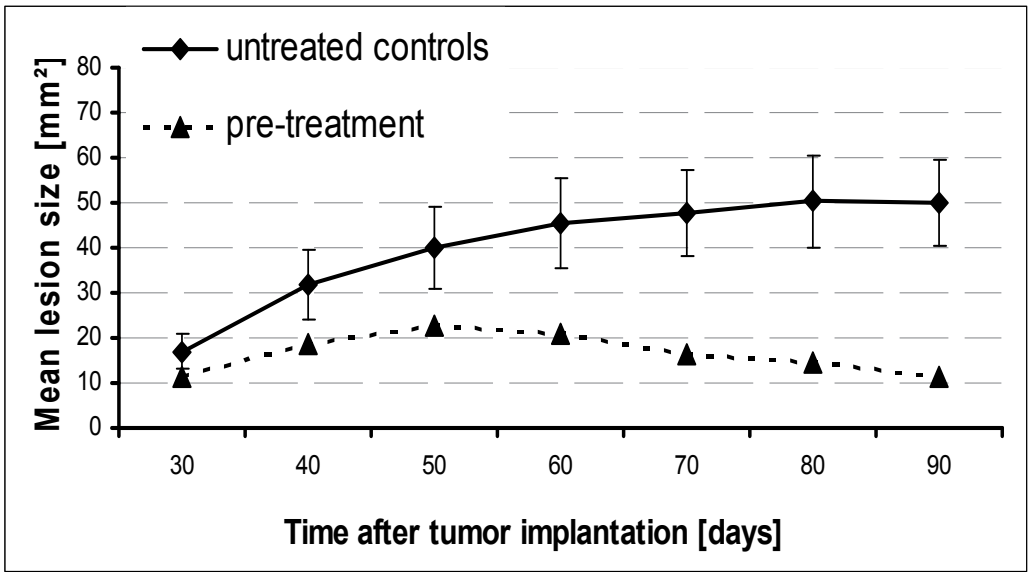


Fig. 5a. Results of *in vivo* experiments. Comparison of the mean lytic lesion sizes of untreated control rats and animals receiving pre-treatment for 48 h (group 3).

4.2 Late treatment

The mean lesion size of animals receiving $ErPC_3$ (group 5, Table 2) increased from day 30 (24 mm^2) to day 60 (63 mm^2) after tumor implantation, and then reached a plateau (Fig. 5b). There was no statistically significant difference to untreated control rats during the observation time. The mean lytic lesion size of animals treated with 10 mg/kg anti-BSPII increased from day 30 (10 mm^2) to day 60 (39 mm^2) after tumor implantation (group 6, Table 2; Fig. 5c). Thereafter, the average lesion size decreased significantly to a minimum size at day 90 (29 mm^2, $p<0.05$) in comparison to untreated controls. The average daily growth rate was 0.32 mm^2 compared to 0.55 mm^2 for the controls (Table 3).

The average lytic lesion size of animals receiving both, $ErPC_3$ and anti-BSPII IgY (group 7, Fig. 5c) increased minimally from 28 mm^2 to 32 mm^2 at day 60 and to 34 mm^2 at day 90 after tumor cell implantation. The average growth per day was 0.1 mm^2 (Table 3). At days 70 and 80, the mean lytic lesion size was significantly smaller than that of untreated control animals (30 mm^2 and 26 mm^2, $p<0.05$). Animals receiving late treatment with the antibody alone or in combination showed fewer complications such as circular bone lysis or soft tissue metastasis caused by the growth of lytic lesions in comparison to untreated control rats (Table 3).

The suitability of the anti-BSPII antibody for a combination therapy was tested by co-administering the antibody with the alkylphosphocholine $ErPC_3$. This agent has been shown to reduce proliferation, colony formation and migration in MDA-MB-231^{GFP+} cells *in vitro*. In nude rats with bone metastasis, this drug alone caused no reduction in lytic lesion size,

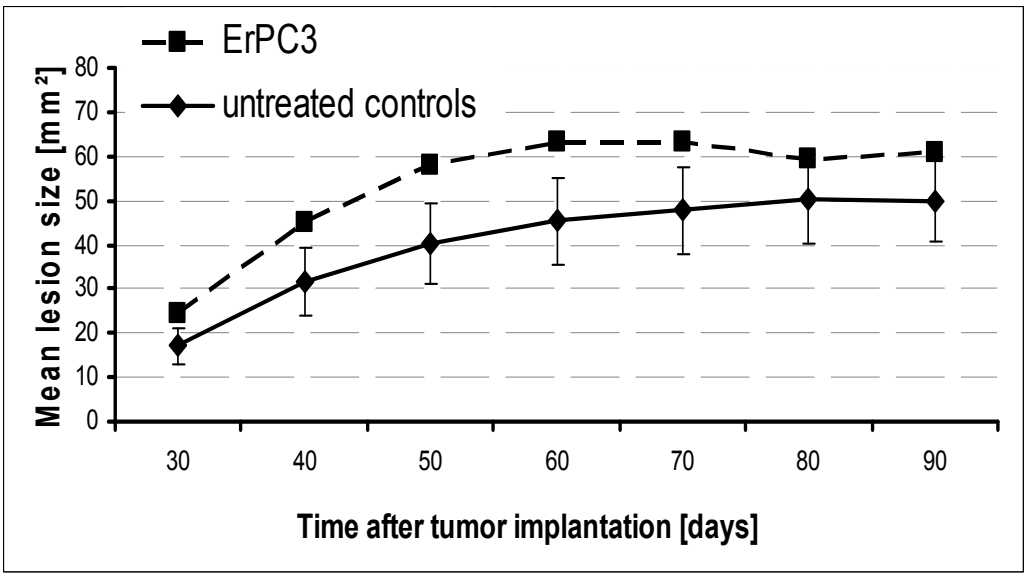


Fig. 5b. Results of *in vivo* experiments: Comparison of the mean lytic lesion sizes of untreated control rats and animals receiving late treatment with $ErPC_3$ (group 5).

but the combination with anti-BSPII IgY resulted in a significant decrease in mean osteolytic lesion size. Also, new bone formation was observed in rats treated with anti-BSPII IgY + $ErPC_3$. This *de novo* bone formation resulted in almost complete remissions of lytic lesions as well as in stabilization of pathologically fractured bones in some rats (Fig. 6, Line VII c, d).

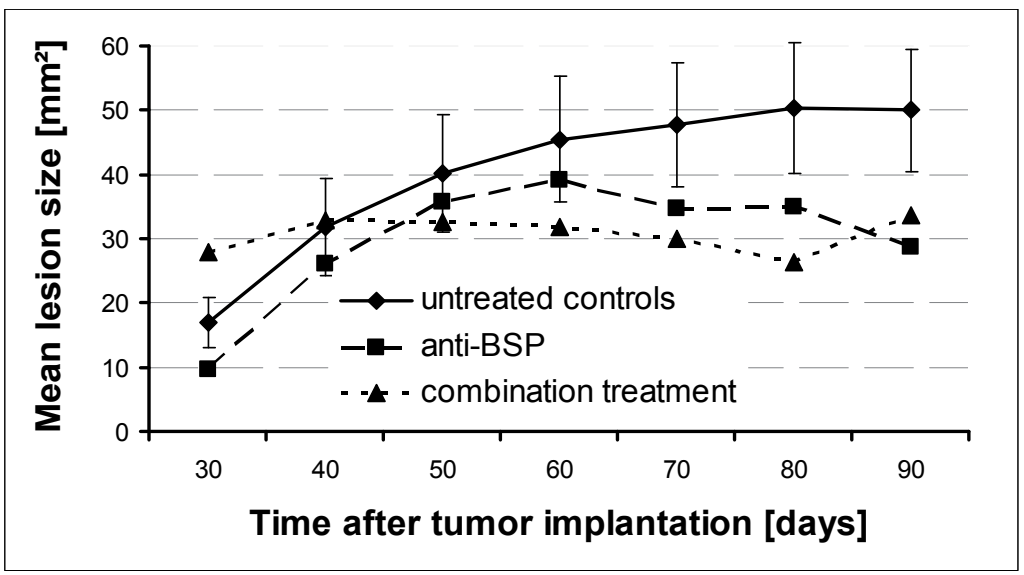


Fig. 5c. Results of *in vivo* experiments. Comparison of the mean lytic lesion sizes of untreated control rats and animals receiving the anti-BSPII IgY alone (group 6) or in combination with $ErPC_3$ (group 7).

Parameter observed	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
T/C% [a] (day 30)	100	37,.6[b]	67.2[b]	48.6[b]	143.1	56.1	164.1
T/C% [a] (day 60)	100	18.7[b,f]	45.7[b]	29.9[b,f]	138.7	86.1	70.3
T/C% [a] (day 90)	100	[c]	28.8[b,f]	[c]	118.6	69.6	52.5 [f]
T/C% [a] (day 90)	100	[c]	22.4[b,f]	[c]	121.2	57.3 [f]	67.5
Rats with circular defects of cortical bone (%)[d]	9 (36)	0 (0)[f]	0 (0) [f]	0 (0)[f]	3 (37.5)	2 (25)	2 (25)
Rats with soft tissue metastasis (%)	8 (32)	0 (0)[f]	0 (0) [f]	0 (0) [f]	3 (37.5)	2 (25)	1 (12.5)
No. of rats without visible metastasis (%)/	---	1 (11.1)	1 (9.1)	2 (20)	---	---	---
No. of complete remissions (%)	2 (7.4)	---	---	---	2 (25)	1 (12.5)	0 (0)
Average daily growth rate of lytic lesions[e] (day 30-90) (mm^2)	0.55	0.07 [f]	-0.01 [f]	0.18f	0.61	0.32 [f]	0.10 [f]

[a] Mean lytic lesion size of experimental rats in percent of the corresponding lesion size of untreated controls. [b] Mean value excluding rats without any visible metastasis.
[c] Group terminated at day 60 after tumor implantation.
[d] Circular defects of femur or tibia potentially resulting in a bone fracture; circular defects of the fibula were excluded.
[e] Average daily growth rate from day 30 to 90 after tumor implantation for groups 1, 3, 5-7 and from day 30 to 60 for groups 2 and 4. [f] Significant difference versus control rats ($p<0.05$).

Table 3. Results of *in vivo* experiments.

Consequently, reduced osteolytic lesion sizes were observed in rats that had been treated with the antibody against BSPII before or after the appearance of skeletal metastasis. Beyond that, in rats with overt lytic lesions, at days 60 - 65 new bone formation was observed in the femur and tibia. On computed tomography reconstructions (Fig. 6) of this animal, the new bone formation in response to anti-BSPII treatment can be seen with greater plasticity. Remarkably, formation of new bone has previously not been described after treating overt osteolytic lesions. Even bisphosphonates being the most widely used treatment for patients with breast cancer bone metastasis are only able to delay the growth and progression of skeletal lesions by inhibiting osteoclast-mediated bone resorption.

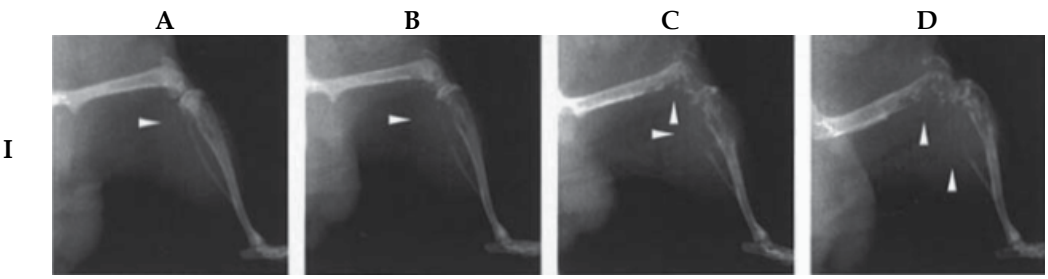


Fig. 6. (Continued)

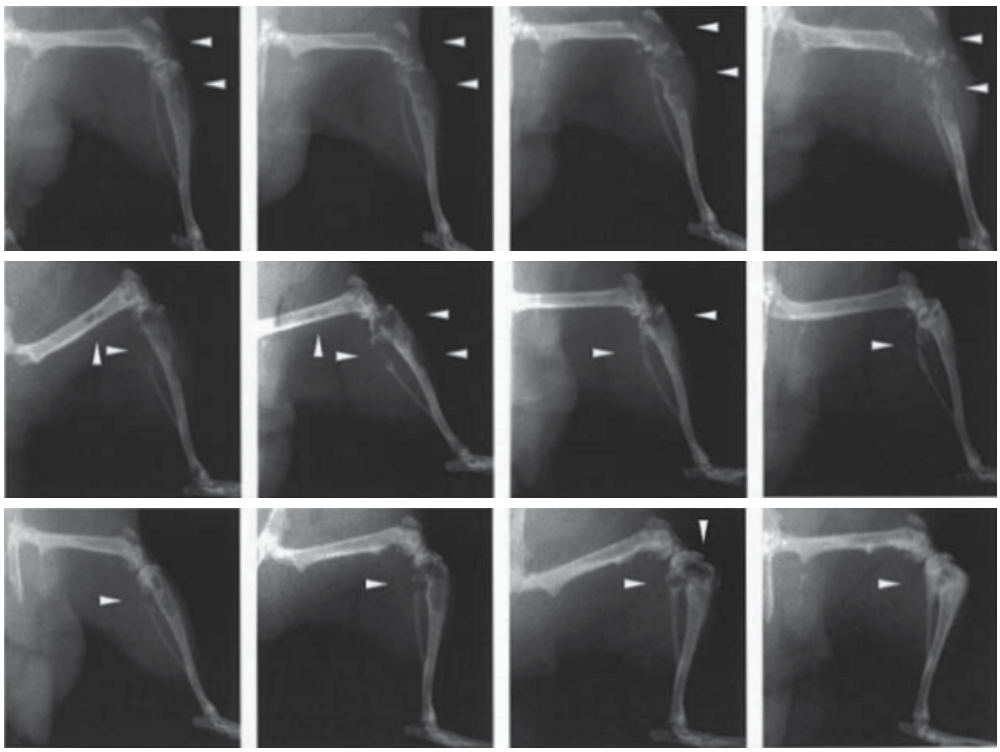


Fig. 6. Radiographic comparison of lytic lesions in the right hind leg of nude rats (lesions are indicated by arrows). Serial X-rays of experimental rats taken after 30-35 days (A), 40-45 days (B), 60-65 days (C) and 80-95 days (D) after tumor cell implantation.
Roman numbers denote individual animals, they correspond also to their group numbers and these rats are typical for the whole group. These include an untreated control rat (I, group 1), a rat (V) treated with $ErPC_3$ (group 5), a rat (VI) treated with anti-BSPII IgY antibody, and a rat (VII) treated with $ErPC_3$ plus the IgY antibody (group7).

5. Combination treatment of lytic skeletal metastasis with the bisphosphonate zoledronate and the anti-BSPII IgY

Over the last decades, bisphosphonates have become an essential part of the treatment of bone metastasis. They bind with high affinity to hydroxyapatite crystals and therefore accumulate in the skeleton. There they are potent inhibitors of osteoclast bone resorption and thus prevent or reduce the development of osteolytic lesions caused by breast cancer cells (28). Besides this established efficacy, N-containing bisphosphonates have been shown to inhibit the activity of farnesyl diphosphonate synthase, a key enzyme in the mevalonate pathway (29). In addition, these third generation bisphosphonates have been shown to exert cytostatic and pro-apoptotic effects on breast cancer cells *in vitro* (30). Nevertheless, treatment with bisphosphonates is considered as a palliative measure and therefore various drug combinations have been examined to improve the overall anti-neoplastic effect in breast cancer patients with skeletal metastasis (31-33). Among various options that can be envisaged the combination with an agent targeting the pathophysiology of skeletal lesions seems therapeutically attractive.

In addition to these studies demonstrating that an IgY-antibody against BSPII was effective in reducing proliferation, colony formation and migration of MDA-MB-231^{GFP+} cells *in vitro* as well as *in vivo* it could be shown that this treatment induced new bone formation in the aforementioned nude rat model (34, 35).

Therefore it was the aim of this study to combine a potent bisphosphonate, e.g., zoledronic acid, with the IgY antibody for treating the mammary carcinoma cell line MDA-MB 231^{GFP+}, which causes osteolytic lesions *in vivo*. The combination of agents with unrelated mechanisms of action was expected to result at least in an additive combination effect.

In order to explore a possible synergistic effect in lytic skeletal metastasis caused by MDA-MB-231^{GFP+} cells, the rats were treated with combined exposure to zoledronic acid and the IgY antibody against bone sialoprotein II alone or in simultaneous combination.

The experimental design is shown in Table 4. An untreated control group (group 1) was compared with five different treatment groups. The untreated control group was monitored radiographically over a period of 100 days after tumor cell inoculation. A possible preventive effect of zoledronic acid was tested on a group of ten rats (group 2) by administering 60 µg/kg (150 nmol/kg) subcutaneously on days 14 and 7 prior to tumor cell inoculation. This group was examined over a period of 70 days following tumor cell inoculation. Furthermore, zoledronic acid alone was used in an early treatment scheme prior to the development of lytic lesions (group III; n = 10; 60 µg/kg/week [150 nmol/kg/week] zoledronic acid, s.c.; d7-d28 after tumor cell injection) as well as in combination with the anti-BSPII IgY (group IV; n = 10; 60 µg/kg/week (150 nmol/kg/week) zoledronic acid, s.c.; d7-d28 after tumor cell injection plus 20 mg/kg anti-BSPII IgY, s.c.; d1, d3, d5 after tumor cell injection). These groups were also examined over a period of 70 days.

In addition, two groups of rats (n = 10 each) with established lesions were treated according to a late treatment scheme. One of these groups (group V) received a late single drug treatment (60 µg/kg/week [150 nmol/kg/week] zoledronic acid, s.c.; d35–d84 after tumor cell inoculation) and the other group (group VI) was treated with a late combination treatment (60 µg/kg/week [150 nmol/kg/week] zoledronic acid, s.c.; d35–d84 after tumor cell inoculation and 10 mg/kg/week anti-BSPII IgY, s.c.).

Group no.	Animal no. (% total study)	Mode of treatment	Agent	Concentration/ dosage	Time period of exposure/ treatment schedule
1	20 (28.57)	Untreated control rats	---	---	---
2	10 (14.28)	Pre-treatment of rats	zoledronic acid	60 µg/kg/week s.c.	Day 14, 7 before cell inoc.
3	10 (14.28)	Early single treatment	zoledronic acid	60 µg/kg/week s.c.	Day 7, 14, 21, 28 after cell inoc.
4	10 (14.28)	Early combination treatment	zoledronic acid + Anti-BSPII IgY	60 µg/kg/week s.c. 20 mg/kg s.c.	Day 7, 14, 21, 28 after cell inoc. Day 1, 3, 5 after cell inoc.[a]
5	10 (14.28)	Late single treatment	zoledronic acid	60 µg/kg/week s.c.	Once weekly during 35d-84d[b]
6	10 (14.28)	Late combination treatment	zoledronic acid + Anti-BSPII IgY	60 µg/kg/week s.c. 10 mg/kg s.c.	Once weekly during 35d-84d following cell inoc.

[a] before the appearance of lytic lesions. [b] after the appearance of lytic lesions.

Table 4. Design of *in vivo* experiments.

5.1 Pre-treatment

The mean lytic lesion size in animals receiving preventive treatment was significantly smaller than the mean lesion size of untreated controls at days 30–60. The increase in size of existing lesions was significantly lower (average daily growth rate: 0.3 mm^2/d vs. 45.3 mm^2/d) as compared to untreated controls. The incidence of lesions was significantly reduced to 50%. Soft tissue metastases occurred in 20% (2/10) of rats which corresponds to half the incidence observed in untreated control rats (8/20; 40%). The significant reduction in lesion size was corroborated by analyzing the manifestation of lesions in the involved skeleton. Lesions in the femur and fibula were reduced by 55 and 65%, respectively, whereas lesions of the tibia were present in each animal affected. Also, the number of rats with periosteal defects of the cortical bone was significantly smaller as shown by a 60% incidence in the treated group versus 95% in the control group (Table 5).

5.2 Early treatment

The mean lesion areas of the early single drug and combination treatment schedules were almost identical. The initial mean lytic lesion areas were significantly smaller than the respective control area (4.0 and 4.8 mm^2 vs. 25.6 mm^2). Remarkably, the respective T/C% values of 15.5 and 18.6 at day 30 decreased with time to 5.5 and 6.5 at day 60. The average daily growth rate (mm^2/d) was significantly reduced from 45.3 mm^2/d in controls to 0.2 mm^2/d in animals receiving single drug treatment and to 0.1 mm^2/d in those receiving the combination treatment. The incidence of lytic lesions was significantly reduced to 30% with both early treatment schemes corresponding to a 70% reduced incidence as compared with the control group. Periosteal defects of cortical bone were prevented totally with the early combination treatment and were reduced to 33% with the early single drug treatment. The appearance of lytic lesions was decreased by both early treatment schemes in the femur and fibula, the latter being significant ($P \leq 0.05$). Soft tissue metastasis was not observed following the single drug treatment whereas the combination treatment was associated with a reduced incidence of 20% compared to 40% in the control group (Table 5).

5.3 Late treatment

With both late treatment schemes the mean lesion areas were significantly reduced as measured at 60 and 90 days following tumor cell inoculation. The late combination treatment improved the effect of zoledronic acid. For example, the mean lesion area at day 60 following tumor cell inoculation was 93.5 mm^2 in the control group, 31.3 mm^2 in the late single drug treatment group and 19.5 mm^2 in the late combination treatment group. The average daily growth rate of lytic lesions significantly decreased from 45.3 mm^2/d (control group) to 0.3 mm^2/d (late single drug treatment group), and to 0.2 mm^2/d (late combination treatment group) after 60 days following tumor cell inoculation and from 22.7 mm^2/d (controls) to less than 0.01 mm^2/d (single drug treatment) and 0.05 mm^2/d (combination treatment) after 90 days following tumor cell inoculation. The remission rate of lytic lesions was significantly increased to 40% (single drug treatment) and 30% (combination treatment) compared to the control group (5%, $P = 0.015$ and $P = 0.057$, respectively). The appearance of lytic lesions was reduced in all affected bones (femur, tibia and fibula) with both late treatment schemes. For lesions of the femur and fibula the combination treatment was slightly more effective than zoledronic acid alone, reaching almost significance ($P = 0.06$) for lesions of the femur. Similarly, the development of periosteal defects of cortical bone was significantly reduced in all three bones ($P < 0.05$) except for the femur following treatment

with zoledronic acid. Here, the late single drug treatment caused a reduction by 35% whereas the late combination treatment reduced this parameter by even 55%. In both late treatment groups just half as many rats developed soft tissue metastasis as compared to the control group (Table 5).

The clinical dose of zoledronic acid in oncology is 4 mg i.v. every 3 – 4 weeks, which corresponds to about 110 µg/kg. Thus, a single experimental dose corresponded to about 60% of a single clinical dose. When considering total dosages, the preventive regimen with a total of 120 µg/kg administered in two weeks is within the clinical range given within 3 – 4 weeks. The early and late treatment regimens summed up to 240 and 480 µg/kg within 4 and 8 weeks, respectively. These doses are about two fold higher than the equivalent clinical doses (4 and 8 mg within 4 and 8 weeks, respectively). Of note, the experimental doses are even lower than the clinically used doses if they are compared on the basis of body surface area. In this case the rats received single doses of about 0.4 mg/m^2 body surface area as compared to 2.2 mg/m^2 in patients. The corresponding total doses in rats were 0.8 mg/m^2 (pre-treatment), 1.6 mg/m^2 (early treatment) and 3.2 mg/m^2 (late treatment). The latter value is to be compared to 4.4 mg/m^2 in the clinical situation for a period of 8 weeks. Thus, overall the dosages used in rats were equivalent to those in patients although tumors usually grow faster in rats than in humans. Therefore the single doses were given more frequently in rats in order to produce relevant results.

The antiosteolytic effect of zoledronic acid in nude rats was only marginally increased by the IgY antibody. The therapeutic advantage was best discernible in the incidence of femoral osteolytic lesions when comparing zoledronic acid alone (80%) with the combination treatment (40%), but was less pronounced when comparing periosteal defects of cortical bone (60% vs. 40%). The main difference to the *in vitro* design was a lower dose of zoledronic acid in vivo (60 µg/kg) which already caused a maximum therapeutic effect and thus this effect could not be improved significantly by addition of the IgY antibody. A reason for this observation could be the pharmacokinetic property of bisphosphonates to accumulate in bone. The affinity of bisphosphonates for bone has been correlated with a long half-life and is similar to that observed for tetracyclines, strontium or fluoride. Thus, the skeletal concentration of zoledronic acid, together with its potency, was presumably too high to allow survival of the majority of osteoclasts and therefore caused the maximum antiosteolytic effect possible. In this scenario the IgY antibody could only show additional activity if it exerted a direct tumoricidal action or a stimulatory effect on osteoblasts. Both properties have been demonstrated in previous studies. Since zoledronic acid has tumoricidal properties as well, a clear difference between zoledronic acid and the combination would be hard to identify. Therefore, we hypothesized that a direct effect on osteoblasts may be the basis for the differences favoring the combination that were seen in response to the late treatment groups.

It is unclear why the combination effect could best be observed in the femur of the animals. Recently, it has been described that osteolytic lesions induced by human MDA-MB-435 breast cancer cells in nude mice were associated with a greater than 90% reduction in the number of osteoblasts. Based on this observation we speculate that the size of osteolytic lesions might be indicative for the extent of this effect, in that smaller lesions might be associated with a lower reduction in osteoblast counts as opposed to larger lesions. In that case those bones with initially smaller or less frequent osteolytic lesions such as the femur could be left with a higher number of osteoblasts which would be able to recalcify osteolytic lesions upon appropriate stimulation.

Observed Parameter		Time period (day)[a]	Controls	Pre-treatment (Zoledronic acid)	Early treatment		Late treatment	
					Zoledronic acid	Combination	Zoledronic acid	Combination
Mean area (mm^2) ± SD		30	25.6 ± 10.8	5.3 ± 4.1*	4.0 ± 2.8*	4.8 ± 4.0*	23.3 ± 18.7	12.9 ± 13.6
		60	93.5 ± 52.8	13.7 ± 8.2*	5.1 ± 3.2*	6.1 ± 5.8*	31.3 ± 32.3*	19.5 ± 19.7*
		90	106.2 ± 57.3				23.4 ± 25.7*	15.6 ± 15.8*
growth rate of lytic lesions (mm^2/d)[b]		30–60	45.3	0.3*	0.2*	0.1*	0.3*	0.2*
		30–60	22.7				≤ 0.01*	0.05*
Incidence of lytic lesions		30–60	100% (20/20)	50% (5/10)*	30% (3/10)*	30% (3/10)*	100% (10/10)	100% (10/10)
Rate of lytic lesions remission[c,d]		30–60	5% (1/20)	0% (0/5)	0% (0/3)*	0% (0/3)*	40% (4/10)*	30% (3/10)*
Distribution of lytic lesions	Femur	30–60	75% (15/20)	20% (1/5)*	33% (1/3)	33% (1/3)	80% (8/10)	40% (4/10)
	Tibia	30–60	100% (20/20)	100% (5/5)	100% (3/3)	100% (3/3)	90% (9/10)	90% (9/10)
	Fibula	30–60	85% (17/20)	20% (1/5)*	0% (0/3)*	0% (0/3)*	70% (7/10)*	60% (6/10)*
Rats with circular defects of cortical bone	Total	30–60	95% (19/20)	60% (3/5)*	33% (1/3)*	33% (0/3)*	60% (6/10)*	40% (4/10)*
	Femur	30–60	50% (10/20)	0	0	0	40% (4/10)	10% (1/10)*
	Tibia	30–60	85% (17/20)	60% (3/5)	33% (1/3)	0	50% (5/10)*	30% (3/10)*
	Fibula	30–60	80% (16/20)	0	0	0	30% (3/10)*	30% (3/10)*
Rats with soft tissue metastasis		30–60	40% (8/20)	20% (2/10)	0	20% (2/10)	20% (2/10)	20% (2/10)

[a] Days following tumor cell inoculation. [b] Average daily growth rate.
[c] Percentage based on all animals that were observed only or treated after the occurrence of lytic skeletal lesions. [d] Remission = Decrease in size ≥ 50% of initial size. *Significant effect versus control ($P \leq 0.05$).

Table 5. Effect of administering zoledronic acid alone and in combination with anti-IgY BSPII treatment.

In conclusion, the combination of zoledronic acid and the anti-BSPII antibody caused only a low therapeutic advantage over zoledronic acid alone, probably due to a maximum anti osteolytic effect caused by the bisphosphonate alone. Nevertheless, an almost significant effect was observed in parameters indicating recalcification such as the reduction of osteolytic lesions and the prevention of periosteal defects of cortical bone. These observations may favor using the IgY-antibody in addition to zoledronic acid in order to stimulate osteoblast induced recalcification.

6. Treatment of lytic skeletal metastasis with antisense oligonucleotides against OPN and BSPII

Here, we report on the selection of antisense oligonucleotides (ASOs), which are effective in reducing their protein levels. We considered the two proteins to be potential targets for treatment in order to slow down or suppress the formation of bone metastasis. For a specific treatment, we identified antisense oligonucleotides that are capable of reducing the expression levels of OPN and BSPII. The activity of these ASOs was determined by Western blot and by inhibition of colony formation as well as of metastasis formation of pre-exposed MDA-MB-231^{GFP+} human mammary carcinoma cells (36, 37).

Selection of ASOs

The suitability of 10 different ASOs per gene was predicted by using the HUSAR program ''Mfold'', which takes RNA folding into account. Application of this program onto the cDNA sequence of OPN (acc. no. gi:3360431) and BSPII (acc. no. gi:11435526) resulted in the recognition of RNA stretches that probably contain bulges or loops, that are preferentially single stranded and thus allow access of DNA antisense structures. ASOs of 20 base pair length were selected against these single-stranded regions and synthesized with a phosphorothioate backbone, to increase stability against degrading enzymes (Table 6). Controls included a nonsense oligonucleotide (NSO) derived from the HBV genome that served as a control for unspecific effects of a 20 bp long phosphorothioate oligomer. In addition, its base composition differed only slightly from the mean composition of all ASOs used (A:3 versus 4.1; C:4 versus 4.4; G:6 versus 4.8; T:7 versus 6.6). Sequence-specific effects were controlled by using ASOs differing by three base pairs with regard to the respective cDNA sequence. Finally, the use of 10 ASOs per target gene allowed to include sequences that were scrambled (identical in base composition) except for two bases that were permutated.
For determining the potential secretion of OPN and BSPII, an MDA-MB-231^{GFP+} subline was generated, which was selected by continuously reducing the FBS content of the medium, until the cells kept growing without FBS. This cell line was denoted as MDA-MB-231^{F}. All cells were kept in log-phase, and passaged 1 – 3 times per week depending of their growth rate, and maintained under standard conditions (37°C, humidified atmosphere, 5% CO_2).
Stock solutions of ASOs in distilled water were diluted to appropriate concentrations with phosphate-buffered saline (PBS). For transfecting cells with ASOs, lipofectamine (Lipofectin, Invitrogen, Karlsruhe, Germany) or electroporation was used.

$ErPC_3$

For treating MDA-MB-231^{F} cells, $ErPC_3$, which was kept as stock solution in ethanol and PBS (10mM; ratio of diluents 1:1; V:V), was diluted in PBS resulting in final concentrations of 10, 14 and 20 mM. The medium containing $ErPC_3$ was changed after 24 hours in all experiments and the cells were further grown for 48 – 72 hours without $ErPC_3$ (for details of sequential exposure see below).

No.	(OPN)[a] cDNA	(BSPII)[a] cDNA
ASO-01	bp 73–92	bp 46–65
	5'-CTC ATG GTA GTG AGT TTT CC-3'	5'-TGA TTG CTT CCT CTG GCA GT-3'
ASO-02	bp 661–680	bp 53–73
	5'-TTC AGG TCC TGG GCA ACG GG-3'	5'-ATT TTG GTG ATT GCT TCC TC -3'
ASO-03	bp 662–681	bp 328–347
	5'-GTT CAG GTC CTG GGC AAC GG-3'	5'-CTT CAT TGT TTT CTC CTT CA-3'
ASO-04	bp 1343–1362	bp 331–350
	5'-CTA ACT TAA AAA ACA AAA GA-3'	5'-ATT CTT CAT TGT TTT CTC CT-3'
ASO-05	bp 1346–1365	bp 505–524
	5'-ACA CTA ACT TAA AAA ACA AA -3'	5'-CTT CAT CAC TTT CCT TCT CT-3'
ASO-06	bp 193–212	bp 546–565
	5'-GTG GCC ACA GCA TCT GGG TA -3'	5'-GCT TTC TTC GTT TTC ATT TC-3'
ASO-07	bp 193–212	bp 706–725
	5'-GTG GCC ACA GCA TCT GGG TA-3'	5'-TTC CGG TCT CTG TGG TGT CT-3'
ASO-08	bp 195–214	bp 711–730
	5'-ATG TGG CCA CAG CAT CTG GG-3'	5'-CTG CCT TCC GGT CTC TGT GG -3'
ASO-09	bp 556–575	bp 1003–1022
	5'-GGT CTG CGA AAC TTC TTA GA-3'	5'-ACT GGT GGT GGT AGT AAT TC-3'
ASO-10	bp 559–578	bp 1005–1024
	5'-TCA GGT CTG CGA AAC TTC TT-3'	5'-TCA CTG GTG GTG GTA GTA AT-3'

The following sequence from HBV was used as a nonsense control (NSO): 5′-GCG AGG GAG TTC TTC TTC TA-3′. [a]The combination of the abbreviation of the gene and the number of a given ASO was used for its naming.

Table 6. Selected antisense oligonucleotides against OPN and BSPII.

Electroporation

Pending on the duration of treatment, $5x10^5$ – $3x10^6$ cells were suspended in 1ml RPMI 1640 medium without phenol red containing 10 mM concentrations of the respective ASO or NSO. For transfection, the cells were pulsed two or three times for 2ms at 450 or 350V, respectively, in an 800 ml cuvette using an electroporation impulse generator (EPI2500, Dr L Fischer, Heidelberg, Germany) with the capacitance set at 1200 mF. Thereafter, the cells were plated onto six-well plates and grown for 48 – 72 hours.

Lipofectamine

Pending on the duration of treatment, $1x10^5$ – $3x10^5$ cells were transferred onto six-well plates and grown for 24 hours. For transfection, ASO stock solutions were prepared in medium without FBS. According to the protocol of the manufacturer, the respective ASO stock solution was mixed with lipofectamine to allow ASO–liposome complexes to form. To 200 ml medium containing ASO quantities resulting in 10 mM (Western blot and in vivo model) and in 5, 10, or 20 mM (clonogenicity assay) final ASO concentrations, an equal volume of medium without FBS was added containing 5, 10 or 20 ml lipofectamine, respectively. In addition to the ASO treatment, an NSO control (20 mM final concentration with 20 ml lipofectamine) and a lipofectamine control (20 ml) were used. Based on the equilibrium of the ASO–liposome complex formation, the relatively low amount of

lipofectamine used resulted in effective ASO concentrations that were at least by a factor of 10 lower than those in the medium used for transfection. After 12 hours the medium was changed, and cells were further grown for 48 – 72 hours. In the case of a sequential combination treatment this first cycle was repeated with the respective combination partner.

Combination treatment

Sequential combination treatment consisted of two or three treatment cycles. The two-cycle treatment started either with the ASO and was followed by $ErPC_3$ or was performed with the reverse sequence. The three-cycle treatment started either with two cycles of ASO followed by $ErPC_3$ or an initial exposure to $ErPC_3$ was followed by two cycles with an ASO.

Verification of the ASOs treatment by Western blotting

Transfected cells were incubated, harvested and washed. Thereafter $2x10^6$ cells were counted and prepared for Western blotting. The protein concentration of the lysate was determined using the BCA protein assay from Pierce (Rockford, IL) according to the manufacturer's recommendation. After the cell lysates were separated by electrophoresis and transferred to a PVDF membrane, the membrane was incubated with the respective first antibody (OPN: rabbit–antihuman polyclonal or mouse–antihuman monoclonal antibody; BSPII chicken–antihuman or rabbit–antihuman polyclonal (all from Immundiagnostik, Bensheim, Germany)). An HRP-conjugated anti-mouse, anti-rabbit or anti-chicken secondary antibody (all from Immundiagnostik, Bensheim, Germany) and ECL (Amersham Biosciences, Freiburg, Germany) were used to detect the respective proteins by exposing the membrane to an X-ray film. To control for variations in loading, the membranes were stripped for 30 minutes at 56°C in stripping solution. Thereafter, they were reprobed with an antibody against β-actin (mouse monoclonal antibody, Santa Cruz.CA), and a secondary goat anti-mouse polyclonal antibody (Santa Cruz.CA).

Target protein	**OPN**			**BSPII**		
Type of exposure	Single[a]	Twofold[a]	Single[a]	Single[b]	Single[a]	Twofold[a]
ASO-01	25	34	45	78	58	16
ASO-02	45	60	38	94	94	---
ASO-03	32	38	97	44	93	---
ASO-04	16	23	73	55	109	---
ASO-05	43	80	27	63	101	49
ASO-06	45	68	33	16	79	19
ASO-07	31	69	58	25	37	22
ASO-08	48	97	62	34	101	25
ASO-09	---	74	35	46	84	---
ASO-10	---	86	33	40	30	---
NSO	99.5	99	100	99	100	97
Control	100	100	100	100	100	100

[a] Transfection with lipofectamine. [b] Transfection with electroporation.
The used cell line was MDA-MB 231^F. X-ray films documenting chemoluminescence bands were scanned, digitized bands of the respective protein were expressed in relation to untreated control (100%).

Table 7. Inhibition of OPN and BSPII expression in MDA-MB 231^F cells following exposure to a series of antisense oligonucleotides.

The X-ray films were automatically processed and scanned by using an imaging program. The digitized bands of the respective proteins were given as percent of control and corrected for differences in loading by referring to the intensity of the β-actin band. The Western blot results of protein expression after exposing mammary carcinoma cells to ASOs directed against OPN and BSPII mRNA are shown in Table 7. Systematic comparisons showed that ASO-OPN-04 and ASO-BSPII-06 were the most effective structures within the respective series. Single exposure of cells was enough to reduce the OPN expression by 84% in response to ASO-OPN-04. This effect was not increased by repeating the ASO exposure, but the protein concentration was kept at a comparable level (77%). A similar efficacy was obtained for ASO-BSPII-06, which caused an 84% reduced bone sialoprotein II expression following single exposure and an 81% reduced protein level following two-fold exposure.

In vivo model

In order to induce loco regional bone metastasis, and to investigate a preventive effect of the ASOs, $1x10^5$ MDA-MB 231^F cells (control or pre-exposed to ASOs) were injected into a branch of the femoral artery of a nude rat, as described above. After an average of 28 days following the inoculation of control cells, lytic metastases could be detected by X-rays. The size of these lesions, which exclusively occurred in the femur, tibia and fibula of the animals, was recorded for up to 10 weeks.

The results of the *in vivo* experiments are shown in Table 8. Exposure to the ASO-OPN-04 and ASO-BSPII-06 for 3 days before implantation into nude rats caused a significantly reduced tumor take rate, as assessed by the appearance of osteolytic lesions following exposure to ASO-OPN-04, and a reduced size of osteolytic lesions in the X-ray-positive rats following both agents.

Group no.	Animal no.	Agent[a]	Observation period (weeks)	Incidence of metastasis	Size of lesion[b]	Mean	T/Cx100[c]
1	4	ASO-OPN-04	4	1/4	0, 0, 0, 34	8.5(717)[d]	3.9
			6	1/4	0, 0, 0, 46	11.5(723)	3.8
			8	1/4[e]	0, 0, 0, 60[f]	15(730)	4.3
2	4	ASO-BSPII-06	4	1/4	0, 0, 0, 14	3.5(77)	1.6
			6	1/4	0, 0, 0, 35	8.8(718)	2.9
			8	2/4	0, 0, 20, 106[f]	31.5(751)	9.1
3	2	NSO	4	2/2	106, 240	173(134)[g]	78
			6	2/2	162, 411	287(249)	95
			8	2/2	242, 728	485(486)	140
4	2	Untreated cells control rats	4	2/2	314, 127	221(187)	100
			6	2/2	386, 218	302(168)	100
			8	2/2	425, 266	346(159)	100

[a] MDA-MB-231^F cells were exposed to ASOs or NSO (transfected with lipofectamine), before transplantation to nude rats. [b] Product of pixel number and mean black intensity in individual rats as determined by image analysis from the X-ray radiograph. [c] Mean lesion size of treated over control rats times 100. [d] Numbers in brackets denote SD. [e] P = 0.0028 vs. control groups 3 and 4.
[f] P = 0.05 vs. control groups 3 and 4. [g] Numbers in brackets denote range.

Table 8. Results of in vivo experiments.

Nude rats that received 1x10^5 untreated or NSO-treated MDA-MB-231^F breast cancer cells developed bone metastasis in four of four cases (Table 8). The metastatic lesions were detectable for the first time after 4 weeks and were nearly doubled in size until week eight. Three of four rats that received 1x10^5 MDA-MB-231^F breast cancer cells pre-treated with the ASO, directed against OPN, developed no discernible metastasis within the observation period (P=0.028). One of the four rats developed lytic metastasis, but this lesion was distinctly smaller in size than those in control rats (week 4; P=0.05). Similarly, two of four rats that received 1x10^5 MDA-MB-231^F breast cancer cells pre-treated with the ASO directed against BSPII remained free of visible metastasis within the observation period of 8 weeks; one rat showed lytic metastasis after 4 weeks already, another rat developed a metastasis after 8 weeks. Thus, exposure to both, ASO-OPN-04 and ASO-BSPII-06, was associated with a significantly reduced lesion size, and the former agent caused a significantly reduced incidence of lesions, as well.

7. Biodistribution and efficacy of polymeric nanoparticles containing OPN and BSPII antisenses in a mammary carcinoma rat model

The initial steps in metastasis include invasion of tumor cells into normal tissue, traversion of small blood vessel walls and thus access to the circulation (38). Cancer cells that survive these initial steps can enter the sinusoids of the bone marrow and migrate across the sinusoidal wall to the endosteal bone surface (39). For homing into the bone compartment, tumor cells have to develop specific functions that differ from those in the normal tissue and they presumably express several proteins that assist in this process (40). Osteopontin and bone sialoprotein II, have both been characterized as promising targets for a therapy by antisense oligonucleotides directed against the RNA of these proteins, thus preventing or reducing lytic skeletal metastasis (36, 37). Efficient and specific delivery of antisenses (ASOs) and a protection of the sequences from degradation are the crucial conditions for ASO therapeutic efficiency. Due to their large molecular size and high negative charge density, the resulting low cellular permeability of ASOs is a major problem encountered with their therapeutic use (41-43). To improve cellular delivery of ASOs several methods, employed in DNA delivery, have been developed including viral vectors, liposomes, and other delivery systems (44-46).

Especially one of these nanocarrier systems, the nanoparticles (NP), has been increasingly employed as drug delivery devices of small molecules and particular nucleic acids (47). The nanocarriers are utilized to protect molecules from biodegradation as well as to modify their pharmacokinetics and biodistribution (48).

It was hypothesized that effective and safe ASO delivery could be achieved by polymeric nanoparticles (NP) fabricated from the biocompatible and biodegradable PLGA. Poly(lactic-co-glycolic acid) copolymers (PLGA) are among the few synthetic biocompatible biodegradable polymers approved for human gene therapy use (49). Due to their nano-size range and small negative charge, polymeric nanospheres containing ASOs can overcome the absorption barrier of the cell membrane by penetrating inside the cell *via* endocytosis (50, 51). Moreover, the controlled release delivery mode offers increased resistance to nuclease degradation, sustained duration of ASO administration, and consequently, prolonged antisense action.

In this section we report about the characteristics and therapeutic efficiency of a controlled release nanoparticle (NP) delivery system for ASO sequences, designed against OPN and BSP II. The ASO-NP efficiency in the inhibition of metastatic bone lysis was evaluated in the rat animal model of mammary carcinoma (52, 53).

ASO NP preparation and characterization

A double emulsion system and the solvent evaporation technique were used to incorporate OPN-ASO sequences in PLGA (53). At the end of production finished NP were lyophilized and the dry NP were stored in a vacuum desiccator at 4°C. The amount of ASO entrapped in the NP was analyzed and determined using the fluorescence "Oligreen assay kit" (Molecular Probes, Eugene, OR) and by UV spectroscopy at λ = 260 nm.
NP size and morphology were evaluated by dynamic light scattering, ALV (NIBS/HPPS GmbH, Langen, Germany), and a transmission electron microscope (TEM CN12, Philips, Eindhoven, The Netherlands). Average size and size distribution of empty NP and NP loaded with ASO sequences were measured before and after lyophilization. A zeta-sizer (Malvern Instruments Ltd, UK) was used for evaluation of NP charge (Tab. 9).
Several types of NP have been prepared for the various studies, empty NP (blank, serving as control), NP loaded with NS (control), NP containing OPN or BSPII ASO (ASO-NP); and fluorescent NP including, NP loaded with FITC-ASO (FITC-NP), fluorescent empty NP (PLGA-rhodamine, Rhodamine-NP), and double labeled NP (ASO-FITC and PLGA-rhodamine).

	Size distribution (nm)			Loading	
NP content	**Before lyophilization**	**After lyophilization**	**Surface charge (ζ potential, mV)**	**Yield (%)**	**Concentration (μg AS/mg polymer)**
OPN[a]	234 ± 72	281 ± 85	3.06 ± 0.84	51 ± 9	4.80 ± 0.68
BSPII[b]	236 ± 73	294 ± 98	3.88 ± 0.74	55 ± 14	4.56 ± 1.07
NS	233 ± 70	280 ± 90	2.37 ± 0.47	49 ± 13	6.91 ± 2.82
Empty	291 ± 85	324 ± 104	2.6 ± 0.06	---	---

Mean ± SD, each batch was measured in triplicate. [a]OPN = ASO-OPN-04 and [b]BSPII = ASO-BSPII-06 (Tab. 6)

Table 9. The physicochemical properties of AS NP examined.

7.1 Biodistribution pilot study in healthy rats

In a pilot study we tried to clarify the distribution of the nanoparticles in the organism (rat). Therefore the ASO-OPN-04 sequence (Tab. 6) was internally labeled with ^{33}P (54).
The used rats were randomly assigned to treatment or control groups. Naked ASO-OPN-04 (n = 4) or ASO-OPN-04-NP (n = 5) were injected into a rat tail vein at average $13.2x10^6$ DPM/rat. Rats injected with saline were used as a control group (n = 2). The rats were kept in metabolic cages and their excrements were collected every day. The animals were euthanized 24 h, 72 h, and 7 days post-injection. One milliliter of blood, 0.5 ml of urine, whole organs, samples of skeletal muscles, and feces were taken from each rat for analysis. The radioactivity (DPM = disintegrations per minute) in the samples was counted by means of a liquid scintillation analyzer (Packard, Tri-carb 2900TR, USA) against a calibration curve of $R^2 = 0.986$. The radioactivity was normalized to the animal weight and an organ accumulation was expressed as a percentage of total injected doses (Fig. 7a-c).
Following the above experiment in certain tissues a quantitative examination of the whole body biodistribution was performed comparing naked and NP encapsulated ASO.
Levels of naked ASO increased considerably in most tissues after 24 h, reaching highest values in the liver and spleen. Naked ASO was also found at higher levels in blood, in

comparison to NP encapsulated sequences (9.3 ± 1.0% and 7.7 ± 1.2% naked ASO vs. 3.2 ± 0.5% and 1.9 ± 0.4% ASO-NP after 24 h and 72 h, respectively), but the naked ASO amount was insignificant (0.3 ± 0.0%) after 7 days, when ASO-NP level in blood started to increase (4.0 ± 0.9%). Throughout the time-course of the study, ASO-NP was distributed mainly in the liver and spleen. The total recovery from these two organs was as much as 46.4 ± 3.0% and 74.2 ± 7.4%, 24 h and 72 h after the treatment, respectively, and slightly decreased to 59.3 ± 8.9% after 7 days. In contrast, naked ASO sequences accumulated in the spleen to a much lower extent (2.8% ± 0.4% after both 24 and 72 h, and 1.6% ± 0.1% after 7 days). Although relatively high amounts of naked ASOs reached the liver during the first half of the study (33.8% ± 1.7% and 26.7% ± 4.8%, after 24 and 72 h, respectively), only 8.1% ± 1.4% were found in this organ after 7 days (Figure 7c). Throughout the study naked ASOs accumulated to a small extent in the thymus (2.8% ± 0.9%, 3.8% ± 4.8%, and 1.8% ± 0.3% after 24 h, 72 h, and 7 days, respectively). Relatively low levels of ASO, both naked and NP

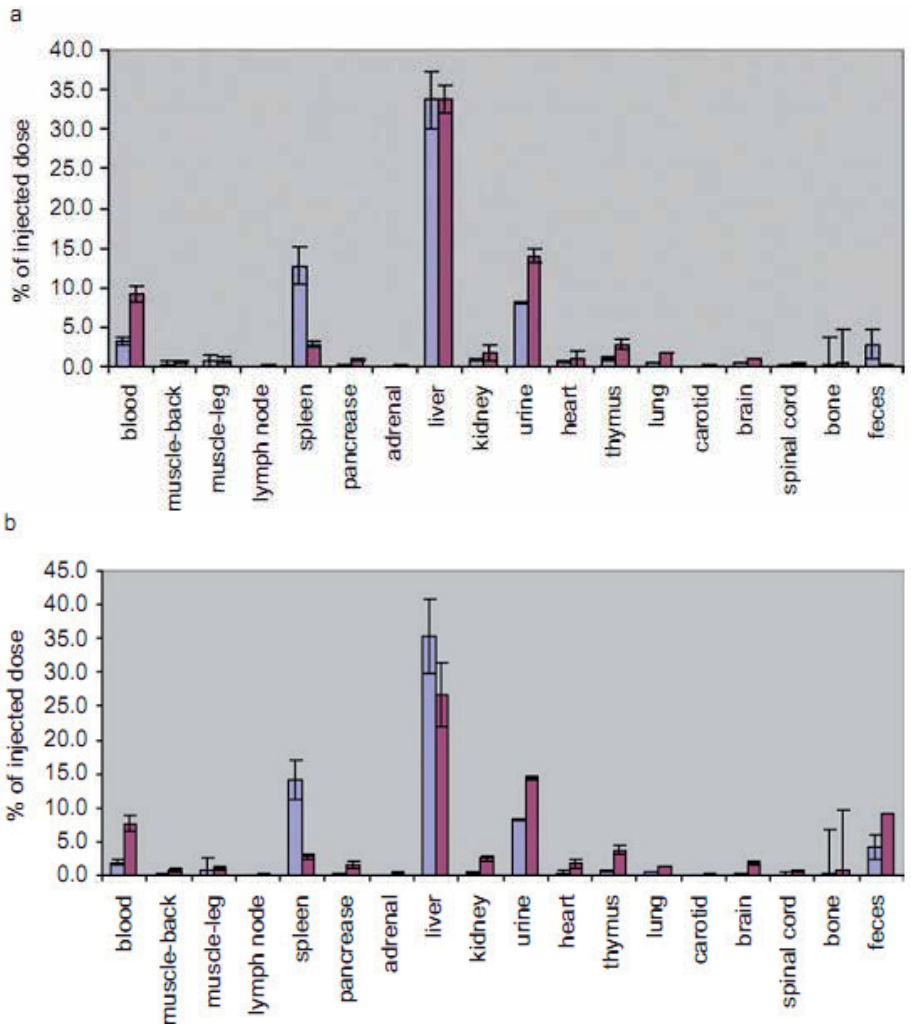


Fig. 7. (Continued)

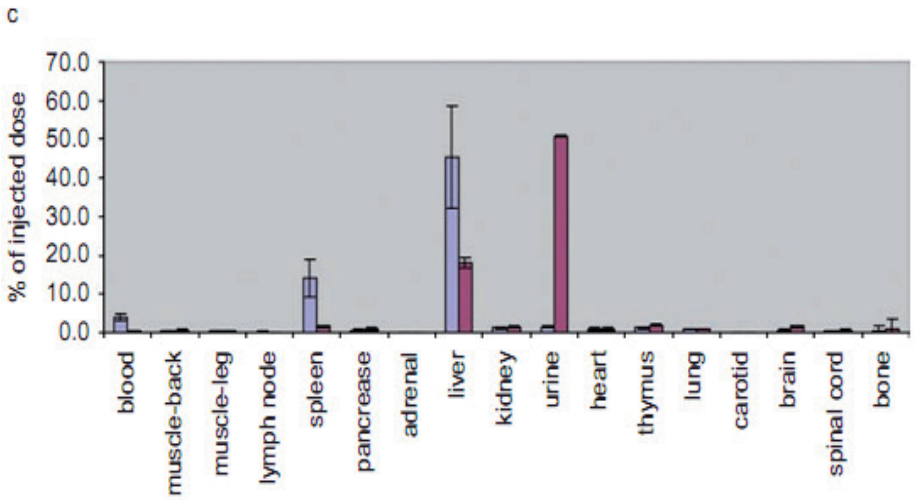


Fig. 7a-c. The quantitative biodistribution of ASO-NP in whole body organs.
Naked (n = 4) or NP (n = 6) encapsulated sequences were internally labeled with ^{33}P ATP and were injected into a healthy male rats (350–400 g) tail vein (average 13.2×10^6 DPM/rat). Rats injected with saline were used as the control group (n = 2). Biodistribution was evaluated 24 h (a), 72 h (b), and 7 days (c) after treatment by beta-counter analysis of blood, organs, and excrement samples. The radioactivity was normalized to the animal weight and organ accumulation was expressed as a percentage of total injected dose (mean ± SD). Bars: blue – ASO NPs, red – naked ASO.

encapsulated, were found in the kidneys at all time points, but always the amount of naked ASO in the kidneys was higher than that of NP encapsulated sequences (1.7% ± 1.0%, 2.5% ± 0.3%, and 1.4% ± 0.3% naked AS vs. 0.8% ± 0.1%, 0.5% ± 0.1%, and 1.2% ± 0.2%, ASO-NP after 24 h, 72 h, and 7 days, respectively). At all time points of the experiment, the accumulation of NP encapsulated ASO was negligible in other organs harvested. Mostly ~1% or less of the injected dose was found in the skeletal muscles, lymph node, pancreas, adrenals, brain, spinal cord, heart, and lungs. Naked ASO was taken up by those tissues to a higher extent than ASO-NP.

Naked ASO and ASO-NP had different clearance profiles. Throughout the study course naked ASO was excreted mainly and rapidly in urine, due to the highly hydrophilic nature of the ASO and its degradation products. Already after 7 days more than half of the naked ASO dose injected was cleared in the urine. In contrast, ASO-NP was excreted in the urine to a lower extent, and a significant amount was also found in the feces at all time points. After 7 days, the clearance of ASO-NP was mostly in feces, whereas only a small ASO amount was found in the urine. The divergent clearance suggests different degradation rate and extent of naked and encapsulated ASO. Following the treatment with naked ASO, all the sequences can be rapidly degraded and cleared in urine. In ASO-NP formulation only the released ASO is exposed to degradation, but encapsulated ASOs remains intact and generally can be found in tissues or excreted in the feces. Moreover, most of the encapsulated ASO was released inside the cells, because of the rapid cellular uptake of NP; hence it is less exposed to degradation by nucleases existing in the extracellular matrix.

It can be concluded that ASO-NP protects the ASO from degradation and provides efficient ASO delivery to the tumor tissue. Moreover, administration by the NP delivery system minimizes ASO accumulation in intact organs due to the ASO sustained release profile, and the favorable NP physicochemical properties.

7.2 NP effects on cells *in vitro*

NP effect on cells' viability

HeLa cells (1.5 x 10^4/chamber) were seeded in a Lab-Tek chambered cover glass system and incubated for 24 hr. The cells were treated with empty NP (10 mg/ml) and were incubated for 24 or 48 hr. At each time point, the cells were washed with PBS and harvested. The total cells' number and amount of living and dead cells were counted, using a hemocytometer. Trypan blue was used for staining dead cells. Non-treated cells were used as a control group. The percentage of living and dead cells was calculated. Statistical differences between NP treated and non-treated groups were tested at each time point by the one-tailed unpaired Student's test. Differences were considered statistically significant at $p < 0.05$.
The average cells' number (living and dead) was similar in treated and non-treated groups 24 hr after treatment (living cells, 95.0% and 96.3%; and dead cells, 5.0% and 5.2% in NP treated and non-treated groups, respectively), as well as after 48 hr (living cells, 94.8% and 95.6%; dead cells, 5.2% and 4.4% in NP treated and untreated groups, respectively).

In vitro visualization of cellular uptake

The cellular uptake of fluorescent ASO sequences encapsulated in rhodamine-labeled PLGA-NP by HeLa and MDA-MB-231 tumor cells was examined. MDA-MB-231 and Hela cells (1.5 x 10^4/chamber) were seeded in a Lab-Tek chambered cover glass system (Nunc International Corp.) and incubated for 24 hr. Fluorescent NP loaded with FITC-ASO or naked FITC-ASO sequences were added to the cells and incubated for 4, 24 or 48 hr.
After methanol fixation the cells were mounted with fluorescence microscopy mounting media. ASO-NP uptake in comparison to naked AS was observed and recorded by means of confocal microscopy (Zeiss LSM 410, Germany). Nontreated cells and cells incubated with empty NP were determined as background. Confocal cross-sections of cells taken at 24 and 48 hr after treatment were used to verify intracellular (cytoplasmic) localization of the NP and the ASO.
Empty fluorescent NP were shown to internalize and accumulate in the cytoplasm of both HeLa and MDA-MB-231 cancer cells, reaching maximal uptake within the first 24 hr (figure not shown). No significant auto-fluorescence was observed in HeLa cells chosen for further uptake evaluation experiments. Similarly to the empty NP, ASO loaded NP were internalized by the cells and accumulated in the cellular cytoplasm. High NP internalization was achieved by 24 hr. In contrast, naked ASO uptake was decreased overtime. Confocal cross-section images of HeLa cells 24 and 48 hr after treatment with ASO-NP verified cell internalization and cytoplasm localization of the NP.
Cells treated with empty NP exhibited a relatively high uptake of 13.6 ± 0.01% already after 4 hr, followed by a gradual increase and thus reaching maximum uptake after 24 hr (44.02 ± 0.68%), and a slight decrease after 48 hr (39.65 ± 0.02%). Treatment with naked ASO resulted in a significant uptake (17.18 ± 0.83% of the cells) already 4 hr after treatment. The naked ASO internalization rate increased to its maximum level after 24 to 48 hr (35.22 ± 19.19% and 30.40 ± 0.11%, respectively). In spite of a relatively rapid NP internalization, fluorescent

signal from FITC-ASO encapsulated in NP was detected in less than 10% of the cells after 8 hr. In contrast, the number of cells loaded with ASO was markedly increased after 24 hr, reaching 58.81 ± 25.75%.

Extent and intensity of NP endocytosis: The representative experiments demonstrate the continued uptake over time of ASO-NP. After 24 hr, a significant increase of NP uptake was exhibited in cells treated with empty rhodamine-NP. ASP-NP internalization (encapsulated FITC ASO) was also observed after 4 hr, but to a lesser extent than naked ASO. In contrast, the fluorescence of FITC-ASO in NP was markedly increased after 24 hr, and was much higher than that of naked ASO. Internalization of naked ASO was significant already after 4 hr (41.48 ± 0.03). The AS accumulated inside the cells, reaching 63.22 ± 0.01 and 74.45 ± 0.11, after 24 and 48 hr, respectively. In contrast, empty NP uptake was slower, 21.12 ± 0.01 after 4 hr, increasing gradually to 46.43 ± 0.01 after 24 hr. The uptake of rhodamine-NP was decreased after 48 hr. Following treatment with ASO encapsulated in non-fluorescent NP, relatively low levels of ASO were detected inside the cells after 4 and 8 hr, increasing significantly after 24 hr (150.25 ± 0.87).

7.3 Bioactivity – Inhibition of metastatic bone lysis by OPN and BSPII ASOs

The tumor cells were inoculated as described above (see chapter 2). Resulting bone metastases and their mean lytic lesion size was followed by X-ray examinations once weekly for 12 weeks. Treatment results were observed in groups of 4–5 rats at each arm of the studies. The lesion size was measured from X-ray pictures using image analysis software, and the mean lesion size and lesion ratio (mean lesion size in treated group in percent of control) were calculated. The mean lesion size was presented in relative units (RU), corresponding to the image analysis of the pixel's count.

The results were expressed as mean ± SD. Statistical differences between groups in the metastatic lesion size were assessed by the one way ANOVA test.

The treatment with NP, loaded with ASOs (OPN or BSPII), was administered immediately after tumor inoculation into the same vessel as the cells (superficial epigastric artery, Fig. 2). The amount of NP (100 mg in 500 μl PBS, total dose of 600 μg of NSO or ASO) was divided: two thirds of the dose was injected into the vessel and one third (using the same needle) was injected loco regionally, into the muscle surrounding the knee joint (in order to avoid clogging of the artery).

The inhibition of metastatic bone lysis by OPN and BSPII ASO-NP was evaluated in a rat mammary carcinoma metastasis model. Most animals treated with nonspecific NSO delivered by NP (4 of 5 rats) developed bone metastasis (Tab. 10) and multiple lesions in the femur, tibia and fibula. The metastatic lesions were detectable for the first time after 4 weeks (mean lesion size of the control group, 16 ± 14 RU, doubled in size until week 8 (38 ± 36) and continued to grow in all rats till the end of the experiment (week 12).

Treatment with OPN ASO and BSPII ASO loaded NP resulted in a significant decrease in tumor bone metastasis incidence, as assessed by the reduced appearance of osteolytic lesions (Table 10) and in a reduced size of the lesions in metastasis-positive rats. Two of the 4 rats developed lytic metastasis following the treatment with OPN NP, and 3 of 5 with BSPII NP, but the lesions in the ASO NP treatment groups were distinctly smaller in size than those in the control group of NSO-NP (Table 10). Both ASO sequences successfully prevented metastasis incidence and even caused tumor regression in most animals; 3 of 4 and 3 of 5 animals were found free of visible metastasis at the end of the

observation period (week 12) following the treatment with OPN and BSPII NP, respectively (Table 10). It was found that the BSPII ASO had a response by the inhibition of metastasis, but it was somewhat less effective than OPN ASO in bone lysis reduction, when delivered by NP.

Treatment type	Time (weeks)	Metastasis incidence (tumor bearing rats / animals)	Mean lesion size[1]	Lesion ratio T/C x 100[2]
NSO[3]	4	4 / 5	16 ± 14	100
	6	4 / 5	38 ± 36	100
	8	4 / 5	56 ± 62	100
	10	4 / 5	72 ± 81	100
	12	4 / 5	85 ± 97	100
ASO-OPN-04	4	2 / 4*	7 ± 8	44
	6	2 / 4*	9 ± 12	24
	8	2 / 4*	11 ± 18	20
	10	1 / 4*	7 ±13	9
	12	1 / 4*	4.5 ± 9	5
ASO-BSPII-06	4	3 / 5	9 ± 8	56
	6	3 / 5	17 ± 17	45
	8	3 / 5	20 ± 22	36
	10	3 / 5	14 ± 17	19
	12	2 / 5*	12 ± 18*	14

[1] Product of pixel number and mean black intensity (±SD) in individual rats as determined by image analysis from the X-ray radiograph.
[2] Mean lesion size of treated over control rats x100.
*Statistically significant vs. control group (χ^2 test), $p < 0.01$.
[3] Nonsense oligonucleotide (NSO) were used as control group.

Table 10. Inhibition of metastatic bone lysis by OPN and BSPII antisenses delivered by nanoparticles.

It may be concluded that ASO delivery by NP is a promising therapeutic modality providing stability of the encapsulated ASOs and a sustained release. These drugs differ from small-molecule pharmaceuticals in that instead of binding to a pocket of the protein to block certain activities, they prevent the protein from being expressed altogether. This minimizes side effects, and increases the effectiveness of the drug.

8. Refined method for tumor cell application and – detection

The basic procedures used for anesthesia and tumor cell implantation were as described above (see chapter 2) except for the following aspects:
Subsequently to preparing the femoral artery as well as their related arteries (saphenous artery, popliteal artery, descending genicular artery, superficial epigastric artery), they were freed from the surrounding fat tissue and mobilized (Fig. 8a, b).

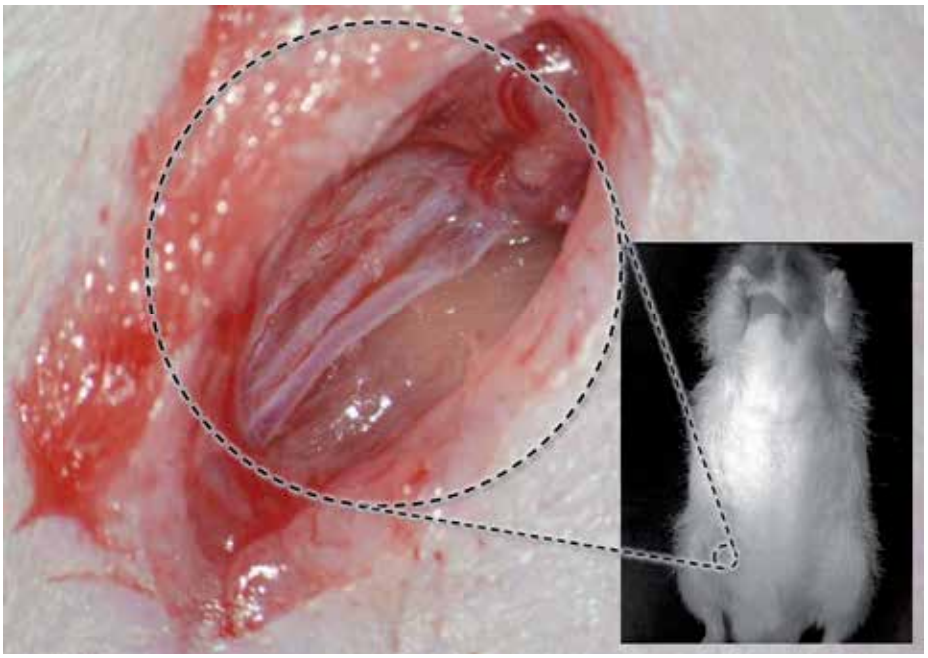


Fig. 8a. Region prepared for tumor cell implantation.

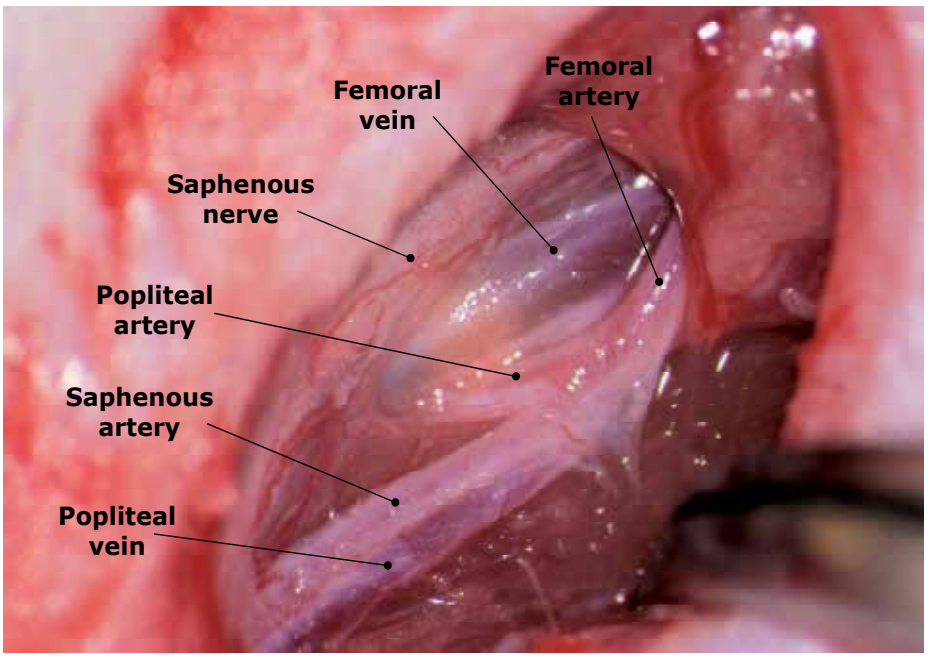


Fig. 8b. Mobilized arterial branches.

Then the femoral artery was separated from the neighboring vein and -nerve in the area in which the saphenous and superficial epigastric arteries branch off the femoral artery. These vessels were exposed to a 1% papaverin solution to inhibit their contraction. After an incubation time of one minute, the femoral artery as well as the saphenous and popliteal arteries were temporarily occluded by ligating them with three surgical threads (Prolene 7-0, Ethicon) (Fig. 9).

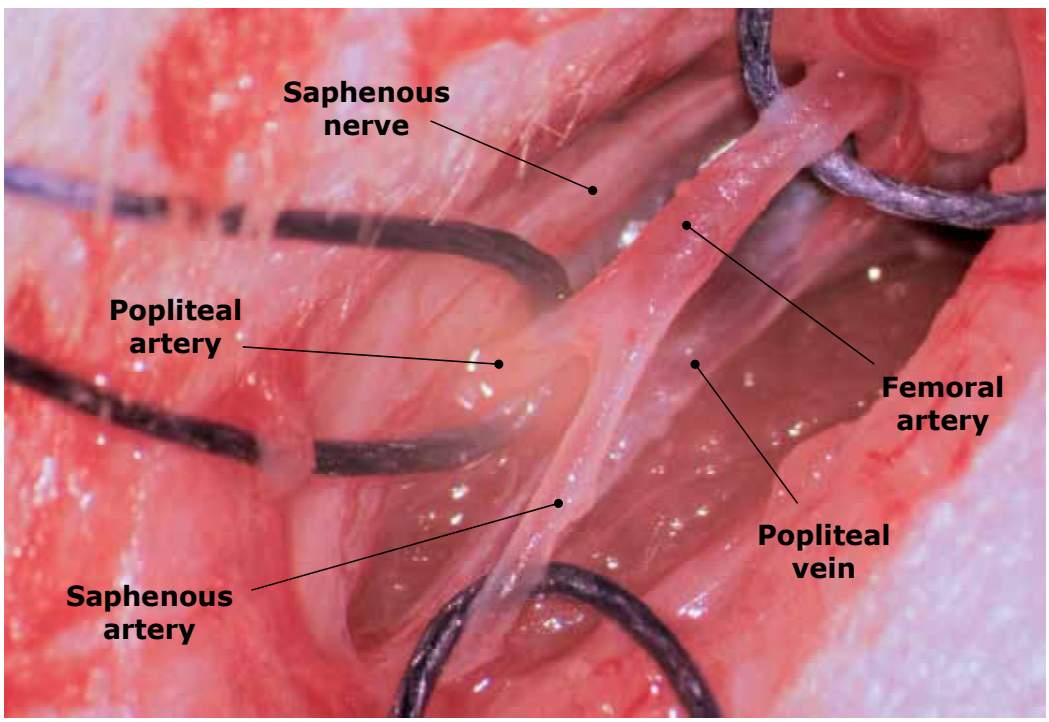


Fig. 9. Arterial branches prepared for tumor cell injection.

This enabled to control the blood flow as well as the direction of inoculated tumor cells. To that purpose a cannula (27G x ½") was inserted into the dilated lumen of the saphenous artery. Due to the persisting blockade of the femoral and saphenous arteries the tumor cells ($1x10^5$ cells in 0.2 ml PBS) and the blood stream were directed only into the popliteal artery (Fig. 10a, b).

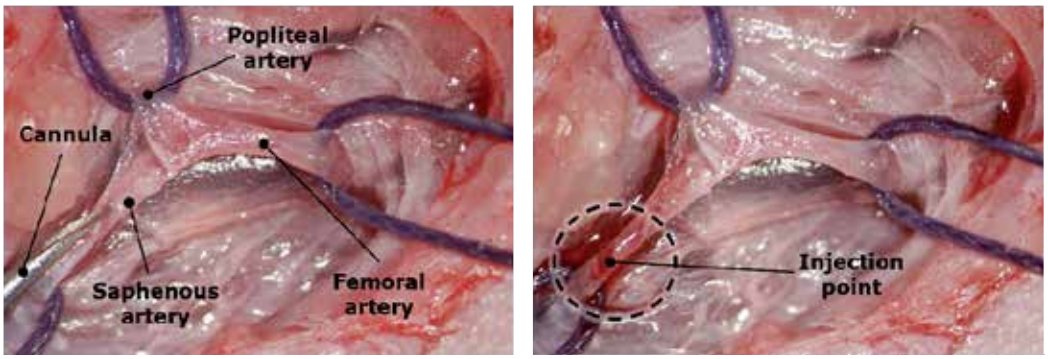


Fig. 10. a. Placement of the cannula into the saphenous arterty, b. Puncture site after withdrawal of the cannula.

For better visualization, trypan blue was injected to demonstrate the successful direction of the injected volume by the colored vessel walls (Fig. 11 a). After withdrawal of the cannula, the perforation of the arterial wall was sealed by using a piece of fat tissue which was pressed on the puncture site by sterile cotton swabs until the bleeding halted (Fig. 11. b). Subsequently, the operation site was closed as described above.

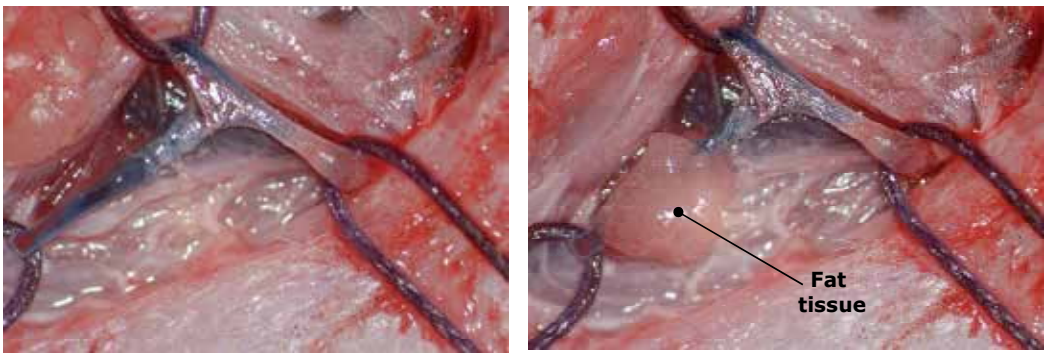


Fig. 11. a. Colored vessel walls show the direction of the blood stream, b. the puncture site has been closed by fat tissue.

Advantage of the refined method for tumor cell application.
The refined method allows to re-use the same artery after tumor cell injection, e.g. for locally administering therapeutics. In addition, it qualifies by a smaller surgical wound and a reduced time required for the surgical procedure.
Both methods result in the loco-regional application of tumor cells causing local tumor growth, only. The resulting osteolytic lesions caused in femur, tibia and fibula of the corresponding hind leg can be monitored by CT as early as two to four weeks after tumor cell implantation. In addition, the soft tissue tumor can be recorded by MRT for lesions ≥ 1 mm^3 (corresponding to 1 x 10^6 tumor cells). The need to observe disease progression of tumor cells in animal models has led to the development of various imaging techniques.
For monitoring the state of tumor cells directly after their intra-femoral application, the tumor cells have been stably transfected with the reporter genes (firefly-) luciferase and

(cherry-) RFP. The luciferase marker gene allows a non-invasive detection of tumor cells down to 1 x 10^3 tumor cells per hind leg (Fig. 12 a, b).
The intraperitoneal injection of luciferin (500 µl of a potassium-D-luciferin solution (1.5mg/rat)) results in a light emission which can be detected by a sensitive CCD camera (IVIS Imaging System – Series 100).

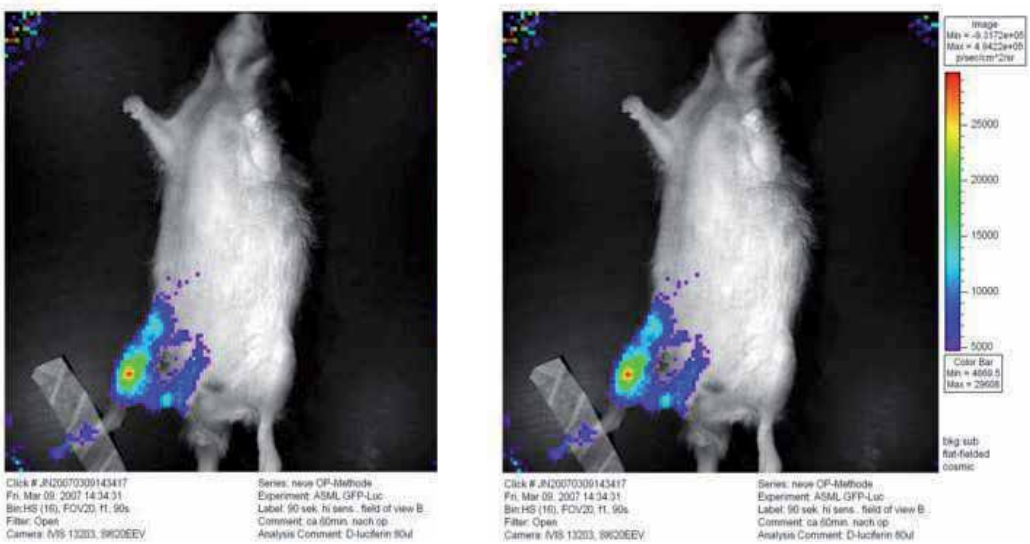


Fig. 12. a. 10 Minutes after implantation of 1 x 105 tumor cells and injection of 1.5 mg luciferin, b. 60 Minutes after implantation of 1 x 105 tumor cells and re-injection of 1.5 mg luciferin.

Bioluminescence Imaging (BLI) is an extremely sensitive method for detecting metastases. Already micro-metastatic signals from a few thousand luciferase positive MDA-MB-$231^{RFP/Luci+}$ cells were detectable. However, a crucial drawback of the bioluminescence method is the relatively poor spatial resolution and the fact that luciferase-transfected cells are indispensably needed. A comparison of the three methods for cell monitoring and their sensitivities is given in Figure 13.

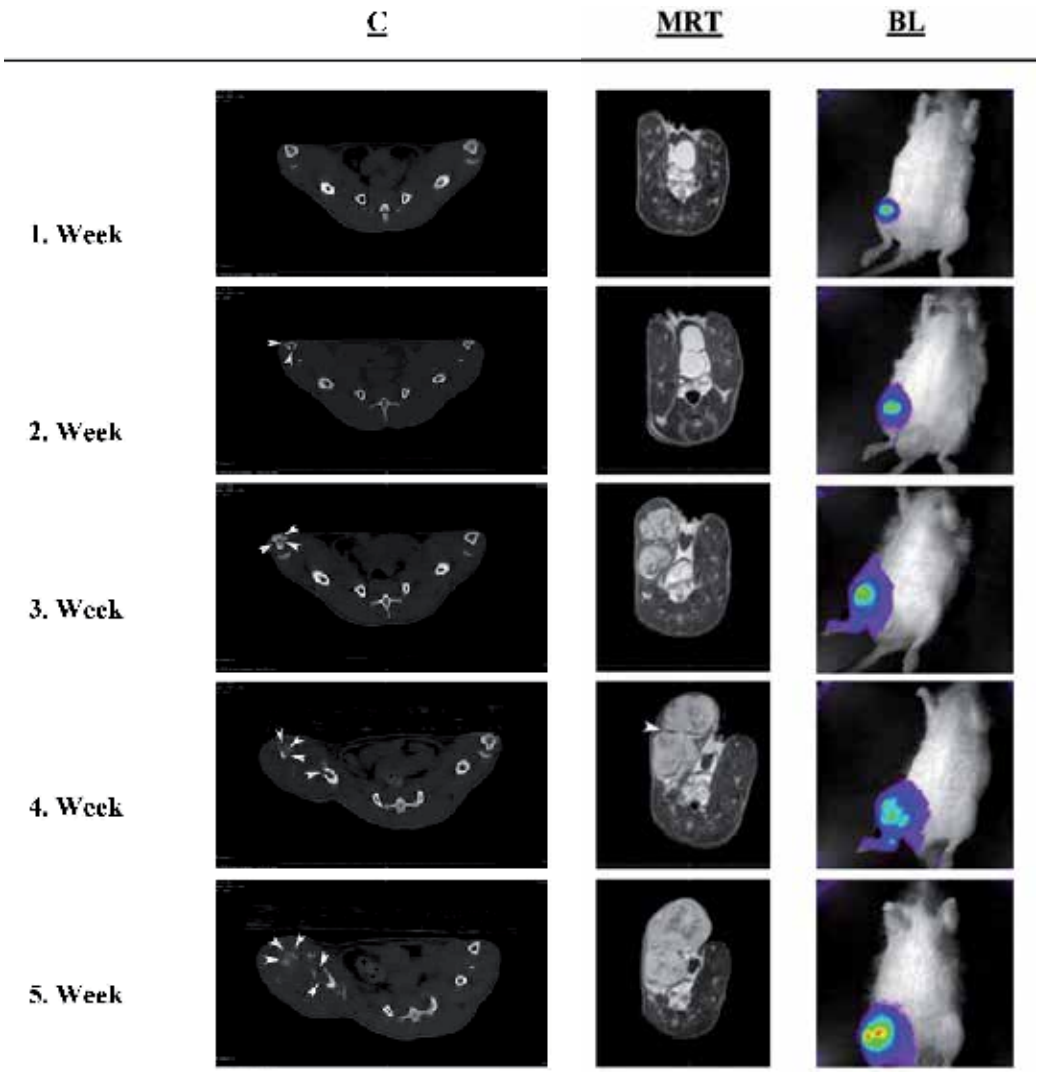


Fig. 13. Comparison of three different methods for monitoring lytic lesions of an untreated control rat.

CT: Bone lesions were detected two weeks after tumor cell injection. Owing to contrast, bone can be differentiated from soft tissues like muscles or fat. As for MRT, a three dimensional reconstruction can be generated. However, there is exposure to radiation and this technique as well as MRT is distinctly more complex and expensive than BLI.

MRT: One advantage of this method is the possibility to measure the volume of the lesions. Another advantage is related to the high contrast found for soft tissues, including their pathologic alterations. This is due to an extremely high resolution.

BLI: As soon as one week after inoculation the MDA-MB231-cells were detected by light emission in response to luciferin administration. With time, the signal increased in size regarding the emitting area as well as its intensity. BLI was the most sensitive method for detecting bone metastases.

9. Acknowledgments

These studies were supported in part by the BMBF BioDisc Cooperation (DKFZ-MOS), Grant No. 0315014/G401 as well as by the company Immundiagnostik AG, Wiesenstrasse 4, 64525 Bensheim, Germany.

10. References

[1] Guise TA: Molecular mechanisms of osteolytic bone metastases. Cancer 88 (Suppl 12): 2892-2898, 2000.
[2] Coleman RE: Skeletal complications of malignancy. Cancer 80 (Suppl 8): 1588-1594, 1997.
[3] Clohisy DR and Mantyh PW: Bone cancer pain. Cancer 97 (Suppl 3): 866-873, 2003.
[4] Withold W, Armbruster FP, Karmatschek M and Reinauer H: Bone sialoprotein in serum of patients with malignant bone diseases. Clin Chem 43: 85-91, 1997.
[5] Bellahcene A, Antoine N, Clausse N, Tagliabue E, Fisher LW, Kerr JM, Jares P and Castronovo V: Detection of bone sialoprotein in human breast cancer tissue and cell lines at both protein and messenger ribonucleic acid levels. Lab Invest 75: 203-210, 1996.
[6] Diel IJ, Solomayer EF, Seibel MJ, Pfeilschifter J, Maisenbacher H, Gollan C, Pechterstorfer M, Conradi R, Kehr G, Boehm E, Armbruster FP and Bastert G: Serum bone sialoprotein in patients with primary breast cancer is a prognostic marker for subsequent bone metastasis. Clin Cancer Res 5: 3914-3919, 1999.
[7] Fedarko NS, Jain A, Karadag A, van Eman MR and Fisher LW: Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. Clin Cancer Res 7: 4060-4066, 2001.
[8] Bellahcene A, Menard S, Bufalino R, Moreau L and Castronovo V: Expression of bone sialoprotein in primary human breast cancer is associated with poor survival. Int J Cancer 69: 350-353, 1996.
[9] Ibrahim T, Leong I, Sanchez-Sweatman O, Khokha R, Sodek J, Tenenbaum HC, Ganss B and Cheifetz S: Expression of bone sialoprotein and osteopontin in breast cancer bone metastases. Clin Exp Metastasis 18: 253-260, 2000.
[10] Waltregny D, Bellahcene A, De Leval X, Florkin B, Weidle U and Castronovo V: Increased expression of bone sialoprotein in bone metastases compared with visceral metastases in human breast and prostate cancers. J Bone Miner Res 15: 834-843, 2000.
[11] Carlinfante G, Vassiliou D, Svensson O, Wendel M, Heinegard D and Andersson G: Differential expression of osteopontin and bone sialoprotein in bone metastasis of breast and prostate carcinoma. Clin Exp Metastasis 20: 437-444, 2003.
[12] Fisher LW, Torchia DA, Fohr B, Young MF and Fedarko NS: Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. Biochem Biophys Res Commun 280: 460-465, 2001.
[13] Fisher LW, Jain A, Tayback M and Fedarko NS: Small integrin binding ligand N-linked glycoprotein gene family expression in different cancers. Clin Cancer Res 10: 8501-8511, 2004.
[14] Ganss B, Kim RH and Sodek J: Bone sialoprotein. Crit Rev Oral Biol Med 10: 79-98, 1999.
[15] Fisher LW, Whitson SW, Avioli LV and Termine JD: Matrix sialoprotein of developing bone. J Biol Chem 258: 12723-12727, 1983.

[16] Bianco P, Fisher LW, Young MF, Termine JD and Robey PG: Expression of bone sialoprotein (BSP) in developing human tissues. Calcif Tissue Int 49: 421-426, 1991.
[17] Tye CE, Hunter GK and Goldberg HA: Identification of the type I collagen-binding domain of bone sialoprotein and the mechanism of interaction. J Biol Chem 280: 13487-13492, 2005.
[18] Ross FP, Chappel J, Alvarez JI, Sander D, Butler WT, Farach-Carson MC, Mintz KA, Robey PG, Teitelbaum SL and Cheresh DA: Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin alpha v beta 3 potentiate bone resorption. J Biol Chem 268: 9901-9907, 1993.
[19] Somerman MJ, Fisher LW, Foster RA and Sauk JJ: Human bone sialoprotein I and II enhance fibroblast attachment in vitro. Calcif Tissue Int 43: 50-53, 1988.
[20] Fedarko NS, Fohr B, Robey PG, Young MF, Fisher LW: Factor H binding to bone sialoprotein and osteopontin enables tumor cell evasion of complement-mediated attack. J Biol Chem. 2000 Jun 2; 275(22):16666-72.
[21] Rudland PS, Platt-Higgins A, El-Tanani M, et al. (2002): Prognostic significance of the metastasis-associated protein osteopontin in human breast cancer. Cancer Res.; 62:3417–3427.
[22] Tuck AB, Chambers AF (2001): The role of osteopontin in breast cancer: Clinical and experimental studies. J Mammary Gland. Biol Neoplasia. 6:419–427.
[23] Senger DR, Perruzzi CA and Papadopoulos A: Elevated expression of secreted phosphoprotein I (osteopontin, 2ar) as a consequence of neoplastic transformation. Anticancer Res 9: 1291-1299, 1989.
[24] Barry ST, Ludbrook SB, Murrison E and Horgan CM: Analysis of the alpha4beta1 integrin-osteopontin interaction. Exp Cell Res 258: 342-351, 2000.
[25] Smith LL and Giachelli CM: Structural requirements for alpha 9 beta 1-mediated adhesion and migration to thrombin-cleaved osteopontin. Exp Cell Res 242: 351-360, 1998.
[26] Weber GF: The metastasis gene osteopontin: a candidate target for cancer therapy. Biochim Biophys Acta: 1552: 61-85, 2001.
[27] Rosol TJ, Tannehill-Gregg SH, LeRoy BE, Mandl S, Contag CH (2003): Animal models of bone metastasis. Cancer; 97:748–57.
[28] Rogers MJ, Watts DJ, Russell RGG (1997): Overview of bisphosphonates. Cancer 80(8):1652–1660.
[29] Green JR (2002): Bisphosphonates in cancer therapy. Current opinion in oncology: 14:609–615.
[30] Green JR (2003): Antitumor effects of bisphosphonates. Cancer 97(3Suppl):840–847.
[31] Ross JR, Saunders Y, Edmonds PM, Patel S, Broadley KE, Johnston SRD (2003): Systematic review of role of bisphosphonates on skeletal morbidity in metastatic cancer. BMJ 327:469.
[32] Yoneda T, Michigami T, Bing Y, Williams PJ, Niewolna M, Hiraga T (2000): Actions of bisphosphonate on bone metastasis in animal models of breast carcinoma. Cancer 88:2979–2988
[33] Hiraga T, Ueda A, Tamura D, Hata K, Ikeda F, Williams PJ, Yoneda T (2003): Effects of oral UFT combined with or without zoledronic acid on bone metastasis in the 4T1/luc mouse breast cancer. Int J Cancer 106:973–979.
[34] Bäuerle T, Adwan H, Kiessling F, Hilbig H, Armbruster FP, Berger MR (2005): Characterization of a rat model with sitespecific bone metastasis induced by MDA-MB-231 breast cancer cells, and its application to the effects of an antibody against bone sialoprotein. Int J Cancer 115:177–186.

[35] Bäuerle T, Peterschmitt J, Hilbig H, Kiessling F, Armbruster FP, Berger MR (2006): Treatment of bone metastasis induced by MDA-MB-231 breast cancer cells with an antibody against bone sialoprotein. Int J Oncology 28:573–583.
[36] Adwan H, Bäuerle TJ, Berger MR. (2004): Downregulation of osteopontin and bone sialoprotein II is related to reduced colony formation and metastasis formation of MDA-MB-231 human breast cancer cells. Cancer Gene Ther; 11:109–20.
[37] Adwan H, Bäuerle TJ, Najajreh Y, Elazer V, Golomb G, Berger MR (2004): Decreased levels of osteopontin and bone sialoprotein II are correlated with reduced proliferation, colony formation, and migration of GFP-MDA-MB-231 cells. Int J Oncology 24: 1235-1244.
[38] Kakonen SM, Mundy GR (2003): Mechanisms of osteolytic bone metastases in breast carcinoma. Cancer; 97:834–9
[39] Mundy GR. et al. (1997): Mechanisms of bone metastasis. Cancer; 80:1546–56.
[40] Mundy GR. et al. (2002): Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer; 2:584–93.
[41] Akhtar S, Juliano RL (1992): Cellular uptake and intracellular fate of antisense oligonucleotides. Trends Cell Biol; 2:139–44.
[42] Patil SD, Rhodes DG, Burgess DJ: DNA-based therapeutics and DNA delivery systems: a comprehensive review. AAPS J 2005; 7:E61–E77.
[43] Kurreck J. et al. (2003): Antisense technologies. Improvement through novel chemical modifications. Eur J Biochem; 270:1628–44.
[44] Mahato RI, Takakura Y, Hashida M (1997): Development of targeted delivery systems for nucleic acid drugs. J Drug Target; 4:337–57.
[45] Smith AE (1995): Viral vectors in gene therapy. Annu Rev Microbiol; 49:807–38.
[46] Ledley FD et al. (1994): Non-viral gene therapy. Curr Opin Biotechno; 5:626–36.
[47] Zhang L, Gu FX, Chan JM, Wang AZ, Langer RS, Farokhzad OC (2008): Nanoparticles in medicine: therapeutic applications and developments. Clin Pharmacol Ther; 83:761–9.
[48] Li SD, Huang L (2008): Pharmacokinetics and biodistribution of nanoparticles. Mol Pharm; 5:496–504.
[49] Shive MS, Anderson JM (1997): Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Deliv Rev; 28:5–24.
[50] Cohen H, Levy RJ, Gao J, Fishbein I, Kousaev V, Sosnowski S, Slomkowski S, Golomb G (2000): Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. Gene Ther; 7: 1896–905.
[51] Cohen-Sacks H, Najajreh Y, Tchaikovski V, Gao G, Elazer V, Dahan R, Gati I, Kanaan M, Waltenberger J, Golomb G (2002): Novel PDGFbetaR antisense encapsulated in polymeric nanospheres for the treatment of restenosis. Gene Ther; 9:1607–16.
[52] Elazar V., Adwan H, Rohekar K, Zepp M, Lifshitz-Shovali R, Berger MR, Golomb G: Biodistribution of antisense nanoparticles in mammary carcinoma rat model. Drug Delivery, 2010, 1–11, Early Online
[53] Elazar V., Adwan H, Bäuerle TJ, Rohekar K, Golomb G, Berger MR (2010): Sustained delivery and efficacy of polymeric nanoparticles containing osteopontin and bone sialoprotein antisenses in rats with breast cancer bone metastasis. Int J Cancer; 126(7):1749-60.
[54] Bishop JS, Guy-Caffey JK, Ojwang JO, Smith SR, Hogan ME, Cossum PA, Rando RF, Chaudhary N. (1996): Intramolecular G-quartet motifs confer nuclease resistance to a potent anti-HIV oligonucleotide. J Biol Chem; 271:5698–703.

23

Inhibition of Tumor Growth and Metastasis by a Combination of Anti-VEGF-C and Enhanced IL-12 Therapy in an Immunocompetent Mouse Mammary Cancer Model

Masa-Aki Shibata[1], Junji Morimoto[2], Eiko Shibata[3], Mariko Harada-Shiba[3] and Shigekazu Fujioka[1]
[1]*Laboratory of Anatomy and Histopathology, Faculty of Health Science, Osaka Health Science University, Temma, Osaka*
[2]*Laboratory Animal Center, Osaka Medical College, Osaka*
[3]*Department of Molecular Innovation in Lipidology, National Cerebral & Cardiovascular Center Research Institute, Osaka*
Japan

1. Introduction

Breast cancer represents a major health problem in women, with more than 1,000,000 new cases and 370,000 deaths yearly worldwide [1]. Perhaps more worrisome is an apparently increasing incidence of breast cancer among younger women under 40 years of age recently reported in many countries worldwide [2-4]. The lethality of breast cancer is largely due to metastasis, preferentially to the lymph nodes, lungs and bones [5]; in order to delay the progression of breast cancer and prolong patient life, more effective chemopreventive and antimetastatic treatments and less toxic chemotherapeutic agents are desperately required.
Vascular endothelial growth factor-C (VEGF-C) is expressed in a variety of malignant tumors including mammary cancer [6] and over-expression of VEGF-C has been reported to be associated with lymph node metastasis and poor prognosis in breast cancer patients [7,8]. A number of animal studies using cell lines [9-11] and transgenic mice[12] have been conducted in an attempt to demonstrate that VEGF-C over-expression is able to promote cancer metastasis. Using a 'RNA interference' approach with an immunocompetent mouse mammary cancer model, we previously demonstrated that inhibition of VEGF-C or VEGF-A by gene silencing using vectors expressing short interfering RNA (siRNA) leads to suppression of lymphatic and/or hematogenous metastasis [13].
The cytokine interleukin-12 (IL-12), a heterodimer composed of p35 and p40 subunits, is produced primarily by dendritic cells, macrophages/monocytes, and neutrophils and functions in enhancing the activity of cytotoxic T lymphocytes and NK cells. Both subunits are necessary to exert biological activity [14]. IL-12 plays an important role in the induction of a cell-mediated immune response [15]. This cytokine is also involved in the differentiation of native T cells to the Th1 subset, and induces production of interferon-γ (IFNγ) in both T and NK cells. In addition, IL-12 has been shown to exert a potent anti-neoplastic effect in a

variety of tumors in animal models [16-19]. The anti-tumor activity of IL-12 is considered to be due to anti-angiogenic effects as well as to induction of immune response [19-21]. The number of $CD8^+$ T cells and dendritic cells is significantly elevated in induced murine mammary tumors stably transfected with VEGF-C siRNA, suggesting that VEGF-C modulates the immune response [22]. Based on the above evidence, we chose to use an immunocompetent mammary cancer model in this study.

2. Materials and methods

2.1 BJMC3879 cell line

Mouse mammary tumor virus (MMTV), isolated and purified from medium in which Jyg-MC cells (established from mammary tumors of the Chinese wild mouse) were grown, was inoculated into the inguinal mammary glands of female BALB/c mice, resulting in the development of mammary carcinomas [23]. The BJMC3879 mammary adenocarcinoma cell line was subsequently derived from a metastatic focus within a lymph node from one of the inoculated mice and the cell line continues to show a high metastatic propensity, especially to lymph nodes and lungs [19,24,25]. We maintain the BJMC3879 cell line in either RPMI-1640 medium or Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum supplemented with streptomycin/penicillin in an incubator at 37°C under a 5% CO_2 atmosphere.

2.2 Animals

Forty female 6-week-old BALB/c mice were used in this study (Japan SLC, Inc., Hamamatsu, Japan). The animals were housed no more than 5 per plastic cage on wood chip bedding with free access to water and food and maintained under conditions of controlled temperature (21 ± 2 °C), humidity (50 ± 10 %), and lighting (12 h-12 h light-dark cycle). All animals were held for a 1-week acclimatization period before study commencement. This animal experiment was approved by the Animal Experiment Committee of Osaka Medical College. Husbandry was in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals at Osaka Medical College, the Japanese Government Animal Protection and Management Law (No.105) and the Japanese Government Notification on Feeding and Safekeeping of Animals (No.6).

2.3 Vectors for VEGF-C siRNA and IL-12 expression

We used short hairpin RNAs (shRNA) targeting mouse VEGF-C to generate siRNA. The previously determined mouse VEGF-C siRNA sequence, 5'-GCATGAACACCAGCACAGGTT*ccaagag*AACCTGTGCTGGTGTTCATGC-3', [13] contains a 21-nucleotide sequence in sense and antisense orientation separated by a 7-nucleotide spacer (indicated above by small letters in italics). The complementary oligonucleotide was annealed and ligated into a *Bbs*I/*Bbs*I-digested psiRNA-h7SKGFP-zeo vector (InvivoGen, Inc., San Diego, CA, USA). This vector contains the human 7SK promoter (an RNA polymerase III promoter), which can generate high amounts of shRNAs [26]. We identified positive clones by restriction digestion and confirmed by sequencing.

The plasmid, pORF-mIL-12 (InvivoGen, Inc., San Diego, CA, USA), encodes for the mouse IL-12 gene; it is an active fusion of the p35 and p40 subunits linked by bovine elastin motifs to express IL-12 as a single peptide with the signal sequence in the p35 subunit. This vector

is regulated by the elongation factor –1α (EF-1α)/human T cell leukemia virus type 1 (HTLV-1) long terminal repeat hybrid promoter and has previously shown anti-neoplastic effects [19]. To produce the empty control vector, we deleted the *IL-12* gene from pORF-mIL-12 via digestion with *NcoI/NheI*.

2.4 *In vivo* gene therapy using VEGF-C siRNA and/or IL12 expression vectors

BJMC3879 cells (5 x 10^6 cells/0.3 ml in phosphate buffered saline) were inoculated into the right inguinal region of the 40 female BALB/c mice and the animals randomly allocated into 4 groups - pVec (control), psiVEGF-C, pIL12, and psiVEGF-C+pIL12 - of 10 mice each. Two weeks post-inoculation, when the tumors had reached 0.2–0.4 cm in diameter, we injected either psiVEGF-C, pIL12 or psiRNA-VEGF-C+pIL12, or the pVec control directly into the tumors of mice in the appropriate treatment groups. The vectors were injected using a 27-guage needle at a concentration of 0.5μg/μl in sterile saline while the animals were under isoflurane anesthesia. A total volume of 150 μl was introduced into larger tumors (more than 0.6 cm in maximum diameter), while smaller tumors of 0.6 cm in maximum diameter were infused until we detected leakage of the vector solution. Immediately after vector injection, we performed *in vivo* gene electrotransfer by applying a conductive gel (Echo Jelly; Aloka., Co., Ltd., Tokyo, Japan) topically to the unshaved skin over the injected tumor. Electric pulses were delivered directly to the tumor via "forceps" platinum plate electrodes (CUY650-10; Nepa Gene Co., Ltd., Ichikawa, Japan) using a CUY21EDIT square-wave electropulser (Nepa Gene Co., Ltd.). We had previously determined the parameters for optimal gene electrotransfer: for intratumoral injection of 50-75 μg plasmid (dependent on tumor size as mentioned above), 8 pulses with a pulse length of 20 milliseconds at 100 volts proved to be most efficient [13,24,27].

Using calipers, we measured the size of each treated mammary tumor weekly and calculated tumor volumes using the formula *maximum diameter x (minimum diameter)*^{2} *x 0.4* [28]. Individual body weights were also recorded at weekly intervals. All surviving animals received 50 mg/kg 5-bromo-2'-deoxyuridine (BrdU; Sigma Co., St. Lois, MO, USA) i.p. at 1 h prior to sacrifice.

2.5 Histopathological analysis

After 8 weeks of treatment and observation/ tumor measurement, all mice were euthanized under isoflurane anesthesia and the mammary tumors and certain lymph nodes (specifically, nodes from axillary and femoral regions, as well as any that appeared abnormal) were removed. We then immediately fixed a portion of each tissue sample in 10% phosphate-buffered formalin. Lungs were routinely inflated with the fixative, excised, and immersed back into the phosphate-buffered formalin. We subsequently trimmed and examined all lobes for metastatic foci before processing all tissues through to paraffin blocks, after which they were cut into 4-μm-thick sections and stained with hematoxylin and eosin (H&E) for histopathological examination or left unstained for immunohistochemistry.

2.6 Immunohistochemical analysis of mammary tumors for microvascular density and dilated lymphatic vessels

To quantitatively assess blood and lymphatic microvessel density in the primary mammary carcinomas, we used the avidin-biotin immunohistochemical complex method (LSAB kit;

DakoCytomation) with a rabbit polyclonal antibody against CD31 (Lab Vision Co., CA, USA), a specific marker for blood vessel endothelium, and a hamster anti-podoplanin monoclonal antibody (AngioBio Co., Del Mar, CA, USA) targeted to lymphatic endothelium. The number of CD31-positive blood microvessels was counted as previously described [29]; briefly, we scanned slides at low-power (X100) magnification to identify those areas having the highest number of vessels and the 5 areas of highest microvascular density were then selected and counted at higher (X200–400) magnification to obtain mean ± SD values. We counted the number of podoplanin-positive lymphatic vessels containing intralumenal tumor cells and expressed the numbers of immunopositive structures as an average ± SD.

2.7 Statistical analyses

We analyzed significant differences in the quantitative data between groups using the Student's *t*-test via the method of Welch, which provides for insufficient homogeneity of variance. The differences in metastatic incidence we examined by Fisher's exact probability test, with $P<0.05$ or $P<0.01$ considered to represent a statistically significant difference.

3. Results

3.1 Body weights and tumor growth

No mortality was observed in this study. At experimental weeks 2 through 6, body weights in mice receiving pIL12 and the combination psiVEGF-C + pIL12 treatment began to decrease compared to both control and psiVEGF-C alone. Though mild (less than 5% reduction compared to the controls), weight loss was consistently significant in the combination group during this 4-week period, but the pIL12 group showed statistically lower weights intermittently at weeks 2 and 4 (Fig. 1A). The general condition of the animals was good throughout the experiment. At conclusion of the study at week 8, body weights were roughly equivalent across treatment groups; however, as can be seen in Figure 1B, the tumor volumes of all 3 treatment groups were significantly suppressed from experimental weeks 3 to termination as compared to the pVec controls. The average tumor volumes at week 8 were as follows: pVec control group, 1715 ± 662 mm^3; psiVEGF-C group, 954 ± 470 mm^3; pIL12 group, 756 ± 343 mm^3; psiVEGF-C + pIL12 group, 860 ± 437 mm^3.

3.2 Tumor morphology and metastases

Histopathologically, all mammary carcinomas proved to be moderately differentiated adenocarcinomas. Representative histologic morphologies of lymph node and lung metastases are illustrated in Figures 2A-H. Both the metastatic incidence and multiplicity in lymph node and lung was markedly reduced in all treatment groups as compared to control; as illustrated in Figures 3A and B for lymph node metastasis and Figures 3C and D for lung metastasis, the reductions were statistically significant within the parameters of overall node and lung metastatic incidence and in the number of larger metastatic lung foci >250 μm. Treatment with pIL12 alone appears to be more effective in inhibiting tumor spread than psiVEGF-C, but by all criteria evaluated, the psiVEGF-C + pIL12 combination yielded the greatest reductions in metastatic spread and severity over either psiVEGF-C alone, pIL12 alone, or pVec control (Figures.3B and D).

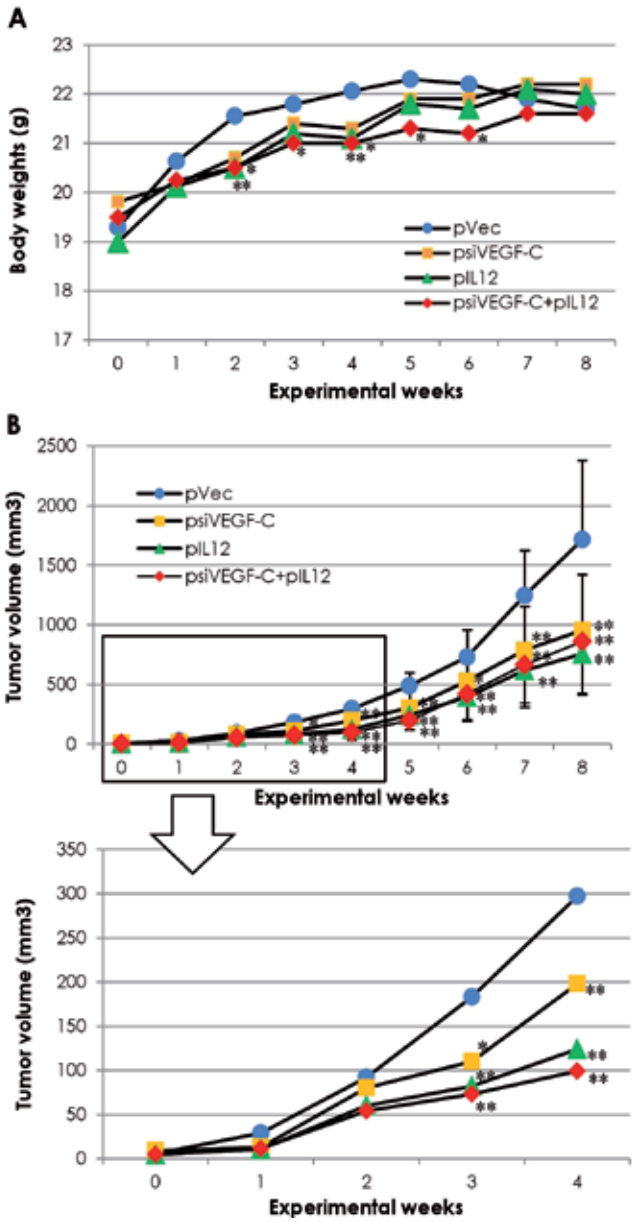


Fig. 1. Body weights (**A**) and mammary tumor volumes (**B**) in female BALB/c mice treated with pVec (control), psiVEGF-C, pIL12, and the psiVEGF-C+pIL12 combination vector. (**A**) Body weights were significantly lower in the pIL12 group at weeks 2 and 4, and in the psiVEGF-C+pIL12 combination group from weeks 2 through 6, as compared to the pVec group. (**B**) Increases in tumor volume were significantly suppressed in mice transfected with either psiVEGF-C alone, pIL12 alone, or combined psiVEGF-C+pIL12 at weeks 3 – 8 (experiment termination) compared to pVec -treated control mice. The data for body weights at weeks 0 – 4 are magnified. Data represent mean ± SD. *P<0.05; **P<0.01 compared with pVec controls.

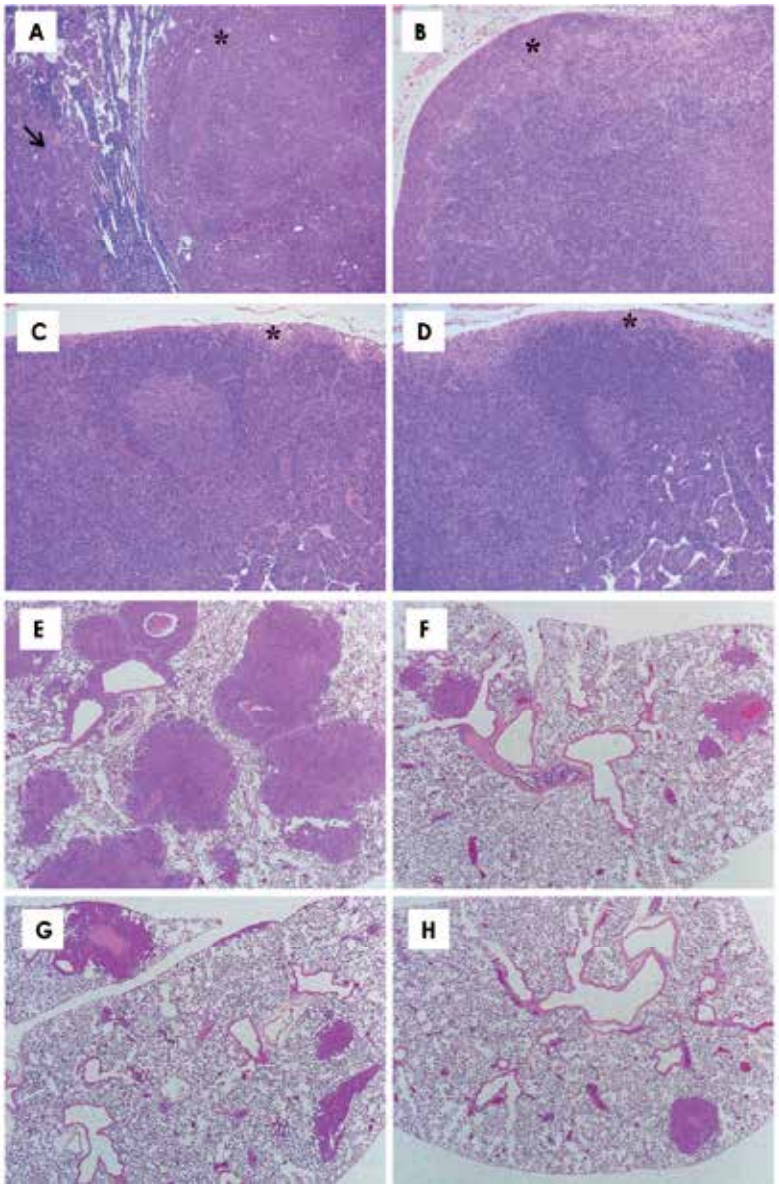


Fig. 2. Metastasis to a lymph node (**A-D**). (**A**) Metastatic carcinoma cells fill the sinusoidal space (arrow) in a control mouse. (**B**) A lymph node from a tumor injected with psiVEGF-C. Metastatic carcinoma cells filled the subcapsular sinus (asterisk). (**C**) and (**D**) No metastatic cells are observed in the subcaspsular sinus of a lymph node from a mouse in the pIL12 group (**C**), or from an animal in the combination psiVEGF-C+pIL12 group (**D**), but histiocytes are acummulating here in each case (asterisks). (**E**) Metastatic foci in the lung of a control (pVec) mouse. Many metastatic foci and small to large nodules were seen. (**F-H**) Metastatic foci tended to be smaller in mice receiving psiVEGF-C (**F**), pIL12 (**G**), and the combination vector (**H**) than those observed in the pVec group (**E**). H&E staining. Magnification: **A-D**, x100; **E-H**, x40.

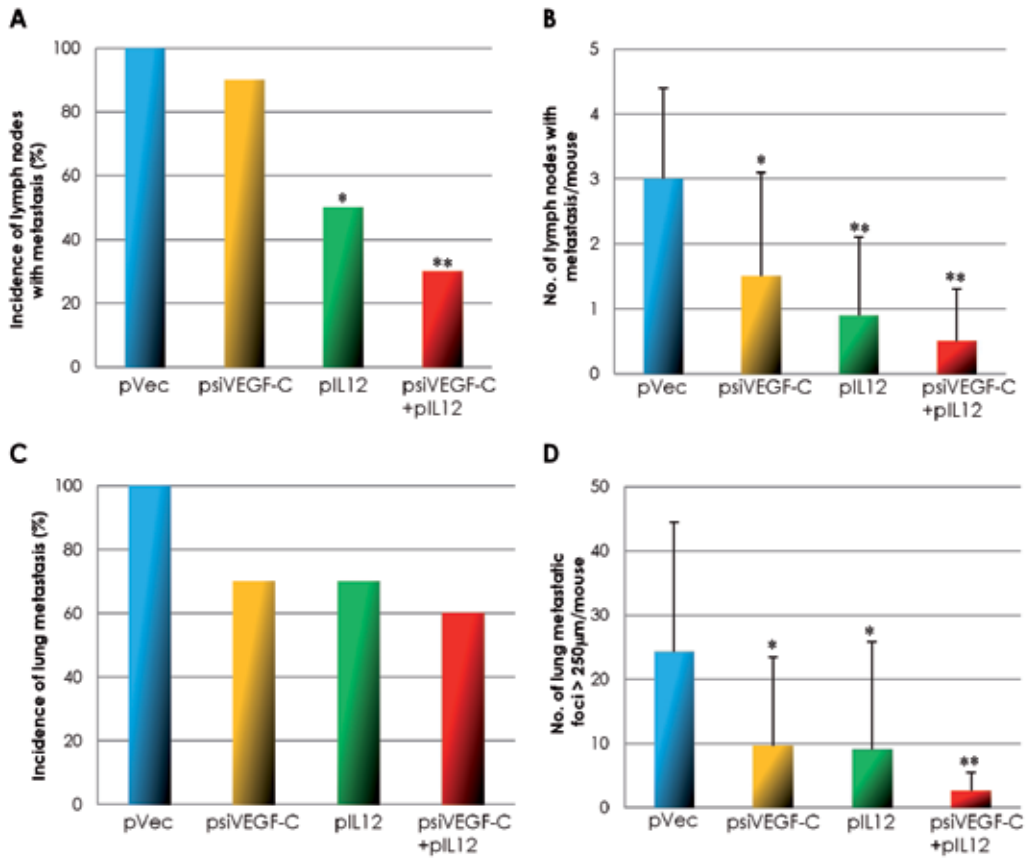


Fig. 3. Quantitative analysis of lymph node metastasis (**A** and **B**) and lung metastasis (**C** and **D**) in mice treated with pVec (control), psiVEGF-C alone, pIL12 alone, or combined psiVEGF-C+pIL12. (**A**) The incidence of lymph node metastasis was 100% in the pVec group, while the incidence was 90% in the psiVEGF-C group, 50% in the pIL12 group and 30% in the psiVEGF-C+pIL12 group; these incidences were significantly lower with pIL12 alone and pIL12 combine with psiVEGF-C. (**B**) Similarly, the number of lymph nodes with metastases per mouse was also significantly decreased in all groups receiving therapeutic treatment. (**C**) The incidence of lung metastasis tended to decrease in all therapeutic groups, but the decrease was not statistically significant. (**D**) However, the number of lung metastatic nodules >250 µm was significantly lower in all groups receiving therapeutic treatment. Data represent mean ± SD. *P<0.05; **P<0.01

3.3 Angiogenesis as measured by microvessel density

The immunohistochemical appearance of microvessels immunopositive for CD31, which is specific for the endothelium of blood vessels, is represented in Figures 4A and B. Tumor angiogenesis, as determined by the number of stained microvessels within the tumors themselves, was significantly lower in all therapeutic groups when compared to the pVec control group (Figure 5A).

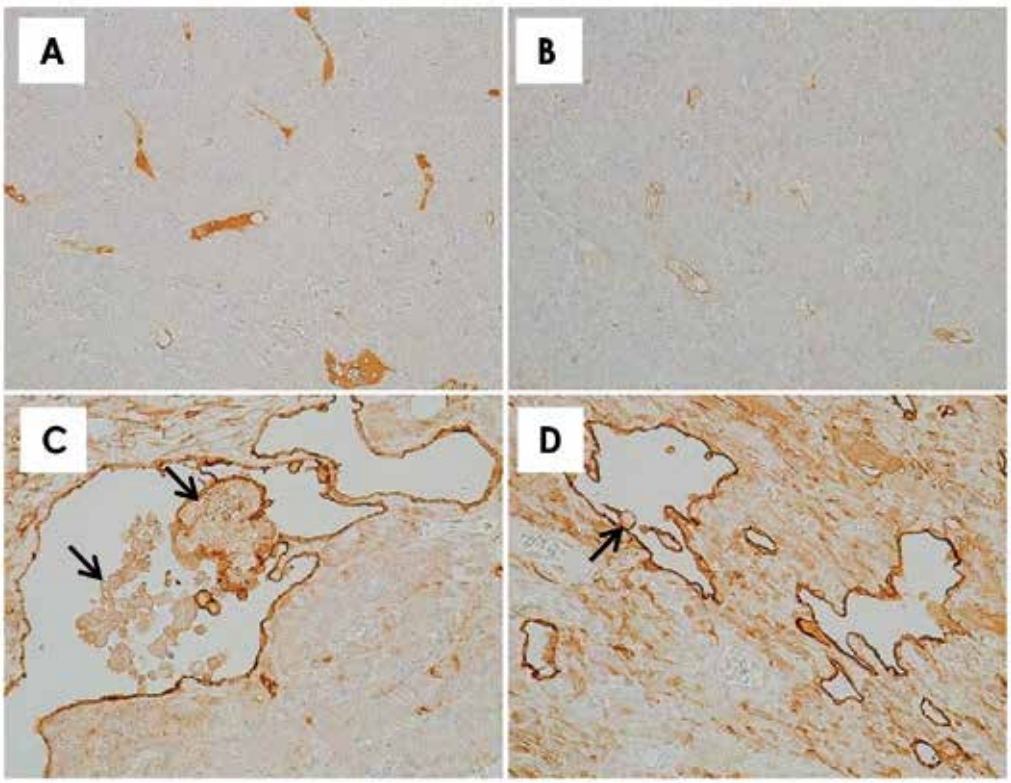


Fig. 4. Immunohistochemical analysis of angiogenesis (**A** and **B**) and lymphangiogenesis (**C** and **D**) in mammary tumors transfected with pVec (control), psiVEGF-C alone, pIL12 alone, or combined psiVEGF-C+pIL12. (**A**) A section representative of control tumors show a higher density of well-developed CD31-positive microvessels, whereas in tumors transfected with psiVEGF-C alone, pIL12 alone, or combined psiVEGF-C+pIL12 (**B**), few immunopositive vessels are seen. (**C**) Lymphatic vessels were often dilated and frequently contained migrating tumor cells within the lumina (arrows, pVec-transfected tumor). (**D**) The numbers of lymphatic vessels containing intralumenal tumor cells were lower in tumors transfected with psiVEGF-C alone, pIL12 alone, or combined psiVEGF-C+pIL12 (arrow). **A** and **B**, anti-CD31 immunohistochemistry; **C** and **D**, anti-podoplanin immunohistochemistry. Magnification: **A-D**, x400.

3.4 Dilated lymphatic vessels

The relative decrease in the number of dilated lymphatic vessels containing intraluminal tumor cells indicates migratory inhibition of cancer cells via the lymphatics of the tumor. Anti-podoplanin staining of the lymphatic microvessels in mammary tumors is demonstrated in Figures 4C and D. In all groups, these lymphatic microvessels were well developed in the outer, superficial layers of the mammary tumors in a somewhat hexagonal network pattern. We frequently observed tumor cells within the lumina of dilated lymphatic vessels in tumors of both control (Figure 4C) and treated animals (Figure 4D). However, as shown in Figure 5B, the number of lymphatic vessels carrying detached cancer cells was lower in both the pIL12 and psiVEGF-C + pIL12 groups, but this difference was statistically

significant only in the mice receiving pIL12 alone. The data from mice treated with psiVEGF-C alone showed large variations.

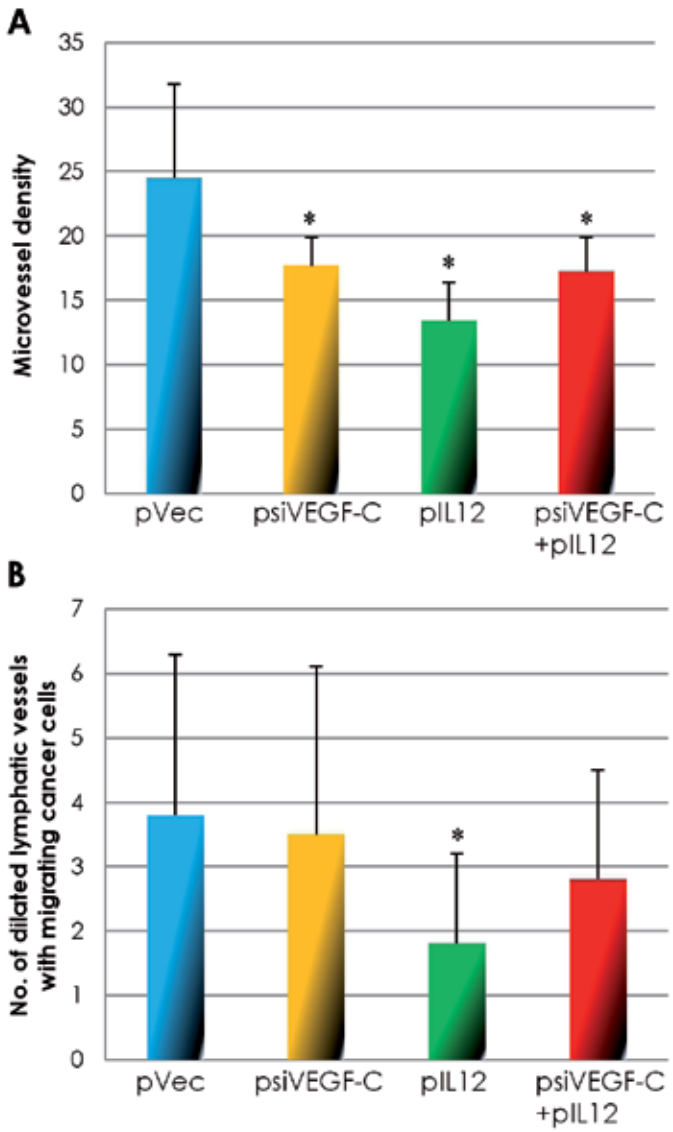


Fig. 5. Microvessel density (**A**) and frequency of lymphatic vessels containing migrating cancer cells (**B**) from tumors transfected with pVec (control), psiVEGF-C alone, pIL12 alone or combined psiVEGF-C+pIL12. (**A**) Microvessel density was significantly lower in tumors of mice receiving therapeutic vectors compared to the pVec control. (**B**) The number of lymphatic vessels containing intraluminal cancer cells was lower in the tumors transfected with pIL12 alone and combined psiVEGF-C+pIL12 as compared with the pVec controls, but significant differences were observed in the pIL12 alone group only. Data represent mean ± SD. *P<0.05.

4. Discussion

Since metastasis seems to be the biggest prognostic factor for lethality in most cancers, finding therapies that control or totally inhibit tumor spread is of paramount importance. A variety of mechanisms may contribute to the dissemination of primary cancer cells: local tissue invasion, systemic metastasis via tumor blood vessels to distant organs, and lymphatic metastasis via tumor lymphatic vessels to the sentinel lymph node, distal lymph nodes, and from there to distal organs. In general, the most common pathway of initial dissemination is via the lymphatics, with patterns of spread via afferent ducts [30]. The lymphatic capillaries present in tissues and tumors provide entrance into the lymphatic system, allowing cancer cell migration to the lymph nodes. In this study, lymph node metastasis was significantly decreased by exposure to vectors expressing siVEGF-C, IL-12, or a combination vector expressing both. We also observed a significant decrease in the number of lymphatic vessels containing tumor cells intraluminally in tissues from mice receiving pIL12 alone and the combination of psiVEGF-C/pIL12, suggesting an inhibitory effect on migration into tumor lymphatic vessels that supports the significant reduction in lymph node metastasis in these groups.

VEGF-C expression has been shown to correlate with lymph node metastasis in a variety of human cancers, including breast [6,31]. In many animal models of cancer, VEGF-C has been shown to enhance tumor lymphangiogenesis, the metastatic spread of tumor cells to lymph nodes and, in some cases, to distant organs [32]. Downregulation of VEGF-C using siRNA has been shown to reduce lymph node and lung metastases in murine mammary cancer models [13,22]. In 2009, an endogenous soluble isoform of VEGFR-2 (sVEGFR-2) that sequesters VEGF-C was identified and shown to be the first endogenous specific inhibitor of lymphatic vessel growth [33]. Endogenous sVEGFR-2 is a truncated form of 230 kDa membrance-bound form of VEGFR-2 resulting from alternative splicing. Subsequently, it has been shown that endogenous sVEGFR-2 suppresses tumor growth and lymph node metastasis in a mouse mammary cancer model [34]. This molecule significantly inhibits lymphangiogenesis, but not angiogenesis, in mammary tumor tissues [34]. In addition, VEGFR-3, the VEGF-C receptor, is predominantly expressed on lymphatic endothelial cells [35], and VEGF-C-dependent activation of VEGFR-3 stimulates the growth of lymph endothelial cells and lymphatics [36]. Blockade of VEGFR-3 signaling by soluble VEGFR-3 inhibits lymphangiogenesis and lymph node metastasis in experimental animal cancer models [11,37,38].

Cancer cells metastasize to distal sites via the vascular system as well as via the lymphatic system. Significant decreases in microvessel density were observed in the tumors we injected with psiVEGF-C, pIL12, and the combination vector. VEGF-C has been reported to stimulate angiogenesis under certain experimental conditions [39]. The biosynthesis of VEGF-C involves proteolytic processing that gives intermediate forms along with a 21kDa mature form [36]. The intermediate forms predominantly bind to VEGFR-3, whereas the mature form can bind to both VEGFR-3 and VEGFR-2 to induce angiogenesis [36], which explains the inhibition of angiogenesis observed with exposure to psiVEGF-C [36,40]and which is in agreement with our previous VEGF-C siRNA experiment [13].

In contrast, IL-12 has also been shown to strongly inhibit angiogenesis in mouse corneal neovascularization [20] and in several tumor models [19,21]. IL-12 itself has no direct action on vascular endothelial cells; however, IL-12 induction of IFNγ can apparently suppress angiogenesis on Matrigel-cultured human umbilical vein endothelial cells [19]. But IFNγ

does not seem the only player in angiogenesis inhibition; the cytokine IP-10 (IFNγ-inducing protein-10) has also been reported to be a potent antiangiogenic factor *in vivo* [41]. The exact mechanism of angiogenic suppression induced by IL-12 is therefore another avenue to explore in tumor therapeutics.

And the means of administration may also affect the efficacy of IL-12 as an anti-angiogenic/anti-metastatic agent. In a phase I clinical trial, recombinant IL-12 stimulated significant immunological activity in cancer patients [42]. However, despite initial enthusiasm for recombinant IL-12 as a potential anti-tumor agent, severe systemic toxicities have repeatedly been reported in clinical trials, limiting its use [43,44]. In contrast to direct cytokine administration, *IL-12* gene therapy using an adenoviral vector in animal cancer models has been shown to be as effective as protein exposure, but avoids the systemic toxicity seen in human trials [45-47]. One of the major advantages of gene transfer compared with the administration of recombinant proteins is the quicker achievement of steady-state levels of circulating protein [48]; administration of recombinant proteins leads first to a concentration peak, which may be within the zone of toxicity and responsible for adverse effects, followed by a rapid fall to sub-therapeutic levels.

The administration of either psiVEGF-C, pIL12, or a combination of both psiVEGF-C + pIL12 vectors significantly suppressed tumor growth and metastasis in our immunocompetent metastatic mammary cancer model. Since Carter *et al.* have reported the chance of tumor recurrence and/or metastasis increases dramatically once breast cancers reach 4 cm or larger [49], this reduction in tumor volume induced by decreasing VEGF-C and increasing IL-12 expression could be clinically significant; the fact that the treatment with a combined psiVEGF-C and pIL12 vector showed an enhanced inhibitory effect not only on tumor growth but also on metastasis is of particular importance when considering therapeutic strategies in breast cancer treatment. In conclusion, treatment with psiVEGF-C and pIL12 exerted combinational effects for suppression of tumor growth and metastasis in mouse mammary cancer model, suggesting a potentially significant clinical option in the treatment of metastatic human breast cancer.

5. Abbreviations

BrdU, 5-bromo-2'-deoxyuridine; *EF-1α/HTLV-1*, elongation factor –1α/human T cell leukemia virus type 1; *H&E*, hematoxylin and eosin; *IFNγ*, interferon-γ; *IL-12*, interleukin-12; *IP-10*, IFNγ-inducing protein-10; *MMTV*, mouse mammary tumor virus; *shRNA*, short hairpin RNAs; *siRNA*, short interfering RNA; *VEGF-C*, vascular endothelial growth factor-C; *VEGFR*, vascular endothelial growth factor receptor

6. Acknowledgements

This investigation was supported by a Grant-in-Aid for Scientific Research (C)(2) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (No. 17591360 to M.A. Shibata).

7. References

[1] Guarneri, V., Conte, P.F. (2004), The curability of breast cancer and the treatment of advanced disease. *Eur J Nucl Med Mol Imaging* 31 Suppl 1, S149-61.

[2] Agarwal, G., Pradeep, P.V., Aggarwal, V., Yip, C.H., Cheung, P.S. (2007), Spectrum of breast cancer in Asian women. *World J Surg* 31, 1031-40.
[3] Brinton, L.A., Sherman, M.E., Carreon, J.D., Anderson, W.F. (2008), Recent trends in breast cancer among younger women in the United States. *J Natl Cancer Inst* 100, 1643-8.
[4] Bouchardy, C., Fioretta, G., Verkooijen, H.M., Vlastos, G., Schaefer, P., Delaloye, J.F., *et al.* (2007), Recent increase of breast cancer incidence among women under the age of forty. *Br J Cancer* 96, 1743-6.
[5] Nguyen, D.X., Massague, J. (2007), Genetic determinants of cancer metastasis. *Nat Rev Genet* 8, 341-52.
[6] Salven, P., Lymboussaki, A., Heikkila, P., Jaaskela-Saari, H., Enholm, B., Aase, K., *et al.* (1998), Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human tumors. *Am J Pathol* 153, 103-8.
[7] Mylona, E., Alexandrou, P., Mpakali, A., Giannopoulou, I., Liapis, G., Markaki, S., *et al.* (2007), Clinicopathological and prognostic significance of vascular endothelial growth factors (VEGF)-C and -D and VEGF receptor 3 in invasive breast carcinoma. *Eur J Surg Oncol* 33, 294-300.
[8] Nakamura, Y., Yasuoka, H., Tsujimoto, M., Imabun, S., Nakahara, M., Nakao, K., *et al.* (2005), Lymph vessel density correlates with nodal status, VEGF-C expression, and prognosis in breast cancer. *Breast Cancer Res Treat* 91, 125-32.
[9] Skobe, M., Hawighorst, T., Jackson, D.G., Prevo, R., Janes, L., Velasco, P., *et al.* (2001), Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 7, 192-8.
[10] Karpanen, T., Egeblad, M., Karkkainen, M.J., Kubo, H., Yla-Herttuala, S., Jaattela, M., *et al.* (2001), Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* 61, 1786-90.
[11] He, Y., Kozaki, K., Karpanen, T., Koshikawa, K., Yla-Herttuala, S., Takahashi, T., *et al.* (2002), Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. *J Natl Cancer Inst* 94, 819-25.
[12] Mandriota, S.J., Jussila, L., Jeltsch, M., Compagni, A., Baetens, D., Prevo, R., *et al.* (2001), Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J* 20, 672-82.
[13] Shibata, M.A., Morimoto, J., Shibata, E., Otsuki, Y. (2008), Combination therapy with short interfering RNA vectors against VEGF-C and VEGF-A suppresses lymph node and lung metastasis in a mouse immunocompetent mammary cancer model. *Cancer Gene Ther* 15, 776-786.
[14] Gubler, U., Chua, A.O., Schoenhaut, D.S., Dwyer, C.M., McComas, W., Motyka, R., *et al.* (1991), Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc Natl Acad Sci U S A* 88, 4143-7.
[15] Colombo, M.P., Trinchieri, G. (2002), Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* 13, 155-168.
[16] Coughlin, C.M., Wysocka, M., Trinchieri, G., Lee, W.M. (1997), The effect of interleukin 12 desensitization on the antitumor efficacy of recombinant interleukin 12. *Cancer Res* 57, 2460-7.
[17] Boggio, K., Nicoletti, G., Di Carlo, E., Cavallo, F., Landuzzi, L., Melani, C., *et al.* (1998), Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice. *J Exp Med* 188, 589-96.

[18] Roy, E.J., Gawlick, U., Orr, B.A., Rund, L.A., Webb, A.G., Kranz, D.M. (2000), IL-12 treatment of endogenously arising murine brain tumors. *J Immunol* 165, 7293-9.

[19] Shibata, M.A., Ito, Y., Morimoto, J., Kusakabe, K., Yoshinaka, R., Otsuki, Y. (2006), In vivo electrogene transfer of interleukin-12 inhibits tumor growth and lymph node and lung metastases in mouse mammary carcinomas. *J Gene Med* 8, 335-352.

[20] Voest, E.E., Kenyon, B.M., O'Reilly, M.S., Truitt, G., D'Amato, R.J., Folkman, J. (1995), Inhibition of angiogenesis in vivo by interleukin 12. *J Natl Cancer Inst* 87, 581-6.

[21] Morini, M., Albini, A., Lorusso, G., Moelling, K., Lu, B., Cilli, M., *et al.* (2004), Prevention of angiogenesis by naked DNA IL-12 gene transfer: angioprevention by immunogene therapy. *Gene Ther* 11, 284-91.

[22] Chen, Z., Varney, M.L., Backora, M.W., Cowan, K., Solheim, J.C., Talmadge, J.E., *et al.* (2005), Down-regulation of vascular endothelial cell growth factor-C expression using small interfering RNA vectors in mammary tumors inhibits tumor lymphangiogenesis and spontaneous metastasis and enhances survival. *Cancer Res* 65, 9004-11.

[23] Morimoto, J., Imai, S., Haga, S., Iwai, Y., Iwai, M., Hiroishi, S., *et al.* (1991), New murine mammary tumor cell lines. *In vitro Cell Dev Biol* 27A, 349-351.

[24] Shibata, M.A., Morimoto, J., Otsuki, Y. (2002), Suppression of murine mammary carcinoma growth and metastasis by HSV*tk*/GCV gene therapy using *in vivo* electroporation. *Cancer Gene Ther* 9, 16-27.

[25] Shibata, M.A., Ito, Y., Morimoto, J., Otsuki, Y. (2004), Lovastatin inhibits tumor growth and lung metastasis in mouse mammary carcinoma model: a p53-independent mitochondrial-mediated apoptotic mechanism. *Carcinogenesis* 25, 1887-1898.

[26] Czauderna, F., Santel, A., Hinz, M., Fechtner, M., Durieux, B., Fisch, G., *et al.* (2003), Inducible shRNA expression for application in a prostate cancer mouse model. *Nucleic Acids Res* 31, e127.

[27] Shibata, M.A., Morimoto, J., Doi, H., Morishima, S., Naka, M., Otsuki, Y. (2007), Electrogene therapy using endostatin, with or without suicide gene therapy, suppresses murine mammary tumor growth and metastasis. *Cancer Gene Ther* 14, 268-278.

[28] Shibata, M.A., Liu, M.-L., Knudson, M.C., Shibata, E., Yoshidome, K., Bandy, T., *et al.* (1999), Haploid loss of *bax* leads to accelerated mammary tumor development in C3(1)/SV40-TAg transgenic mice: reduction in protective apoptotic response at the preneoplastic stage. *EMBO J* 18, 2692-2701.

[29] Gorrin-Rivas, M.J., Arii, S., Furutani, M., Mizumoto, M., Mori, A., Hanaki, K., *et al.* (2000), Mouse macrophage metalloelastase gene transfer into a murine melanoma suppresses primary tumor growth by halting angiogenesis. *Clin Cancer Res* 6, 1647-1654.

[30] Sleeman, J.P. (2000), The lymph node as a bridgehead in the metastatic dissemination of tumors. *Recent Results Cancer Res* 157, 55-81.

[31] Valtola, R., Salven, P., Heikkila, P., Taipale, J., Joensuu, H., Rehn, M., *et al.* (1999), VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathol* 154, 1381-90.

[32] Achen, M.G., Mann, G.B., Stacker, S.A. (2006), Targeting lymphangiogenesis to prevent tumour metastasis. *Br J Cancer* 94, 1355-60.

[33] Albuquerque, R.J., Hayashi, T., Cho, W.G., Kleinman, M.E., Dridi, S., Takeda, A., *et al.* (2009), Alternatively spliced vascular endothelial growth factor receptor-2 is an essential endogenous inhibitor of lymphatic vessel growth. *Nat Med* 15, 1023-30.

[34] Shibata, M.A., Ambati, J., Shibata, E., Albuquerque, R.J., Morimoto, J., Ito, Y., *et al.* (2010), The endogenous soluble VEGF receptor-2 isoform suppresses lymph node metastasis in a mouse immunocompetent mammary cancer model. *BMC Med* 8, 69.

[35] Kaipainen, A., Korhonen, J., Mustonen, T., van Hinsbergh, V.W., Fang, G.H., Dumont, D., *et al.* (1995), Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci U S A* 92, 3566-70.

[36] Joukov, V., Pajusola, K., Kaipainen, A., Chilov, D., Lahtinen, I., Kukk, E., *et al.* (1996), A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 15, 290-98.

[37] Shimizu, K., Kubo, H., Yamaguchi, K., Kawashima, K., Ueda, Y., Matsuo, K., *et al.* (2004), Suppression of VEGFR-3 signaling inhibits lymph node metastasis in gastric cancer. *Cancer Sci* 95, 328-33.

[38] Lin, J., Lalani, A.S., Harding, T.C., Gonzalez, M., Wu, W.W., Luan, B., *et al.* (2005), Inhibition of lymphogenous metastasis using adeno-associated virus-mediated gene transfer of a soluble VEGFR-3 decoy receptor. *Cancer Res* 65, 6901-9.

[39] Cao, Y., Linden, P., Farnebo, J., Cao, R., Eriksson, A., Kumar, V., *et al.* (1998), Vascular endothelial growth factor C induces angiogenesis in vivo. *Proc Natl Acad Sci U S A* 95, 14389-94.

[40] McColl, B.K., Stacker, S.A., Achen, M.G. (2004), Molecular regulation of the VEGF family -- inducers of angiogenesis and lymphangiogenesis. *APMIS* 112, 463-80.

[41] Angiolillo, A.L., Sgadari, C., Taub, D.D., Liao, F., Farber, J.M., Maheshwari, S., *et al.* (1995), Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo. *J Exp Med* 182, 155-62.

[42] Robertson, M.J., Cameron, C., Atkins, M.B., Gordon, M.S., Lotze, M.T., Sherman, M.L., *et al.* (1999), Immunological effects of interleukin 12 administered by bolus intravenous injection to patients with cancer. *Clin Cancer Res* 5, 9-16.

[43] Leonard, J.P., Sherman, M.L., Fisher, G.L., Buchanan, L.J., Larsen, G., Atkins, M.B., *et al.* (1997), Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood* 90, 2541-8.

[44] Cohen, J. (1995), IL-12 deaths: explanation and a puzzle. *Science* 270, 908.

[45] Mazzolini, G., Narvaiza, I., Martinez-Cruz, L.A., Arina, A., Barajas, M., Galofre, J.C., *et al.* (2003), Pancreatic cancer escape variants that evade immunogene therapy through loss of sensitivity to IFNgamma-induced apoptosis. *Gene Ther* 10, 1067-78.

[46] Nakamori, M., Iwahashi, M., Nakamura, M., Ueda, K., Zhang, X., Yamaue, H. (2003), Intensification of antitumor effect by T helper 1-dominant adoptive immunogene therapy for advanced orthotopic colon cancer. *Clin Cancer Res* 9, 2357-65.

[47] Yamazaki, M., Straus, F.H., Messina, M., Robinson, B.G., Takeda, T., Hashizume, K., *et al.* (2004), Adenovirus-mediated tumor-specific combined gene therapy using Herpes simplex virus thymidine/ganciclovir system and murine interleukin-12 induces effective antitumor activity against medullary thyroid carcinoma. *Cancer Gene Ther* 11, 8-15.

[48] Bloquel, C., Fabre, E., Bureau, M., Scherman, D. (2004), Plasmid DNA electrotransfer for intracellular and secreted proteins expression: new methodological developments and applications. *J Gene Med* 6, S11-S23.

[49] Carter, C.L., Allen, C., Henson, D.E. (1989), Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases. *Cancer* 63, 181-187.

Part 5

Drug Resistance

24

Roles and Mechanisms of Estrogen and Estrogen Receptors in Breast Cancer Resistant to Chemotherapy

Weimin Fan[1,2] and Meihua Sui[1,3]
[1]*Program of Innovative Cancer Therapeutics, First Affiliated Hospital of Zhejiang University College of Medicine, Hangzhou*
[2]*Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston*
[3]*Center for Bionanoengineering, Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou*
[1,3]*China*
[2]*USA*

1. Introduction

Breast cancer is the most common malignancy of women in many countries including United States and many European countries. Chemotherapy plays a major role in the treatment of advanced breast cancer, either as an adjuvant to primary therapy or as palliation therapy to improve symptoms and prolong survival. The previous 50 years have seen numerous advances in the properties of chemotherapeutic agents. However, a significant proportion of cancers are inherently unaffected by the administration of anticancer drugs. Furthermore, another considerable proportion of patients undergoing chemotherapy display an initial reduction in tumor size and then relapse with a marked insensitivity to a variety of drugs. Both phenomena are brought about by a resistant phenotype, which presents perhaps the single greatest barrier to successful chemotherapy.
Biological mechanisms contributing to drug resistance may be present *de novo* or arise after exposure to anticancer drugs. At present, drug resistance is considered as a multifactorial phenomenon involving several major mechanisms (1, 2). In general, two main groups of factors contribute to the development of drug resistance. The first group includes pharmacological and physiological factors such as drug metabolism and excretion, inadequate access of the drug to the tumor, inadequate infusion rate and inadequate route of delivery. The second group includes cell- or tissue-specific factors. For example, increased repair of DNA damage, reduced apoptotic cell death, altered metabolism of drugs, increased energy-dependent efflux (*e.g.* ATP-binding cassette transporters) of chemotherapeutic drugs and microRNAs are known factors correlated with the development of anticancer drug resistance (1). In recent years, both clinical observations and experimental studies suggested that steroid hormones and their receptors might also affect the therapeutic efficacy of antineoplastic drugs (3-8).

Traditionally, steroid hormones can be grouped into five groups by the receptors to which they bind: glucocorticoids, mineralocorticoids, androgens, estrogens and progestagens (9-11). Previous studies from our laboratory showed that glucocorticoids, such as dexamethasone, could significantly interfere with the antitumor activities of paclitaxel *in vitro* and *in vivo* (3, 4, 12). Further studies suggest that paclitaxel may induce apoptotic cell death through activation of the NF-κB/IκB signaling pathway, whereas glucocorticoids inhibit paclitaxel-induced apoptosis through induction of IκBα synthesis, which antagonizes paclitaxel-mediated activation of NF-κB and subsequently results in inhibition of paclitaxel-induced apoptosis (4, 13, 14). Considering that cancer patients are routinely pretreated with glucocorticoids (such as dexamethasone) before receiving taxanes (*e.g.* paclitaxel, docetaxel) to prevent taxane-related hypersensitivity reactions or other adverse effects, the finding of glucocorticoid-mediated inhibition of paclitaxel-induced apoptosis raises a clinically relevant question as to whether pretreatment of glucocorticoids might actually interfere with the therapeutic efficacy of paclitaxel. We have recently reviewed the influence and impact of glucocorticoids on drug-induced apoptosis (4). The cuurent article is largely focused on the role of estrogen and estrogen receptors on the resistance to chemotherapy and the potential strategies to reverse the resistance or sensitize ER+ breast tumors to chemotherapy.

2. Estrogen and estrogen receptors in the development and treatment of breast cancer

Estrogens, such as 17-β estradiol (E2) in human, are steroidal sex hormones that are synthesized from cholesterol and primarily secreted by the ovaries. They play a major role in the development and maintenance of the reproductive tract as well as in the development of the mammary glands. Estrogens also maintain bone density and reduce cardiovascular system by regulating cholesterol levels and influence some brain structures (15, 16). However, besides their physiological functions, estrogens are also involved in the development and progression of breast and the uterus cancers and can maintain tumor cell proliferation (15, 16).

Estrogen action is primarily mediated by two types of estrogen receptors (ERs), *i.e.* ERα and ERβ. ERs are members of the superfamily of nuclear receptors (17, 18). ERs in the cell nucleus mediate the effects of the ligand E2 by functioning as transcriptional regulators that access various target gene promoters either by directly binding to specific estrogen response elements (EREs) within the promoter or indirectly by interacting with other transcriptional regulators bound to the promoter. Further, several cases of ligand-independent activation of ERα mediated by its phosphorylation by various signaling pathways have been reported (19). In addition, ERα localized in the extra-nuclear compartment (such as the plasma membrane or cytoplasm ER) of target cells, can also mediate several nongenomic effects of estrogen. These non-genomic actions are associated with the activation of a kinase cascade, such as growth factor receptor kinases (*e.g.* epidermal growth factor receptor). By these means, E2 and ERα facilitate pathways involved in the promotion of cell proliferation, inhibition of apoptosis, stimulation of metastasis, and angiogenesis. Although there is growing evidence that the ERβ may inhibit the action of ERα by heterodimerizing with it, the overall role of ERβ in breast cancer remains to be better clarified. A number of reviews have recently been published on the biological roles of estrogens and molecular activities of ERs (15-20). Unless otherwise specified, ER refers to ERα within this review.

Cumulative analysis of tumor biopsies has shown that ERs present in ~65% of human breast tumors (21, 22). This is consistent with the crucial role of the ERα subtype in breast cancer etiology and progression, and with the role played by estrogens as tumor promoters. It has long been known that breast tumors that express the ERα protein (ER+) behave in a fundamentally different fashion than ER-negative (ER-) tumors with regard to their response to hormonal therapies, given that outcomes are often favorable in ER-positive breast tumors treated by adjuvant endocrine therapy alone (23, 24). Neoadjuvant chemotherapy has a well-established role in the management of early-stage, operable breast cancer, and remains the gold standard downstaging systemic therapy in many centers, regardless of ER status. However, the data from other clinical trials or retrospective analyses suggest that ER status might also affect the efficacy of chemotherapy (5-8). Specifically, it has been observed that some chemotherapeutic agents may be less effective in patients with ER+ tumors than those with ER- tumors.

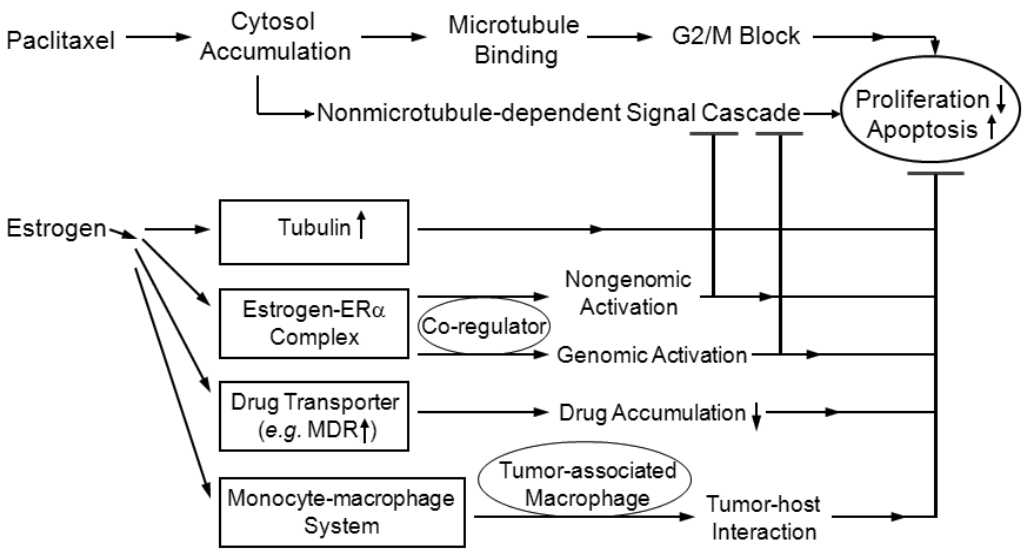


Fig. 1. Hypothesized pathways of estrogen/ER-mediated chemoresistance using paclitaxel as an example. ⊤ and ⋮ represent inhibitory or antagonistic action. MDR, multidrug resistance. There are possible cross-talks between indicated pathways.

3. Current understnding of ER-mediated chemoresistance

More than one decade ago, Lippman ME *et al* first determined the relation between ER and the response rate to cytotoxic chemotherapy in 70 breast cancer patients (6). They found that 34 of 45 patients with low or absent ER values (<10 fmol/mg of cytoplasmic protein) had objective responses to chemotherapy, whereas only 3 of 25 patients with higher ER values (>10 fmol/mg of cytoplasmic protein) responded (p<0.0001). There were no statistically significant differences between the two groups in age, menopausal status, disease-free interval, Karnofsky index or prior therapy. Moreover, differences in sites of involvement or type of combination chemotherapy did not account for the increased response rate in ER- patients. This is the first report suggesting that ER status might be an important predictor of

response to cytotoxic chemotherapy in breast tumors. Since then, evidence from clinical trials or retrospective analyses is accumulating that improvements in chemotherapy disproportionately benefit breast cancer patients with ER- tumors, in which multiple chemotherapeutic regimens have been tested in these studies (6, 25-33), such as taxanes-, anthracycline- and navelbine-containing regimens. More recently, in a retrospective clinical study conducted by us and our collaborators, we found that primary breast cancer patienhts with ER+ tumors achieved significant lower pathologic response than those with ER- breast tumors when treated with preoperative chemotherapeutic regimens including DEC (docetaxel+epirubicin+cyclophosphamide), VFC (vinorelbine/vincristine+5-fluorouracil+ cyclophosphamide) and EFC (epirubicin+5-fluorouracil+cyclophosphamide) (34).

The involvement of ER in chemoresistance has also been confirmed in a number of *in vitro* studies (5, 7, 8, 35-40). For example, ER- breast cancer tissue was found chemosensitive *in vitro* compared with ER+ tissue against six antitumor drugs including carboquone, adriamycin, mitomycin C, aclacinomycin A, cisplatin and 5-fluorouracil (5). When subjected ER+ human breast cancer MCF-7 and ZR-75–1 cells to paclitaxel or to UV irradiation, marked increases in cell apoptosis were induced. However, these responses were significantly reversed by incubation with E2, which was probably mediated through the plasma membrane estrogen receptor (40). Recently, we established several isogenic ER+ cell lines by stable transfection of ERα expression vectors into ER- breast cancer BCap37 cells to investigate the possible influence of ER on the therapeutic efficacy of paclitaxel and *vinca* alkaloids (7, 8). We found that 17-β estradiol significantly reduced the overall cytotoxicity of these antimicrotubule drugs in ERα-expressing BCap37 but had no influence on the ER- parental cells or ER- MDA-MB-468 cells. Further analyses indicate that expression of ERα in BCap37 cells mainly interferes with the apoptotic cell death but not mitotic arrest induced by these drugs. Moreover, we found that the addition of ICI 182,780 (fulvestrant), a selective ER down-regulator, could completely reverse the above resistance observed in ER+ BCap37 cells, and sensitize MCF-7 and T47D cell lines to the treatment of the above drugs (see Fig. 2). These findings further confirmed the correlation between ERα and drug resistance in ER+ tumor cells.

4. Possible mechanisms of ER-mediaed chemoresistance

Estrogens and ERs are well-known for their critical roles in the development and progression of breast tumors, through genomic or non-genomic pathways as described above. Plentiful data also indicate that estrogens and ER are involved in or interact with a number of apoptosis- or proliferation-related signal pathways existed in tumor cells. Therefore, it is believed that through interaction with and/or regulation on specific or various co-regulators or downstream molecules, estrogen/ER induce chemoresistance in tumor cells by promoting tumor growth and/or inhibiting the antitumor effect of chemotherapeutic drugs. Several mechanisms that may contribute to ER-mediated drug resistance are discussed below. It appears that the underlying mechanisms of ER-mediated chemoresistance are quite complicated and specifically related with the tumor models and chemotherapeutic drugs studied.

4.1 Role of apoptosis-related molecules in ER-mediated chemoresistance

Reduced apoptotic cell death or enhanced tumor cell proliferation are major factors involved in drug resistance. Whereas it is not completely understood how estrogen and ER regulate

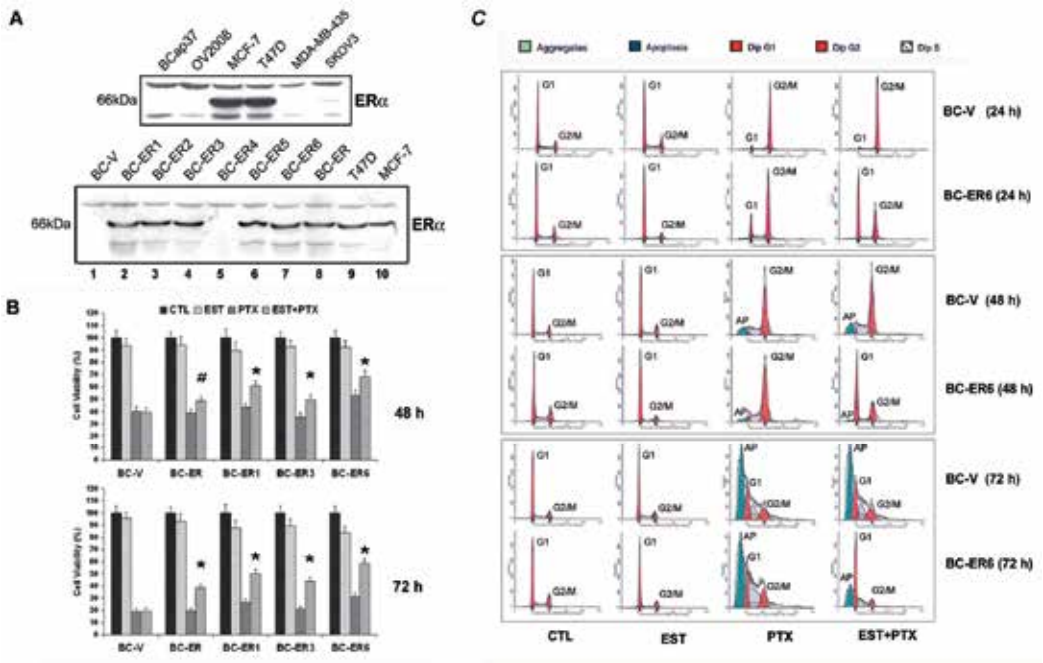


Fig. 2. ERα expression attenuates the anticancer activity of paclitaxel (7). A, protein extracts of BCap37 cells transfected with empty vector (BC-V) or ERα were analyzed by Western blot. T47D and MCF-7 cells were used as positive controls of ERα expression. *B*, cells were treated with 1 nmol/L 17-β estradiol, 50 nmol/L paclitaxel, or the combination treatment in which cells were preincubated with 17- β estradiol for 12 h before paclitaxel treatment. Cell viability was evaluated by MTT assays after both 48 and 72 h of paclitaxel treatment. BC-ER, pooled transfectants of BCap37 transfected with ERα, BC-ER1–7, single clones 1 to 7 of BCap37 transfected with ERα; CTL, control; EST, 17-β estradiol; PTX, paclitaxel. #, $P < 0.05$, when compared with the group treated with paclitaxel alone in the same cell line; *, $P < 0.001$, when compared with the group treated with paclitaxel alone in the same cell line. *C*, cells treated with 1 nmol/L 17-β estradiol, 50 nmol/L paclitaxel, or their combination for indicated time points were harvested, and DNA content stained with propidium iodide for flow cytometric analysis. Peaks corresponding to G1, G2-M, and S phases of the cell cycle and apoptotic cells (AP).

the growth of tumor cells, it is known that hormonal induction of growth factors/receptors such as transforming growth factor α, epidermal growth factor, Her-2 contributes to the proliferative actions of E2 (41-44). Recent studies indicate that several apoptosis-related moleculars or signal pathways, such as bcl-2 and p53, might be involved in E2/ER-mediated resistance to chemotherapy.

Expression of the bcl-2 protein prevents apoptotic cell death induced by a variety of stimuli including most chemotherapeutic agents (45-47). Teixeira C *et al* demonstrated that depletion of estrogen from the medium results in loss of expression of the bcl-2 in MCF-7 cells, whereas reexposure to estrogen markedly induces the bcl-2 expression (48). Moreover, estrogen depletion, the simultaneous treatment of ICI 164,384, or the transfection of bcl-2 antisense significantly sensitized MCF-7 cells to adriamycin, consistent with a decrease in

the bcl-2 levels. Their data suggest that estrogen can promote resistance of ER+ breast cancer cells to chemotherapeutic drugs through a mechanism that involves regulation of the bcl-2, which supports the recent report that bcl-2 expression usually occurs in ER+ breast tumors, whereas ER- breast cancer biopsies tend to lack this protein (48). Another study conducted by Razandi M *et al* showed that in ER+ human breast cancer cells, the apoptosis, activation of c-JNK, phosphorylation of Bcl-2 and Bcl-xl, activation of caspase induced by paclitaxel or UV radiation were significantly reversed by incubation with E2. E2 also independently activated extracellular signal-regulated protein kinase activity, which contributed to the antiapoptotic effects. In addition, our recent studies also demonstrated that E2 significantly inhibited paclitaxel or *vinca a*lkaloids-induced phosphorylations of bcl-2 and c-raf-1, as well as the degradation of IκBα in BCap37 cells transfected with ERα, which was accompanied with decreased sensitivity of BC-ER cells to the above anticancer drugs (7, 8).

In response to various extracellular and intracellular signals, p53 mediates cellular processes, such as apoptosis, cell cycle arrest, and senescence, depending on the signal and the cellular context (49-51). A body of accumulating evidence suggests the possibility of a cross-talk between pathways mediated by ERα and p53. Das GM *et al* demonstrated the direct binding of ERα to p53 both *in vitro* and *in vivo* to endogenous p53 target gene promoters, which subsequently resulted in inhibition of transcriptional activation by p53 (52). They further showed that ERα bound to p53 on endogenous *Survivin* and *MDR1* gene promoters, leading to inhibition of p53-mediated transcriptional repression of these genes. Further, alleviating p53-mediated transcriptional repression of *Survivin* contributes to the ability of ERα to inhibit apoptosis in human breast cancer cells. RNA interference-mediated knockdown of ER resulted in reduced survivin expression and enhanced the propensity of MCF-7 cells to undergo apoptosis in response to staurosporine treatment. These data indicate that countering p53-mediated transcriptional repression of *Survivin* is at least one of the important mechanisms underlying the antiapoptotic function of ERα (53).

4.2 Involvement of tumor growth rate in ER-mediated chemoresistance

Evidence has shown that ER- tumors have a higher growth rate as indicated by a higher labeling index and mitotic index (6). Since many agents used in chemotherapy for breast cancer have some degree of cell-cycle specificity, there might be a correlation between higher growth rate and chemotherapy response (6). Dougherty MK *et al* used three *in vitro* models (MCF-7, T47D and ZR-75) to examine and compare growth rates as well as paclitaxel-induced apoptosis in ER+ and ER- clones with the same originate (54). They found that in T47D and ZR-75 cell lines, loss of ER was associated with a decrease in doubling time and an increase in paclitaxel sensitivity. However, when cell culture conditions were altered to achieve equivalent cell proliferation rates, no difference in paclitaxel sensitivity was observed. Similarly, an ER- clone of MCF-7 cells that did not exhibit an enhanced growth rate compared to its ER+ counterpart also did not show increased paclitaxel sensitivity. In these *in vitro* models, the decreased sensitivity to paclitaxel appears to be correlated closely with the decreased growth rate observed in ER+ breast tumors (54).

4.3 ABC transportors and ER-mediated chemoresistance

The most widely studied phenomenon of drug resistance is multidrug resistance (MDR) that has been linked to overexpression of a membrane associated P-glycoprotein (1, 2), a member of ATP-binding cassette (ABC) transporter family that functions as an efflux pump for

various structurally unrelated anticancer agents, such as the *vinca* alkaloids, anthracyclines and taxanes (1, 2). Several studies have suggested that ABC transporters might be involved in E2/ER-induced drug resistance. For example, E2 increased the cytoplasmic concentration of P-gp in ER+ breast cancer cells that were resistant to doxorubicin treatment (55). In addition, ABCC11 (MRP8) expression is high in high-expressing ER breast cancers, supporting the notion that expression of ABCC11 in ER+ breast cancers may contribute to decreased sensitivity to chemotherapy combinations (56). Interestingly, Sugimoto Y *et al* recently reported that both estrogens (57) and antiestrogens (58) inhibit breast cancer resistance protein (BCRP)-mediated drug resistance. They also found that the physiological levels of E2 down-regulate both endogenous and exogenous BCRP expression in ER+ cells by post-transcriptional mechanisms (59). Moreover, they showed that estrogen decreases P-gp expression in MDR1-transduced, ER+ human breast cancer cells, and this E2-mediated P-gp down-regulation sensitizes tumor cells to vincristine. However, it is possible that the effects of estrogen on P-gp expression may differ in ER+ human breast cancer cells expressing endogenous and exogenous P-gp, which needs to be further assessed in appropriate models (60).

4.4 Enhanced β3-tubulin expression by E2/ER

It has been suggested that certain changes in cytoskeletons, such as tubulin mutations and isoforms, alterations in microtubule-bindings proteins (*e.g.* stathmin, tau), as well as enhanced β3-tubulin expression might be correlated with reduced response to antimicrotubule agent-based chemotherapy or worse outcome in a variety of tumor settings. In *in vitro* studies or in clinical investigations, enhanced expression of β3-tubulin has shown to play a crucial role in the development of chemoresistance to antimicrotubule agents in a variety of tumors such as lung, breast, prostate or orarian cancers (61-66), and has be considered as a predictive marker of paclitaxel resistance (25, 67-70). Nevertheless, the mechanism underlying β3-tubulin expression still remains unclear. In *Drosophila*, β3-tubulin expression is enhanced by an exposure to ecdysone, a steroid hormone, through a transcriptional mechanism (71). Recently, Saussede-Aim J *et al* found that exposure of ER+ MCF-7 cells to estradiol induced β3-tubulin expression in both mRNA and protein levels, while estradiol had no effect on the expression of β3-tubulin in ER- MDA-MB-231 cells (72). They further showed that co-administration of antiestrogens including tamoxifen or fulvestrant, completely abolished the increase of β3-tubulin mRNA levels due to estradiol in MCF-7 cells, implying that estradiol regulates β3-tubulin expression, and thereby induces resistance of ER+ breast tumors to antimicrotubule drugs through an ER-dependent pathway.

4.5 Tumor-host interaction in ER-mediated chemoresistance

Estrogen regulates differentiation, maturation and function of many cell types in monocyte–macrophage system directly or indirectly *via* other cells by autocrine/paracrine mechanisms (73). Estrogen effects on this system are primarily repressive, and mainly mediated by repression of expression of genes for cytokines or modulation of other inflammatory mediators by the ER-dependent or nongenomic pathways. The ER-dependent mechanisms mostly involve modulation of the NF-kappaB pathway for transcriptional regulation of cytokine or other mediator genes. In the context of hormone-regulated cancer, estrogen can influence production of cytokines or other inflammatory mediators by both tumor cells and tumor-invading macrophages (73). The interactions of breast cancer cells with tumor-

associated macrophages, regulation of the monocyte–macrophage system by estrogen and cross-talk between the ER and cytokine-mediated pathways, may play an important role in tumor progression as well as the development of resistance to anticancer treatment (73-75).

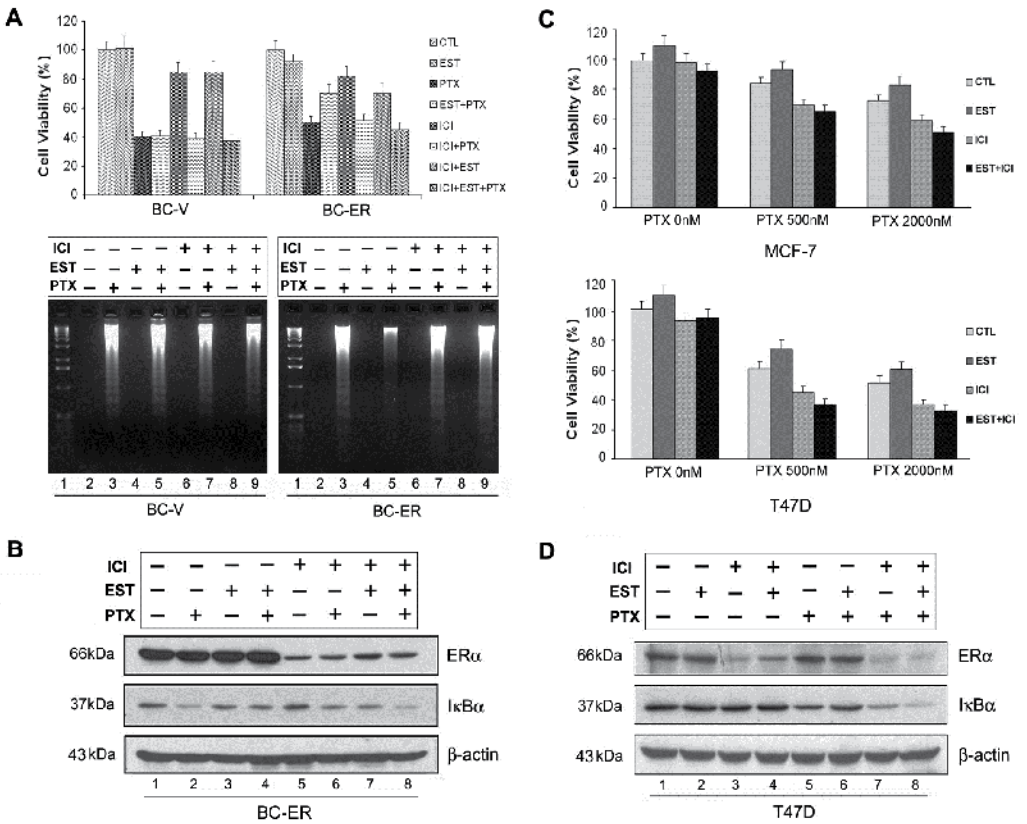


Fig. 3. ICI 182, 780 abrogates the resistance of ERα positive breast tumor cells to paclitaxel (7). BCap37 cells were treated with 1 nmol/L 17-β estradiol, 50 nmol/L paclitaxel, 100 nmol/L ICI 182, 780 or their various combinations. MCF-7 and T47D cells were treated with 100 nmol/L 17-β estradiol, 500 or 2000 nmol/L paclitaxel, 1 μmol/L ICI 182, 780 or their various combinations. *A*, determination of cell viability by MTT assays and apoptosis by DNA fragmentation assays after BC-V and BC-ER cells were exposed to paclitaxel for 48 h. *B*, effect of 17-β estradiol, paclitaxel, ICI 182, 780 and their combinations on the expression of ERα and IκBα in BC-ER cells. *C*, cell viability of MCF-7 and T47D cells after 72 h of paclitaxel treatment with MTT assays. *D*, effect of 17-β estradiol, paclitaxel, ICI 182, 780 and their combinations on the expression of ERα and IκBα in T47D cells. Proteins were extracted from cells after 24 h of paclitaxel treatment.

5. Stratagies to sensitize ER+ breast tumors to chemotherapy

Considering that ERs are expressed in ~65% of human breast cancer, the ER-mediated resistance to chemotherapy has become a big challenge for clinical treatment of breast tumors. Unfortunately, despite the fact that the involvement of ER in drug resistance to

chemotherapy has been observed for more than a decade, very few studies have investigated the potential strategies to reverse the ER-mediated chemoresistance or sensitize ER+ breast tumors to chemotherapy. Because the resistance of ER+ breast tumors to chemotherapy is mainly mediated by activation of estrogen/ER signal pathway, it is logical that agents targeting or inhibiting the ER signal pathway may have the potential to reverse the ER-mediated chemoresistance. Indeed, as described below, a number of studies have shown that antiestrogenic agents in combination with chemotherapeutic drugs are of significant therapeutic benefit in ER+ breast cancer over chemotherapy alone. Moreover, recent investigations indicate that the ER-derived peptide, MicroRNAs specifically targeting ER, as well as agents targeting estrogen-related receptors (ERRs) may hold great promise to sensitize ER+ breast tumors to chemotherapy.

5.1 Sensitization of ER+ breast tumors to chemotherapy by SERMs

Selective estrogen receptor modulators (or SERMs) bind ERs but have a mixed agonist/antagonist profile. Tamoxifen and raloxifene are well-known first and second generations of SERMs, respectively (76-78). New SERMs in clinical development include idoxifene, droloxifene, arzoxifene, acolbifene/EM-800, lasofoxifene, TAT-59, ERA-923, toremifene, GW5638/GW7604, *etc* (76-78). Kurebayashi J *et al* found that concurrent treatment of 5-FU and 4-hydroxytamoxifen (4OHT) additively inhibited the growth of ER+ ML-20 and KPL-1 breast cancer cells but not ER- MDA-MB-231 cells (79). They further demonstrated that 4OHT significantly decreased thymidilate synthase activity, which might increase the antitumor activity of 5-FU (79). However, conflicting observations on the interaction between tamoxifen and chemotherapeutic agents including 5-FU and doxorubicin in terms of antitumor activity have been reported by different laboratories (79-83). In addition, Wu L *et al* showed that arzoxifene and 4OHT can inhibit specifically the repopulation of ER+ MCF-7 and T47D breast cancer cells between courses of weekly treatment with 5-FU or methotrexate (84). Most recently, they further confirmed that combined treatment with arzoxifene given between cycles of 5-FU or paclitaxel can inhibit repopulation of MCF-7 breast cancer xenografts (85). They proposed that scheduling of short-acting antiestrogenic agents between courses of adjuvant chemotherapy for human breast cancer has potential to improve the outcome of treatment. Additionally, the increased etoposide cytotoxicity by tamoxifen as compared to cells treated with either drug alone was observed in brain tumor HTB-14 cells expressing ER, which was accompanied with enhanced inhibition of protein kinase C (PKC) and insulin-like growth factor II (IGF-II) (86).

5.2 Sensitization of ER+ breast tumors to chemotherapy by aromatase inhibitors

One strategy to inhibit the activation of estrogen/ER pathway is to block the conversion of estrogen precursors into estrogen by aromatase inhibitors (AIs) (87). Currently, third-generation aromatase inhibitors, such as the non-steroidal agents anastrozole, letrozole and the steroidal agent exemestane, have been introduced into the market as endocrine therapy in postmenopausal patients, either alone or as part of multiple hormonal therapies (88). In addition to the above AIs, cyclooxygenase (COX) inhibitors also decrease aromatase mRNA expression and enzymatic activity (89). A recent study by Chen D *et al* showed that the combination of paclitaxel with exemestane produced additive antitumor effect in cultured human breast cancer cell lines. Interestingly, this additive effect was independent of ERα expression, but dependent on the presence of androstenedione (90). The effects of AIs on

sensitivity of ER+ breast tumors to chemotherapy remains unclear and need to be further investigated.

5.3 Sensitization of ER+ breast tumors to chemotherapy by SERDs

The pure antiestrogens, also called selective estrogen receptor downregulators (or SERDs), including fulvestrant (ICI 182, 780), ZK-703, ZK-253, RU58668 and TAS-108, act by decreasing the level of ERs through their ubiquitinylation and subsequent targeting to the proteasome (87). Unlike tamoxifen, fulvestrant is a pure antagonist of estrogen-regulated gene expression that could down-regulat ER expression without any concomitant rise in other growth signal pathways, *e.g.*, EGFR or TGF-α (8, 87). Recently, our laboratory demonstrated that pretreatment with fulvestrant significantly prevented E2-induced resistance to paclitaxel and *vinca* alkaloids in human breast cancer BCap37 cells transfected with ER-expressing vector (BC-ER) while down-regulates the protein levels of ERα in BC-ER cells (7, 8). Similar sensitizing effect of fulvestrant was observed in MCF-7 and T47D breast cancer cells expressing endogenous ERα (7, 8). These results provided additional evidence for the correlation between ERα and the resistance of breast tumors to chemotherapeutic drugs such as paclitaxel and *vinca* alkaloids. More recently, through implanted ER- and ER+ BCap37 cells into athymic nude mice, we established isogenic ER- and ER+ xenograft breast tumor models. Subsequently, we demonstrated that co-treatment of fulvestrant could significantly sensitize ER+ breast tumors to paclitaxel (unpublished data). Because fulvestrant has been successfully used in the treatment of ER+ advanced breast tumors, our experimental results may also suggest the clinical strategy to combine fulvestrant with certain chemotherapeutic drugs for the treatment of ER+ breast tumors.

5.4 Other strategies potentially useful for sensitizing ER+ breast tumors to chemotherapy

In addition to the well-known antiestrogens including the SERMs, AIs and SEDMs, studies have been conducted to explore new agents that may interfere the biological responses medicated by E2/ER. One example is the synthesis of ER-derived peptide. Two ER-derived peptides specifically targeting estradiol/ER action, pY-peptide (Ac-Leu-pTyr-Asp-Leu-Leu-Leu-NH2) and Tat-peptide (Ac-EFVCLKSIILLNS-AAA-RKKRRQRRR-NH2) have shown activity to inhibit the growth of ER+ breast tumors *in vitro* and *in vivo* (91, 92). Moreover, accumulating evidence is revealing an important role of MicroRNAs in anticancer drug resistance (93). Adams *et al* reported that MicroRNA (miR)-206 could decrease endogenous ERα in MCF-7 cells *via* two specific target sites within the 3′-untranslated region of the human ER transcript (22, 94). They further found that miR-206 expression was markedly decreased in ER+ human breast cancer tissues, and that the introduction of miR-206 into estrogen-dependent MCF-7 cells led to the suppression of ERα expression and growth inhibition. These data suggest that miR-206 is a key factor for the regulation of ERα expression in breast cancer, which could be a novel candidate for targeting ER (22, 94).

Nuclear receptor estrogen-related receptor (ERR) family, comprising ERRα, ERRβ and ERRγ, are the closest relatives to ERα after ERβ (95) . The ERRs share several biochemical activities with ERs, bind and regulate transcription *via* estrogen response elements (EREs) and extended ERE half-sites termed ERR response elements (ERREs), but do not bind endogenous estrogens. The ERRs act in an analogous fashion as ERα, but the effect of ERRα binding to an ERE or ERRE can be either negative or positive. ERRα likely plays a role in

modulating estrogen responsiveness both by modulating levels of estrogens themselves and expression of estrogen-regulated genes in estrogen target-tissues such as breast cancer. The search for ligands of the ERRs is an active area of research. Targeting ERRs holds great promise and may open new opportunities for the management of breast cancers (95).
As described above, the mechanisms underlying ER-mediated chemoresistance involve ER-coregulatory proteins and cross-talk between plasma membrane-localized ER, nuclear-localized ER and other growth-factor signaling networks, such as EGFR, IGFR, VEGFR and HER2. As a consequence, targeting the ER-coregulators or "cross-talk" pathways may provide opportunities to overcome the ER-mediated chemoresistance, either alone or in combination with agents inhibiting E2/ER activation. However, the mechanisms of ER-mediated chemoresistance need to be further clarified so that effective strategies could be developed to sensitize ER+ breast tumors to chemotherapy.

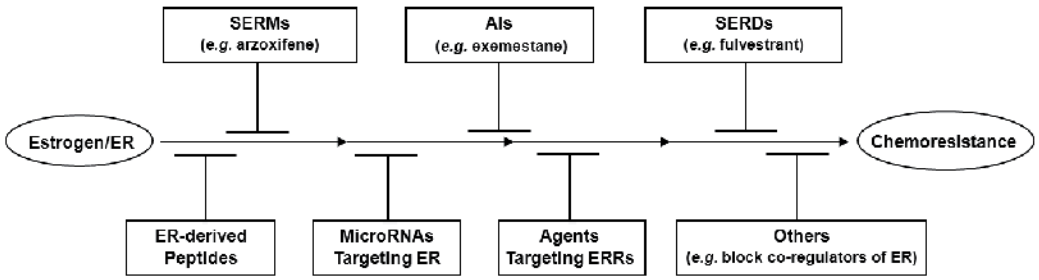


Fig. 4. Possible strategies to reverse ER-mediated chemoresistance or sensitize ER positive tumors to chemotherapy. SERMs, selective estrogen receptor modulators; AIs, aromatase inhibitors; SERDs, selective estrogen receptor down-regulators; ERRs, estrogen-related receptors.

6. Future perspectives

Cumulative data from *in vitro* experiments and clinical investigations have demonstrated the association between ERα expression and the resistance to chemotherapy in breast tumors. However, most of *in vitro* data were based on comparative studies between the tumor cell lines derived from different individuals. Although some paired cell lines were derived under the selective pressure of a low/no estrogen environments, these tumor cells are still not likely to be isogenic because many features, including their proliferative capacity, might have changed due to genetic alterations (54). Thus, it is difficult to elucidate the cellular and molecular mechanisms. The pairs of isogenic breast cell lines generated by stable transfection of ERα or empty vector in our laboratory have provided a valuable model system to investigate the mechanism underlying ERα-mediated breast tumor cell resistance to chemotherapeutic agents. Interestingly, we found that estrogen had marginal effect on microtubule dynamics in breast tumor cells expressing ER (BC-ER) treated with paclitaxel and *vinca* alkaloids, but may decrease the G2-M population through the increase of cells at the G1 phase. This phenomenon is similar to the finding previously reported by Zajchowski *et al.* (96, 97). However, the question still remains whether G1 arrest and decreased G2-M population by estrogen may affect the above drug-induced apoptosis. Further studies are required to elucidate this issue, and it is important to integrate data obtained from breast tumors expressing endogenous ER with those expressing exogenous ER.

Some previous reports about the association between ER status and response to chemotherapy can be confused by the use of chemo-endocrine therapy, where the ER+ population may have responded to the hormonal part of the treatment (98, 99). Moreover, there are heterogeneous in design, in determination of marker and response evaluation, which could be partly responsible for conflicting results about the predictive and prognostic value of these markers (98-103). Therefore, some cautions are required when interpret these results, considering that there are many factors need to be taken into account, such as differences in patient selection, whether the patients were previously untreated with chemotherapy or endocrine therapy, type of chemotherapy, size of the study, follow-up time, different evaluation methods, different cut-off value of ER or other related markers, interactions between combined chemotherapeutic drugs, *etc.*

Compared to the available *in vitro* and clinical reports, very few animal studies have been conducted to determine the role and underlying mechanisms of estrogen and ER in development of chemoresistance, as well as to explorer the potential strategies to reverse the ER-mediated drug resistance. However, appropriate animal models may provide us with easily controlled ways to further evaluate various signal pathways/molecules *in vivo*, to determine the differences between *in vitro* and *in vivo* models, to test chemotherapeutic drugs that we have interests, to investigate agents that may hold promise to sensitize ER+ breast tumors to chemotherapy, either alone or in desirable combinations/sequences. There are less variables need to be taken into account when interpret or analyze the data obtained in animal models compared to clinical patients. The hope to overcome the ER-mediated chemoresistance relies on further clarification of specific pathways or molecules contributing most significantly to the resistance. More exhaustive and systematic attempts to provide this information are essential to reach deeper understandings on ER-mediated chemoresistance in breast tumors. Moreover, it is known that breast cancer patients show a wide range of ER expression levels, and the levels of ER expression in individual patients change during disease progression and/or in response to systemic therapies. Thus, the treatment plan for breast cancer patients might need to be optimized based on the most up-to-date molecular characteristics and responses to therapy in individuals.

7. Acknowledgements

This work was supported in part by Grants NNSF-81071880, NNSF-30973456, ZJ-DST-2008C14079 and NIH CA92880 (to Fan W).

8. References

[1] Raguz, S.;Yagüe, E. Resistance to chemotherapy: new treatments and novel insights into an old problem. *Br. J. Cancer*, 2008, 99(3), 387-391.

[2] Luqmani, Y.A. Mechanisms of drug resistance in cancer chemotherapy. *Med. Princ. Pract.*, 2005, 14 Suppl 1, 35-48

[3] Sui, M.; Chen, F.; Chen, Z.; Fan, W. Glucocorticoids interfere with therapeutic efficacy of paclitaxel against human breast and ovarian xenograft tumors. *Int. J. Cancer.*, 2006, 119, 712–717.

[4] Fan, W.; Sui, M.; Huang, Y. Glucocorticoids selectively inhibit paclitaxel-induced apoptosis: mechanisms and its clinical impact. *Curr. Med. Chem.*, 2004, 403–411.

[5] Maehara, Y.; Emi ,Y.; Sakaguchi, Y.; Kusumoto ,T.; Kakeji, Y.; Kohnoe, S.; Sugimachi, K. Estrogen receptor-negative breast cancer tissue is chemosensitive in vitro compared with estrogen-receptor-positive tissue. *Eur. Surg. Res.*, 1990, 50–55.

[6] Lippman, M.E.; Allegra, J.C.; Thompson, E.B.; Simon, R.; Barlock, A.; Green, L.; Huff, K.K.; Do, H.M.; Aitken, S.C.; Warren, R. The relation between estrogen receptors and response rate to cytotoxic chemotherapy in metastatic breast cancer. *N. Engl. J .Med.*, 1978, 298, 1223–1228.

[7] Sui, M.; Huang, Y.; Park, B.H.; Davidson, N.E.; Fan,W. Estrogen receptor alpha mediates breast cancer cell resistance to paclitaxel through inhibition of apoptotic cell death. *Cancer Res.*, 2007, 67(11), 5337-5344.

[8] Sui, M.; Jiang, D.; Hinsch, C.; Fan, W. Fulvestrant (ICI 182,780) sensitizes breast cancer cells expressing estrogen receptor alpha to vinblastine and vinorelbine. *Breast Cancer Res. Treat.*, 2010, 121(2), 335-345.

[9] McEwen, B.S. Steroid hormones: effect on brain development and function. *Horm. Res.*, 1992, 37, 1-10.

[10] Simons, S.S. What goes on behind closed doors: physiological versus pharmacological steroid hormone actions. *Bioessays*, 2008, 30(8), 744-756.

[11] Dowsett, M.; Folkerd, E.; Doody, D.; Haynes, B. The biology of steroid hormones and endocrine treatment of breast cancer. *Breast*, 2005, 452-457.

[12] Fan, W.; Cheng, L.; Norris, J.S.; Willingham, M.C. Glucocorticoids selectively inhibit taxol-induced apoptosis in a human breast cancer cell line. *Cell Pharmacol.*, 1996, 3, 435–440.

[13] Huang, Y.; Fang, Y.; Wu, J.; Dziadyk, J.M.; Zhu, X.; Sui, M.; Fan,W. Regulation of vinca alkaloid-induced apoptosis by NF-κB/IκB pathway in human tumor cells. *Mol. Cancer Ther.*, 2004, 3, 271–277.

[14] Huang,Y.; Johnson, K.R.; Norris, J.S.; Fan, W. Nuclear factorkappaB/IkappaB signaling pathway may contribute to the mediation of paclitaxel-induced apoptosis in solid tumor cells. *Cancer Res.*, 2000, 60, 4426–4432.

[15] O'Lone, R.; Frith, M.C.; Karlsson, E.K.; Hansen, U. Genomic targets of nuclear estrogen receptors. *Mol. Endocrinol.*, 2004, 18(8), 1859-1875.

[16] Simpson, E.R.; Misso, M.; Hewitt, K.N.; Hill, R.A.; Boon, W.C.; Jones, M.E.; Kovacic, A.; Zhou, J.; Clyne, C.D. Estrogen-the good, the bad, and the unexpected. *Endocr. Rev.*, 2005, 26(3), 322-330.

[17] Cordera, F.; Jordan, V.C. Steroid receptors and their role in the biology and control of breast cancer growth. *Semin. Oncol.*, 2006, 33, 631–641.

[18] Conzen, S.D. Nuclear receptors and breast cancer. *Mol. Endocrinol.*, 2008, 22(10), 2215-2228.

[19] Weigel, N.L.; Zhang, Y. Ligand-independent activation of steroid hormone receptors. *J. Mol. Med.*, 1998, 76, 469–479.

[20] Hanstein, B.; Djahansouzi, S.; Dall, P.; Beckmann, M.W.; Bender, H.G. Insights into the molecular biology of the estrogen receptor define novel therapeutic targets for breast cancer. *Eur. J. Endocrinol.*, 2004, 150(3), 243-255.

[21] Dickson, R.B.; Lippman, M.E. Control of human breast cancer by estrogen, growth factors, and oncogenes. *Cancer Treat. Res.*, 1988, 40, 119-165.

[22] Yamashita, H. Current research topics in endocrine therapy for breast cancer. *Int. J. Clin. Oncol.*, 2008, 13(5), 380-383.

[23] Murphy, L.; Cherlet, T.; Lewis, A.; Banu, Y.; Watson, P. New insights into estrogen receptor function in human breast cancer. *Ann. Med.*, 2003, 35, 614–631.

[24] Ariazi, E.A.; Ariazi, J.L.; Cordera, F.; Jordan, V.C. Estrogen receptors as therapeutic targets in breast cancer. *Curr. Top. Med. Chem*., 2006, 6(3), 181-202.

[25] Pusztai, L. Markers predicting clinical benefit in breast cancer from microtubule-targeting agents. *Ann. Oncol*., 2007, 18 suppl 12: xii15-20.

[26] Cocquyt, V.F.; Schelfhout, V.R.; Blondeel, P.N.; Depypere, H.T.; Daems, K.K.; Serreyn, R.F.; Praet, M.M.; Van, Belle, S.J. The role of biological markers as predictors of response to preoperative chemotherapy in large primary breast cancer. *Med. Oncol*., 2003, 20(3), 221-231.

[27] Faneyte, I.F.; Schrama, J.G.; Peterse, J.L.; Remijnse, P.L.; Rodenhuis, S.; van de Vijver ,M.J. Breast cancer response to neoadjuvant chemotherapy: predictive markers and relation with outcome. *Br. J. Cancer*, 2003, 88(3), 406-412.

[28] International Breast Cancer Study Group (IBCSG). Endocrine responsiveness and tailoring adjuvant therapy for postmenopausal lymph node-negative breast cancer: a randomized trial. *J. Natl. Cancer. Inst*., 2002, 94(14), 1054-1065.

[29] Berry, D.A.; Cirrincione, C.; Henderson, I.C.; Citron, M.L.; Budman, D.R.; Goldstein, L.J.; Martino, S.; Perez, E.A.; Muss, H.B.; Norton, L.; Hudis, C.; Winer, E.P. Estrogen-receptor status and outcomes of modern chemotherapy for patients with node-positive breast cancer. *JAMA,* 2006, 295(14), 1658-1667.

[30] Gianni, L.; Zambetti, M.; Clark, K.; Baker, J. Cronin, M.; Wu, J.; Mariani, G.; Rodriguez, J.; Carcangiu, M.; Watson, D.; Valagussa, P.; Rouzier, R.; Symmans, W.F.; Ross, J.S.; Hortobagyi, G.N.; Pusztai, L.; Shak, S. Gene expression profiles in paraffin-embedded core biopsy tissue predict response to chemotherapy in women with locally advanced breast cancer. *J. Clin. Oncol*., 2005, 23(29), 7265-7277.

[31] Colleoni, M.; Minchella, I.; Mazzarol, G.; Nolè, F.; Peruzzotti, G.; Rocca, A.; Viale, G.; Orlando, L.; Ferretti, G.; Curigliano, G.; Veronesi, P.; Intra, M.; Goldhirsch, A. Response to primary chemotherapy in breast cancer patients with tumors not expressing estrogen and progesterone receptors. *Ann. Oncol*., 2000, 11, 1057-1059.

[32] MacGrogan, G.; Mauriac, L.; Durand, M.; Bonichon, F.;Trojani, M.; de Mascarel, I.; Coindre, J.M. Primary chemotherapy in breast invasive carcinoma: predictive value of the immunohistochemical detection of hormonal receptors, p53, c-erbB-2, MiB1, pS2 and GST pi. *Br. J .Cancer*., 1996, 74, 1458-1465.

[33] Stearns, V.; Singh, B.; Tsangaris, T.; Crawford, J.G.; Novielli, A.; Ellis, M.J.; Isaacs, C.; Pennanen, M.; Tibery, C.; Farhad, A.; Slack, R.; Hayes, D.F. A prospective randomized pilot study to evaluate predictors of response in serial core biopsies to single agent neoadjuvant doxorubicin or paclitaxel for patients with locally advanced breast cancer. *Clin. Cancer Res*., 2003, 9(1), 124-133.

[34] Wang, L.; Jiang, Z.; Sui, M.; Shen, J.; Xu, C.; Fan, W. The potential biomarkers in predicting pathologic response of breast cancer to three different chemotherapy regimens: a case control study. *BMC Cancer*, 2009, 9, 226.

[35] Dittrich, C.; Jakesz, R.; Wrba, F.; Havelec, L.; Haas, O.; Spona, J.; Holzner, H.; Kolb, R.; Moser, K. The human tumour cloning assay in the management of breast cancer patients. *Br. J. Cancer*, 1985, 52(2), 197-203.

[36] Aho, A.J.; Mäenpää, J.U.; Kangas, L.; Söderström, K.O.; Auranen, A.A.; Linna, M. Subrenal capsule assay in human breast cancer. Response to cytostatic drug combinations and correlation with receptor status. *Eur. J. Cancer Clin. Oncol*., 1985, 21(10), 1133-1140.

[37] Kaufmann, M.; Klinga, K.; Runnebaum, B.; Kubli, F. Hormone receptors in breast cancer and response to chemotherapy. *N. Engl. J .Med*, 1979, 300(18), 1052.

[38] Maehara, Y.; Anai, H.; Kusumoto, H.; Sugimachi, K. Poorly differentiated human gastric carcinoma is more sensitive to antitumor drugs than is well differentiated carcinoma. *Eur. J. Surg. Oncol.*, 1987, 13(3), 203-206.
[39] Allegra, J.C.; Lippman, M.E.; Thompson, E.B.; Simon, R. An association between steroid hormone receptors and response to cytotoxic chemotherapy in patients with metastatic breast cancer. *Cancer Res.*, 1978, 38(11 Pt 2), 4299-4304.
[40] Razandi, M.; Pedram, A.; Levin, E.R. Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. *Mol. Endocrinol.*, 2000, 14(9), 1434-1447.
[41] Lee, A.V.; Cui, X.; Oesterreich, S. Cross-talk among estrogen receptor, epidermal growth factor, and insulin-like growth factor signaling in breast cancer. *Clin. Cancer. Res.*, 2001, 7(12 Suppl), 4429s-4435s discussion 4411s-4412s.
[42] Chalbos, D.; Philips, A.; Rochefort, H. Genomic cross-talk between the estrogen receptor and growth factor regulatory pathways in estrogen target tissues. *Semin. Cancer. Biol*, 1994, 5(5), 361-368.
[43] Kato, S. Estrogen receptor-mediated cross-talk with growth factor signaling pathways. *Breast Cancer*, 2001, 8(1), 3-9.
[44] Levin, E.R. Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. *Mol. Endocrinol*., 2003, 17(3), 309-317.
[45] Levine, B.; Sinha, S.; Kroemer, G. Bcl-2 family members: dual regulators of apoptosis and autophagy. *Autophagy*, 2008, 4(5), 600-606.
[46] Adams, J.M.; Cory, S. Bcl-2-regulated apoptosis: mechanism and therapeutic potential. *Curr. Opin. Immunol.*, 2007, 19(5), 488-496.
[47] van Delft, M.F.; Huang, D.C. How the Bcl-2 family of proteins interact to regulate apoptosis. *Cell Res.*, 2006, 16(2), 203-213.
[48] Teixeira, C.; Reed, J.C.; Pratt, M.A. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. *Cancer Res.*, 1995, 55(17), 3902-3907.
[49] Vousden, K.H.; Lu, X. Live or let die: the cell's response to p53. *Nat. Rev. Cancer*, 2002, 2, 594–604.
[50] Vogelstein, B.; Lane, D.; Levine, A.J. Surfing the p53 network. *Nature*, 2000, 408, 307–310.
[51] Hofseth, L.J.; Hussain, S.P.; Harris, C.C. p53: 25 years after its discovery. *Trends Pharmacol. Sci.*, 2004, 25, 177–181.
[52] Liu, W.; Konduri, S.D.; Bansal, S.; Nayak, B.K.; Rajasekaran, S.A.; Karuppayil, S.M.; Rajasekaran, A.K.; Das, G.M. Estrogen receptor α binds p53 tumor suppressor protein directly and represses its function. *J. Biol. Chem.*, 2006, 281, 9837–9840.
[53] Sayeed, A.; Konduri, S.D.; Liu, W.; Bansal, S.; Li, F.; Das, G.M. Estrogen receptor alpha inhibits p53-mediated transcriptional repression: implications for the regulation of apoptosis. *Cancer Res.*, 2007, 67(16), 7746-7755.
[54] Dougherty, M.K.; Schumaker, L.M.; Jordan; V.C.; Welshons W.V.; Curran, E.M.; Ellis, M.J.; El-Ashry, D. Estrogen receptor expression and sensitivity to paclitaxel in breast cancer. *Cancer Biol. Ther.*, 2004, 3(5), 460-467.
[55] Zampieri, L.; Bianchi, P.; Ruff ,P.; Arbuthnot, P. Differential modulation by estradiol of P-glycoprotein drug resistance protein expression in cultured MCF7 and T47D breast cancer cells. *Anticancer Res.*, 2002, 22(4), 2253-2259.
[56] Honorat, M.; Mesnier, A.; Vendrell, J.; Guitton, J.; Bieche, I.; Lidereau, R.; Kruh ,G.D.; Dumontet, C.; Cohen, P.; Payen, L. ABCC11 expression is regulated by estrogen in MCF7 cells, correlated with estrogen receptor alpha expression in postmenopausal breast tumors and overexpressed in tamoxifen-resistant breast cancer cells. *Endocr. Relat. Cancer.*, 2008, 15(1), 125-138.

[57] Imai, Y.; Tsukahara, S.; Ishikawa, E.; Tsuruo, T.; Sugimoto, Y. Estrone and 17-estradiol reverse breast cancer resistance protein-mediated multidrug resistance. *Jpn. J. Cancer Res.*, 2002, 93, 231–235.

[58] Sugimoto, Y.; Tsukahara, S.; Imai, Y.; Sugimoto, Y.; Ueda, K.;Tsuruo, T. Reversal of breast cancer resistance protein-mediated drug resistance by estrogen antagonists and agonists. *Mol. Cancer Ther.*, 2003, 2, 105–112.

[59] Imai, Y.; Ishikawa, E.; Asada, S.; Sugimoto, Y. Estrogen-mediated post transcriptional down-regulation of breast cancer resistance protein/ABCG2. *Cancer Res.*, 2005, 65, 596–604.

[60] utoh, K.; Tsukahara, S.; Mitsuhashi, J.; Katayama, K.; Sugimoto, Y. Estrogen-mediated post transcriptional down-regulation of P-glycoprotein in MDR1-transduced human breast cancer cells. *Cancer Sci.*, 2006, 97(11), 1198-1204.

[61] Rosell, R.; Scagliotti, G.; Danenberg, K.D.; Lord, R.V.; Bepler, G.; Novello, S.; Cooc, J.; Crino, L.; Sanchez, J.J.; Taron, M.; Boni, C.; Da Marinis, F.; Tonato, M.; Marangolo, M.; Gozzelino, F.; Di Constanzo, F.; Rinaldi, M.; Solanga, D.; Stephens, C. Transcripts in pretreatment biopsies from a three-arm randomized trial in metastatic nonsmall-cell lung cancer. *Oncogene*, 22(23), 3548–3553.

[62] Seve, P.; Mackey, J.; Isaac, S.; Tredan, O.; Souquet, P.J.; Perol M, Lai, R.; Voloch, A.; Dumontet, C. Class III beta-tubulin expression in tumor cells predicts response and outcome in patients with non-small cell lung cancer receiving paclitaxel. *Mol. Cancer Ther.*, 2005b, 4(12), 2001–2007.

[63] Shalli, K.; Brown, I.; Heys, S.D.; Schofield, A.C. Alterations of beta-tubulin isotypes in breast cancer cells resistant to docetaxel. *FASEB J.*, 2005, 19(10), 1299–1301.

[64] Ranganathan, S.; Benetatos, C.A.; Colarusso, P.J.; Dexter, D.W.; Hudes, G.R. Altered beta-tubulin isotype expression in paclitaxel-resistant human prostate carcinoma cells. *Br. J. Cancer.*, 1998, 77(4), 562–566.

[65] Kavallaris, M.; Kuo, D.Y.; Burkhart, C.A.; Regl, D.L.; Norris, M.D.; Haber, M.; Horwitz, S.B. Taxol-resistant epithelial ovarian tumors are associated with altered expression of specific beta-tubulin isotypes. *J. Clin. Invest.*, 1997, 100(5), 1282–1293.

[66] Gan, P.P.; Pasquier, E.;Kavallaris, M. Class III beta-tubulin mediates sensitivity to chemotherapeutic drugs in non small cell lung cancer. *Cancer Res.*, 2007, 67(19), 9356–9363.

[67] Tommasi, S.; Mangia, A.; Lacalamita, R.; Bellizzi ,A.; Fedele, V.; Chiriatti, A.; Thomssen, C.; Kendzierski, N.; Latorre, A.; Lorusso, V.; Schittulli, F.; Zito, F.;Kavallaris ,M.; Paradiso, A. Cytoskeleton and paclitaxel sensitivity in breast cancer: the role of beta-tubulins. *Int. J. Cancer*, 2007, 120(10), 2078–2085.

[68] Seve, P.; Dumontet, C. Is class III beta-tubulin a predictive factor in patients receiving tubulin-binding agents? *Lancet Oncol.*, 2008, 9(2), 168–175.

[69] Ferrandina, G.; Zannoni ,G.F.; Martinelli ,E.; Paglia, A.; Gallotta, V.; Mozzetti,S.; Scambia, G.; Ferlini, C. Class III beta-tubulin overexpression is a marker of poor clinical outcome in advanced ovarian cancer patients. *Clin. Cancer Res.*, 2006, 12(9), 2774–2779.

[70] Sève, P., Isaac, S., Trédan, O., Souquet, P.J., Pachéco, Y., Pérol, M., Lafanéchère, L., Penet, A., Peiller, E.L., Dumontet, C. Expression of class III {beta}-tubulin is predictive of patient outcome in patients with non-small cell lung cancer receiving vinorelbine-based chemotherapy. *Clin. Cancer Res.*, 2005, 11(15):5481-5486.

[71] Bruhat, A.; Dreau, D.; Drake, M.E.; Tourmente, S.; Chapel, S.; Couderc, J.L.; Dastugue, B. Intronic and 50 flanking sequences of the Drosophila beta 3 tubulin gene are essential to confer ecdysone responsiveness. *Mol. Cell. Endocrinol.*, 1993, 94(1), 61–71.

[72] Saussede-Aim, J.; Matera, E.L.; Ferlini, C.; Dumontet, C. Beta3-tubulin is induced by estradiol in human breast carcinoma cells through an estrogen-receptor dependent pathway. *Cell Motil. Cytoskeleton*, 2009, 66(7), 378-388.

[73] Härkönen, P.L.; Väänänen, H.K. Monocyte-macrophage system as a target for estrogen and selective estrogen receptor modulators. *Ann. N. Y. Acad. Sci.*, 2006, 1089, 218-227.

[74] Seeger, H., Wallwiener, D., Mueck, A.O. Effects of estradiol and progestogens on tumor-necrosis factor-alpha-induced changes of biochemical markers for breast cancer growth and metastasis. *Gynecol. Endocrinol.*, 2008, 24(10), 576-579.

[75] Kramer, P.R., Kramer, S.F., Guan, G. 17 beta-estradiol regulates cytokine release through modulation of CD16 expression in monocytes and monocyte-derived macrophages. *Arthritis Rheum.*, 2004, 50(6), 1967-1975.

[76] Johnston, S.R. Endocrinology and hormone therapy in breast cancer: selective oestrogen receptor modulators and downregulators for breast cancer - have they lost their way? *Breast Cancer Res.*, 2005, 7(3), 119-130.

[77] Osborne, C.K., Zhao, H., Fuqua, S.A. Selective estrogen receptor modulators: structure, function, and clinical use. *J. Clin. Oncol.*, 2000, 18(17), 72-16.

[78] Howell, S.J., Johnston, S.R., Howell, A. The use of selective estrogen receptor modulators and selective estrogen receptor down-regulators in breast cancer. *Best Pract. Res. Clin. Endocrinol. Metab.*, 2004, 18(1), 47-66.

[79] Kurebayashi, J.; Nukatsuka, M.; Nagase, H.; Nomura, T.; Hirono, M.; Yamamoto, Y,; Sugimoto, Y.; Oka, T.; Sonoo, H. Additive antitumor effect of concurrent treatment of 4-hydroxy tamoxifen with 5-fluorouracil but not with doxorubicin in estrogen receptor-positive breast cancer cells. *Cancer Chemother. Pharmacol.*, 2007, 59(4), 515-525.

[80] Benz, C.; Cadman, E.; Gwin, J.; Wu, T.; Amara, J.; Eisenfeld, A.; Dannies, P. Tamoxifen and 5-fluorouracil in breast cancer: cytotoxic synergism in vitro. *Cancer Res.*, 1983, 43, 5298–5303.

[81] Leonessa, F.; Jacobson, M.; Boyle, B.; Lippman, J.; McGarvey, M.; Clarke, R. Effect of tamoxifen on the multidrug-resistant phenotype in human breast cancer cells: isobologram, drug accumulation, and M(r) 170,000 glycoprotein (gp170) binding studies. *Cancer Res.*, 1994, 54, 441–447.

[82] Osborne, C.K.; Kitten, L.; Arteaga, C.L. Antagonism of chemotherapy-induced cytotoxicity for human breast cancer cells by antiestrogens. *J. Clin. Oncol.*, 1989, 7, 710–717.

[83] Woods, K.E.; Randolph, J.K.; Gewirtz, D.A. Antagonism between tamoxifen and doxorubicin in the MCF-7 human breast tumor cell line. *Biochem. Pharmacol.*, 1994, 47, 1449–1452.

[84] Licun, W.; Tannock, I.F. Selective estrogen receptor modulators as inhibitors of repopulation of human breast cancer cell lines after chemotherapy. *Clin. Cancer Res.*, 2003, 9(12), 4614-4618.

[85] Wu, L.; Tannock, I.F. Effect of the selective estrogen receptor modulator arzoxifene on repopulation of hormone-responsive breast cancer xenografts between courses of chemotherapy. *Clin. Cancer Res.*, 2005, 11(22), 8195-8200.

[86] Ramachandran, C.; Khatib, Z.; Petkarou, A.; Fort ,J.; Fonseca, H.B.; Melnick, S.J.; Escalon, E. Tamoxifen modulation of etoposide cytotoxicity involves inhibition of protein kinase C activity and insulin-like growth factor II expression in brain tumor cells. *J. Neurooncol.*, 2004, 67(1-2), 19-28.

[87] Sola, B.; Renoir, J.M. Antiestrogenic therapies in solid cancers and multiple myeloma. *Curr. Mol. Med.*, 2006, 6(4), 359-368.
[88] Gao, X.P.; Liu, F. New agents in development for breast cancer. *Curr. Opin. Obstet. Gynecol.*, 2007, 19(1), 68-74.
[89] Díaz-Cruz, E.S., Shapiro, C.L., Brueggemeier, R.W. Cyclooxygenase inhibitors suppress aromatase expression and activity in breast cancer cells. *J. Clin. Endocrinol. Metab.*, 2005, 90(5), 2563-2570.
[90] Chen, D.; Hackl ,W.; Ortmann, O.; Treeck, O. Effects of a combination of exemestane and paclitaxel on human tumor cells in vitro. *Anticancer Drugs*, 2004, 15(1), 55-61.
[91] Varricchio, L., Migliaccio, A., Castoria, G., Yamaguchi, H., de Falco, A., Di Domenico, M., Giovannelli, P., Farrar, W., Appella, E., Auricchio, F. Inhibition of estradiol receptor/Src association and cell growth by an estradiol receptor alpha tyrosine-phosphorylated peptide. Mol. Cancer Res., 2007, 5(11):1213-1221.
[92] Castoria, G.; Migliaccio, A.; Giovannelli, P.; Auricchio, F. Cell proliferation regulated by estradiol receptor: therapeutic implications. *Steroids*, 2010, 75(8-9):524-527.
[93] Zheng, T.; Wang, J.; Chen, X.; Liu, L. Role of microRNA in anticancer drug resistance. *Int. J. Cancer*, 2010, 126(1), 2-10.
[94] Adams, B.D.; Furneaux, H.; White, B.A. The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-alpha (ERalpha) and represses ERalpha messenger RNA and protein expression in breast cancer cell lines. *Mol. Endocrinol.*, 2007, 21, 1132–1147.
[95] Ariazi, E.A.; Jordan, V.C. Estrogen-related receptors as emerging targets in cancer and metabolic disorders. *Curr. Top. Med. Chem.*, 2006, 6(3), 203-215.
[96] Zajchowski , D.A.; Sager, R.; Webster, L. Estrogen inhibits the growth of estrogen receptor-negative, but not estrogen receptor-positive, human mammary epithelial cells expressing a recombinant estrogen receptor. *Cancer Res.*, 1993, 53, 5004–5011.
[97] Jiang, S.Y.; Jordan, V.C. Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. *J. Natl. Cancer Inst.*, 1992, 84(8), 580-591.
[98] DiMarco, B.; Aguggini, S.; Bolsi, G.; Cirillo, F.; Filippini, L.; Betri, E.; Bertoli, G.; Alquati, P.; Dogliotti, L. p53 but not bcl-2 immunostaining is predictive of poor clinical complete response to primary chemotherapy in breast cancer patients. *Clin. Cancer Res.*, 2000, 6, 2751-2758.
[99] Makris, A.; Powles, T.J.; Dowsett ,M.; Osborne, C.K.; Trott, P.A.; Fernando, I.N.; Ashley, S.E.; Ormerod, M.G.; Titley, J.C.; Gregory, R.K.; Allred, D.C. Prediction of response to neoadjuvant chemoendocrine therapy in primary breast carcinomas. *Clin. Cancer Res.*, 1997, 3, 593-600.
[100] Gapinski, P.V.; Donegan, W.L. Estrogen receptors and breast cancer: prognostic and therapeutic implications. *Surgery*, 1980, 88, 386-393.
[101] Hilf, R.; Feldstein, M.L.; Gibson, S.L.; Savlov, E.D. The relative importance of estrogen receptor analysis as a prognostic factor for recurrence or response to chemotherapy in women with breast cancer. *Cancer*, 1980, 45, 1993-2000.
[102] Hilf, R.; Feldstein, M.L.; Savlov, E.D.; Gibson, S.L.; Seneca, B. The lack of relationship between estrogen receptor status and response to chemotherapy. *Cancer*, 1980, 46(12 Suppl), 2797-2800.
[103] Minisini, A.M.; Di Loreto, C.; Mansutti, M.; Artico, D.; Pizzolitto, S.; Piga, A.; Puglisi, F. Topoisomerase IIalpha and APE/ref-1 are associated with pathologic response to primary anthracycline-based chemotherapy for breast cancer. *Cancer Lett.,* 2005, 224(1), 133-139.

25

Tamoxifen Resistant Breast Cancer and Autophagy

Grey A. Wilkinson, Adam N. Elwi and Sung-Woo Kim
University of Calgary,
Canada

1. Introduction

1.1 Tamoxifen for the treatment of breast cancer

Tamoxifen was originally developed with the hope of becoming a "morning after" contraceptive. During the 1960s, studies showed that tamoxifen and other anti-estrogens have a profound impact on the fertility of laboratory rats and it was believed that tamoxifen could elicit the same effects in humans. Coincidently, in humans, anti-estrogens were found to improve fertility by inducing ovulation (Jordan, 2006).

The use of tamoxifen for the treatment of breast cancer became apparent when early *in vitro* and *in vivo* studies found that tamoxifen inhibited estradiol (E2) binding to estrogen receptors (ERs) in breast tissue (Jordan, 1976; Jordan & Dowse, 1976; Jordan & Koerner, 1975; Nicholson & Golder, 1975). The inhibition of ERs proved to be a significant finding as the estrogen-stimulated growth and the ovarian dependence of some breast cancers had been known since the later part of the eighteenth century (Beatson, 1896). Prior to the 1970s, and the successful development of drug-based endocrine therapies, attempts to reduce estrogen-stimulated growth of breast cancer include the surgical removal of the ovaries and/or pituitary and adrenal glands (Kennedy, 1965). Unfortunately, for years it was not known which breast tumors would respond favorably to ablative surgery, where only 30% of patients would receive a benefit (Jensen *et al.*, 1971; Kennedy, 1965). By examining proteins from breast tumor biopsies, Jensen *et al.* (1971) showed that if the estrogen receptor is present, tumors will respond to ablative surgical treatment, but if absent, the tumors fail to respond.

Fortunately today, endocrine-based surgical procedures for breast cancer are not necessary for most women as drug-based endocrine therapies have advanced with the use of estrogen antagonists and aromatase inhibitors. Estrogen antagonists, such as tamoxifen or fulvestrant competitively inhibit estrogen binding to ERs. Aromatase inhibitors prevent estrogen production, by inhibiting the aromatase class of enzymes, impeding the conversion of androgens to estrogens and thus reducing the bioavailability of estrogen hormones (Jordan, 1994; Mokbel, 2002).

Currently tamoxifen is typically used as an adjuvant treatment option for early and advanced ER-positive (ER^+) breast cancer in pre- and post-menopausal women (Jordan, 1994). Adjuvant treatment with tamoxifen, has significantly improved disease-free survival and reduced the number of deaths from breast cancer (Early Breast Cancer Trialists' Collaborative, 2005). Tamoxifen may also be used in the neoadjuvant setting and as a

preventative agent for women at high risk of developing the disease, although some patients may feel that when used as a preventative agent the risks may outweigh the benefits (Cuzick *et al.*, 2003; Waters *et al.*, 2010). The most adverse effect of tamoxifen is the increased propensity to develop endometrial cancer that occurs predominantly in post-menopausal women. To subside this effect, tamoxifen, in the adjuvant setting, is administered for at most 5 years. If longer therapy is required, patients are typically switched to aromatase inhibitors or a different anti-estrogen, such as fulvestrant (Perez, 2007).

In breast tissue, tamoxifen acts as an antagonist, binding to ERα, competitively inhibiting E2, and preventing the expression of mitogen, angiogenic, and apoptotic factors. In other tissues such as the endometrium, tamoxifen acts as an agonist and elicits a response similar to estrogen when bound to ERs, potentially leading to the development of endometrial cancer. 'Pure' anti-estrogens, like fulvestrant bind the ER competitively inhibiting estrogen but have no estrogen like effects. In some women, tamoxifen's agonistic actions have been found to have favorable effects on serum cholesterol and protection against bone loss and cardiovascular disease (Hoskins *et al.*, 2009; Osborne, 1998). The tissue dependent agonist and antagonist actions of tamoxifen classify the drug as a selective estrogen receptor modulator (SERM). SERMs modulate the ER signal transduction pathway in ER target tissues through complex and not completely understood mechanisms (Jordan, 2006), although the agonist and antagonist functions of tamoxifen are thought to be mediated through two distinct transactivation domains of ERα, Activating Function-1 (AF-1) close to the N-terminus, and AF-2, in the ligand binding site. In breast tissue, tamoxifen inhibits AF-2 activation and functions as an antagonist of genes that rely on AF-2 transactivation. In genes where AF-2 function is redundant, tamoxifen may function as an agonist and transcription may be driven solely by the AF-1 domain (McDonnell *et al.*, 1995; Ring & Dowsett, 2004; Tzukerman *et al.*, 1994).

1.2 Estrogen receptors

The primary target of tamoxifen is the ER. The ER is a ligand-activated transcription factor that is a critical regulator of breast epithelial cell proliferation, differentiation, and apoptosis. There are two ER isoforms, ERα and ERβ (Dahlman-Wright *et al.*, 2006). The receptors differ in their cellular function, tissue distribution, as well as ligand binding properties. Both ERα and ERβ are expressed in normal and neoplastic tissues. ERα is expressed in 15-30% of the luminal epithelial cells while ERβ is more ubiquitously expressed throughout the mammary tissue. Estrogen dependent proliferation in non-neoplastic breast epithelial cells is thought to occur through paracrine mechanisms where ERα cells promote the proliferation of adjacent ER negative cells (Riggins *et al.*, 2007). In neoplastic breast tissue, ERα is expressed in a greater proportion of cells and growth is thought to occur through both autocrine and paracrine mechanisms (Riggins *et al.*, 2007). The roll of ERβ in breast cancer development is not well understood. ERβ is often down regulated in malignant cells and its presence is often correlated with a better prognosis (Saji *et al.*, 2005; Sugiura *et al.*, 2007). *In vivo* studies in mice have revealed that ERβ expression prevents tumor formation and angiogenesis (Behrens *et al.*, 2007; Paruthiyil *et al.*, 2004). Indeed ERβ may contribute to the pregnancy/lactation-associated protection from breast cancer by disrupting the formation of tight junctions and altering the expression of β-cantenin, traits that have been associated with malignant phenotypes (Riggins *et al.*, 2007). Moreover, ERβ is a marker of improved

response to tamoxifen (Hopp *et al.*, 2004). Thus most endocrine therapies target the actions of ERα.

ERα-positive (ERα$^+$) breast cancers account for at least 75% of the breast cancer patient population. The presence of ERα, assessed immunohistologically, serves as a biomarker for a patient's response to endocrine therapy (Harvey *et al.*, 1999). The majority, 65%, of ERα$^+$ breast cancers also express the progesterone receptor (PR) (Hoskins *et al.*, 2009). The PR, like the ER, is a ligand activated transcription factor that is involved in a wide range of physiological functions, contributing to cell homeostasis and differentiation. A small number of ER-negative but PR-positive tumors also respond favorably to tamoxifen treatment, but this is likely due to a small presence of ERα below the limit of detection (Clarke *et al.*, 2001).

ERα has three distinct pathways of regulating gene expression. The classic model of ERα signaling involves the ligand bound ERα that activates gene expression by direct dimeric binding to DNA response elements in complexes involving co-activators (CoAs) and histone acetyl transferases (HATs). The ER can also influence transcription through protein-protein interactions with other transcription factors, such as activation protein 1 (Ap1) and specific protein 1 (Sp1), which facilitate binding to serum response elements. Lastly, ERs can be activated by downstream signaling events or crosstalk with receptor tyrosine kinases including: epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2, also known as ERBB2), and insulin-like growth factor-1 receptor (IGF-1R) (Kushner *et al.*, 2000; Musgrove & Sutherland, 2009; Ring & Dowsett, 2004; Schiff *et al.*, 2003). Indeed deregulation of estrogen signaling through the estrogen receptors with proliferation through alternative pathways are common mechanisms for tamoxifen resistance (Ali & Coombes, 2002; Musgrove & Sutherland, 2009; Riggins *et al.*, 2007).

1.3 Resistance to tamoxifen

A challenge to nearly all cancer therapies is the development of resistance. Resistance to cancer therapy can be categorized into two major forms, *de novo* and acquired. *De novo* or intrinsic resistance is present in patients that are initially unresponsive to treatment, despite having a phenotypic classification similar to patients that do respond; while acquired resistance is present in patients that respond to treatment initially, but eventually relapse.

Despite tamoxifen's successes, where 70% of ERα$^+$ and/or progesterone receptor positive breast cancer patients respond favorably to treatment, 30-50% of patients will have recurrent disease within 15 years (Clarke *et al.*, 2001; Early Breast Cancer Trialists' Collaborative, 2005). Few strategies besides resorting to invasive chemotherapy, radiation therapy, and surgery have found success in circumventing anti-estrogen resistance. The challenge then, is to identify specific biomarkers that can predict therapeutic response to endrocrine therapies and identify new targets to combat endocrine resistant disease (Musgrove & Sutherland, 2009).

1.4 *De novo* resistance

One of the most prevalent factors contributing to *de novo* tamoxifen resistances is lack of ERα expression (Giacinti *et al.*, 2006). The decreased expression of ERα can be the result of hypermethylation in the CpG islands of the ERα promoter. Studies using MDA-MB-231 ERα-negative breast cancer cells have shown that by re-expressing endogenous ERα with a histone deacelylase (HDAC) inhibitor cells can be re-sensitized to tamoxifen (Sharma *et al.*,

2006). Similar studies have shown that by ectopically expressing ERα in the same cell line, cells do not become sensitive to tamoxifen treatment but have a decreased proliferative and metastatic phenotype when exposed to estrogen (Garcia *et al*., 1992). Thus, the restoration of ERα appears to be a potential therapy for *de novo* resistance or ERα-negative breast cancers. Candidate therapies include the use of demethylating agents or HDAC inhibitors, which could potentially decrease the hypermethylation of CpG island-s within the ERα promoter (Giacinti *et al.*, 2006).

In addition, Hoskins and collogues identified a pharmacokinetic mechanism behind *de novo* tamoxifen resistance (Hoskins *et al*., 2009). Patients carrying inactive alleles of cytochrome P450 2D6 (CYP2D6), or are co-administered drugs that inhibit CYP2D6, fail to convert tamoxifen into its active metabolites endoxifen and 4-hydroxytamoxifen, as a result, patients seem to derive little therapeutic benefit. Interestingly, the inactive alleles of CYP2D6 vary among different ethnic groups, suggesting an ethnic based difference in response to tamoxifen treatment. Approximately 20% of East Asian women, 16% of women with African ancestry, and 6-10% of Caucasian women have decreased CYP2D6 metabolism. The ethnic differences emphasize the importance of patient based therapies and the potential employment of CYP2D6 genotyping prior to tamoxifen treatment (Hoskins *et al*., 2009). Other mechanisms of *de novo* resistance and the mechanisms that lead to lack of ERα expression are not as well understood, although they may be similar to some of the mechanisms that lead to acquired tamoxifen resistance.

1.5 Acquired resistance

Tamoxifen is typically prescribed in the adjuvant setting and administered for a 5 year period. Prolonged exposure may drive the development of acquired resistance that can arise from multiple factors including host-specific differences in immunity, endocrinology, and drug pharmacokinetics. Tumor-specific factors such as alterations in ER isoform ratios or ER transactivation and downstream signaling as well as changes in tyrosine kinases or mitogen growth factor signaling may also contribute to acquired tamoxifen resistance.

In cases where patients acquire resistance to tamoxifen, loss of ERα expression occurs in only 15-20% of breast cancers (Gutierrez *et al*., 2005). Mutations in ERα are also quite rare occurring in <1% of ERα$^+$ tumors (Clarke *et al*., 2003; Herynk & Fuqua, 2004; Riggins *et al*., 2007). Indeed, following the development of tamoxifen resistance, many tumors still respond to treatment with aromatase inhibitors or 'pure' anti-estrogens such as fulvestrant, which also block the effects of estrogen but have no estrogen-like effects, unlike tamoxifen. These observations suggest that estrogen still plays a critical role in tamoxifen-resistant tumors and support the idea that endocrine therapies can lead to the activation of novel signaling pathways that circumvent the effects of anti-estrogens (Lewis & Jordan, 2005; Musgrove & Sutherland, 2009; Riggins *et al*., 2007). In addition, the activation of these pathways may also contribute to *de novo* and acquired resistance to aromatase inhibitors and other anti-estrogens.

In large part, the development of acquired resistance can be attributed to a deregulation of estrogen receptor signaling as well as altered expression and/or modification of several tyrosine kinase growth factor receptors and their downstream signaling targets (Musgrove & Sutherland, 2009; Riggins *et al*., 2007). Signaling from tyrosine kinase receptors such as EGFR, HER2, as well as the IGF-1 receptor (IGF1R) have been associated with the expression of anti-apoptotic, autophagic, and other pro-survival signaling factors that allow for the

development of endocrine resistance. Others have previously discussed the role of the HER2 and EGFR signaling pathways in relation to tamoxifen resistance (reviewed in: Clarke *et al.*, 2003; Musgrove & Sutherland, 2009; Riggins *et al.*, 2007).
In this chapter, we discuss the significance of IGF signaling and present additional results of a previously published RNA interference (RNAi) tamoxifen resistance screen (Ahn *et al.*, 2010). The screen was used to identify new targets to combat tamoxifen-resistant disease or to identify specific biomarkers that predict response to tamoxifen therapy. One of the identified targets, IGF binding protein 5 (IGFBP5) will be discussed in detail, while the other targets have a role in autophagy which has been shown to be essential for cell survival, differentiation, development, and homeostasis in response to stress and nutrient deprivation (Levine & Kroemer, 2008).

2. Methods

RNAi is a powerful tool to inhibit gene expression at the post-transcriptional level and allows the identification of novel cellular mechanisms that regulate gene expression. As well, its manipulation makes high-throughput genetic screens possible. The silencing of genes within mammalian cells is possible by designing small interfering RNA (siRNA) sequences. The siRNAs can be synthesized chemically, then taken up by the cell. Alternatively siRNAs can be produced within the cell, in the form of short hairpin RNAs (shRNAs) from exogenously introduced vectors.

2.1 siRNAs and shRNAs

Since RNA interference (RNAi) was first discovered in the nematode *Caenorhabditis elegans* it has been used to exploit the function of genes in higher organisms (Fire *et al.*, 1998). In particular, large-scale RNAi based techniques have innovated the existing loss-of-function genetic screens in mammalian cells (Elbashir *et al.*, 2001). The use of chemically-synthesized siRNAs has a couple of key advantages including: the constant quality control of reagents that is important for high-throughput settings; and the diverse modification of siRNA molecules that improves stability and delivery to cells. Vice versa, synthetic siRNAs has disadvantages, including its short-life that results in the transient inhibition of gene expression only, along with difficulty in efficiently delivering siRNAs to non-dividing primary cells and their cost in genome-wide high-throughput screens. To circumvent these limitations, several groups have developed vectors that produce short hairpin RNAs (shRNAs) that are processed within the cell into short duplex RNAs having siRNA-like properties (Brummelkamp *et al.*, 2002). Such vectors provide a renewable source of a gene-silencing reagent that can mediate persistent gene silencing after stable integration of the vector into the host-cell genome. Furthermore, the core silencing 'hairpin' cassette can be readily inserted into retroviral, lentiviral, or adenoviral vectors, facilitating delivery of shRNAs into a broad range of cell types (Brummelkamp *et al.*, 2002; Michiels *et al.*, 2002). Additionally, inducible versions of shRNA vectors have been generated and used successfully in genetic screens (Ngo *et al.*, 2006).

2.2 RNAi-screening in breast cancer

The large collections of RNAi libraries have been used in many different ways to identify genes associated with drugs resistance or specific cellular phenotypes such as migration or invasion that act in a cancer-specific genetic context. A list of published screens performed

in breast cancer is presented in Table 1. Using an shRNA library genetic screen, Berns *et al*. (2007) made a significant contribution to breast cancer research when they discovered that PI3K is a determinant in Trastuzumab (Herceptin) resistance for HER2-positive breast cancers. In a siRNA screen with ovarian and breast cancer cells, Swanton *et al.* (2007) revealed that CERT and its client ceramide were integral to paclitaxel-mediated cell death. Furthermore, Swanton and collogues showed that the paclitaxel response metagene is promising as a paclitaxel-specific predictor of pathological complete response in triple-negative breast cancer. Through the genetic screen of tamoxifen resistant breast cancer, Kim and his group identified IGFBP5 as a determinant of its sensitivity (Ahn *et al.,* 2010).

Screening for	**Cell line used**	**shRNA set used**	**Genes identified**	**Reference**
Herceptin resistance	BT-474	Retroviral shRNAs	PI3K pathway	(Berns *et al.*, 2007)
Paclitaxel resistance	MDA-MB-231, MDA-MB-468	siRNAs	PPM1D, CENP, IGF1..	(Bauer *et al.*, 2010)
Paclitaxel resistance	MDA-MB-231, SKOV-3	siRNAs	Ceramide metabolism regulators	(Juul *et al.*, 2010; Swanton *et al.*, 2007)
Tamoxifen resistance	MCF-7	Retroviral shRNAs	IGFBP5	(Ahn *et al.*, 2010)

Table 1. Application of RNAi library screens in breast cancer.

Overall, genetic RNAi screening holds promise in accelerating the development of drug-specific therapeutics or predictive biomarkers, since they target cancer-specific genetic alterations.

2.3 Screening strategies

The screening strategy is shown in schematic form in Fig. 1. Several breast cancer cells, including MCF-7, BT-474 and MDA-MB-231 cells were cultured in DMEM supplemented with 10% fetal bovine serum and treated with tamoxifen (5 μM) to find out which cell line is most sensitive. MCF-7 cells were selected for the RNAi screening as they were shown to be the most sensitive to the drug (Fig. 2). Tamoxifen-resistant MCF-7 cells were established by culturing for 4 wks in 10 μM 4-hydroxytamoxifen. Three shRNA oligonucleotides against each of 8,000 genes were designed and cloned into retroviral vector (pRS) containing a shRNA cassette.

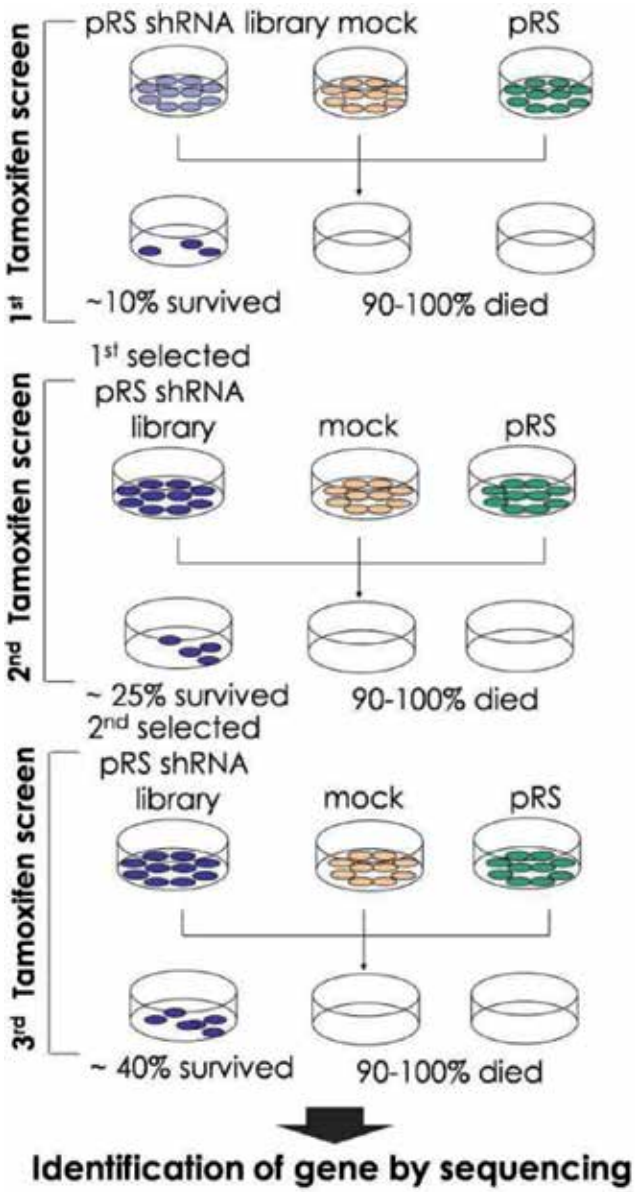


Fig. 1. The screening strategy employed for the identification of tamoxifen resistant genes.

MCF-7 cells were infected with pRS-shRNA library or pRS or mock-infected, selected with puromycin (1 μg/mL) for 2 weeks, and cultured in the presence or absence of tamoxifen (5 μM). After 6 days, genomic DNA was isolated and shRNA inserts were sub-cloned into a pRS vector. Screening was done for two additional rounds to enrich for targets. Targeted genes were identified by sequencing shRNA inserts.

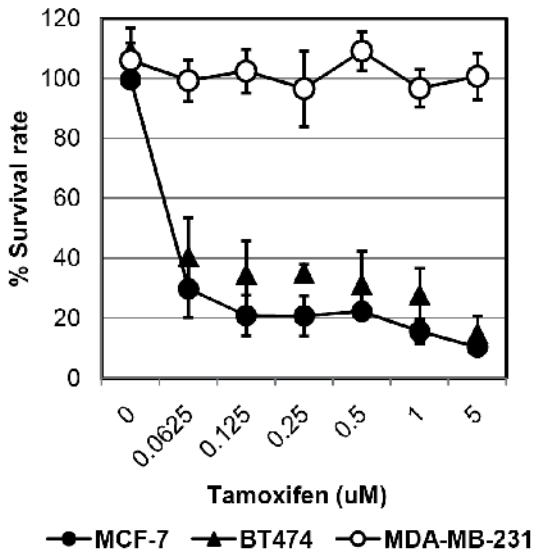


Fig. 2. Various breast cancer cell lines treated with 4-hydroxytamoxifen.

3. Results

Our screen revealed that loss of IGFBP5 contributed to tamoxifen resistance (Ahn *et al.*, 2010). In addition to IGFBP5, we identified several other genes that have a role in regulating autophagy or have a function in the downstream effects of autophagy. Autophagy is the regulated turnover of large components of the cell (e.g., cytosol and organelles) through engulfment in double-membrane sacs, termed autophagosomes, followed by fusion with lysosomes for degradation. These genes include: Vacuolar protein sorting 15 (Vps15), a regulatory subunit of the phosphatidyl inositide 3-kinase (PI3K) complex subunit Vps34 (Lindmo *et al.*, 2008); regulatory-associated protein of mammalian target of rapamycin (mTOR) (Raptor), an inhibitor of mTOR complex kinase activity (Kim *et al.*, 2002); and, interleukin 6 (IL-6), a pro-senescent cytokine (Kuilman *et al.*, 2008). The significance of each of the identified genes will be discussed below.

4. Discussion

4.1 The role of IGFBP5 in tamoxifen resistance

The IGF signaling pathway has important roles in regulating energy metabolism, cellular proliferation, differentiation, and apoptosis (Gluckman *et al.*, 1992; Valentinis & Baserga, 2001). The IGF axis involves a complex regulatory network that operates at a physiological, cellular and sub-cellular level (Pollak *et al.*, 2004). The relationship between IFG signaling and neoplasia has been well documented but the correlation between IGF signaling and endrocrine resistance is not as well understood (Pollak, 2008; Pollak *et al.*, 2004). In general, deregulation of IGF signaling may also correlate with the higher incidences of cancer, in particular breast cancer, among affluent nations due to higher incidences of obesity and a preference for a sedentary lifestyle. Both estrogen and IGFs have a synergistic effect on cell proliferation in MCF-7 breast cancer cell lines. Crosstalk between these two pathways has been implicated in the development of resistance to endocrine therapy and cancer recurrence.

The IGF signaling pathway consists of two peptide growth factors, IGF-1 and IGF-2, which can have characteristics of both circulating hormones and tissue growth factors. IGF-1 and IGF-2 can bind their cell surface receptors, IGF-1R and IGF-2R, as well as six different IGF-binding proteins, IGFBP1 to 6 that influence the binding of IGFs to their receptors. The IGF axis is further regulated by IGFBP proteases, and the proteins involved in intracellular signaling distal to IGF-1R which include members of the insulin receptor substrate family, AKT, the target of rapamycin (TOR), and S6 kinase (Pollak *et al.*, 2004).

Compared to HER2 and EGFR, the role of IGF signaling in anti-estrogen resistance is not as well understood and unlike HER2 and EGFR, data is mixed as to whether IGF-1R levels are elevated or decreased in resistant tumors and cell lines (Brockdorff *et al.*, 2003; Gee *et al.*, 2005; Knowlden *et al.*, 2005). Expression of the ER is controlled by IGF-1 in breast cancer cells (Lee *et al.*, 1997). Conversely, genomic and non-genomic interactions of ER can activate the mitogen-inducing signals of the IGF pathway (Fagan & Yee, 2008). Estrogen signaling also causes the expression of insulin receptor substrate 1 (IRS-1), a scaffolding molecule, which activates downstream signaling of IGF-1 and potentiates its proliferative abilities. Salerno *et al.* (1999) suggest that anti-estrogens, like tamoxifen, down regulated IRS-1 and that this down regulation may be one of the possible mechanisms involved in anti-estrogen response. The importance of IGF-1R is further illustrated with studies that show IGF-1R, in ER-negative MDA-MB-231 breast cancer cells, regulates the migration and adhesion abilities of these cells whereas in ER positive MCF-7 breast cancer cells, IGF-1R can modulate mitogenic stimulation (Bartucci *et al.*, 2001). Crosstalk between the IGF signaling pathway and the ER pathway is not only important in breast cancer development but may allow for the development of therapeutics that targets the interactions and communications between the two signaling pathways.

In our own studies, when screening for genes that confer resistance to tamoxifen, we identified IGFBP5 (Ahn *et al.*, 2010). IGFBP5 is a secreted protein and known to inhibit growth factor binding and signaling through IGF-1R (Beattie *et al.*, 2006). In addition, IGFBP5 has recently been shown to play a critical role in breast cancer progression and metastasis, although the exact mechanism remains obscure (Akkiprik *et al.*, 2008). We showed that knock-down of IGFBP5 resulted in resistance to tamoxifen treatment in MCF-7 cells and in mice tumor xenografts. The IGFBP5 knockdown-induced resistance to tamoxifen occurred potentially via altered IGF signaling and loss of ER expression (Bunone *et al.*, 1996; Kato *et al.*, 1995; Lee *et al.*, 1997). We found that treatment with recombinant IGFBP5 reversed *in vitro* and *in vivo* tamoxifen resistance. We also demonstrated, with a cohort of 153 breast cancer patients, that low IGFBP5 expression is associated with shorter overall survival after tamoxifen therapy. *In vitro,* others have shown that with tamoxifen or fulvestrant treated MCF-7 cells, growth inhibition is associated with increased expression of IGFBP5 and increased IGFBP5 secretion into the cell culture media (Huynh *et al.*, 1996; Parisot *et al.*, 1999; Pratt & Pollak, 1993). Preliminary evidence, in addition to some microarray data, supports the idea that IGFBP5 expression level determines tamoxifen responsiveness (Becker *et al.*, 2005).

4.2 Interleukin-6

Results from our screen indicate that reduced expression of IL-6 can confer tamoxifen resistance *in vitro*. IL-6 is a pleiotropic cytokine that has been found to have both tumor-promoting and tumor-inhibiting functions in breast cancer development (Knupfer & Preiss, 2007). Cytokines can be defined as small protein signaling molecules that tend to be

glycosylated and exert their biological function at low concentrations (pg to ng/mL). Cytokines are secreted predominantly by lymphocytes and macrophages and exert their effect by binding to receptors on the target cell surface, altering the function of the target cell by either paracrine or autocrine signaling. The effects of different cytokines can be additive, synergistic, or antagonistic, and the integration of these effects determines the overall outcome and function of a particular cytokine.

Recently, Gilbert *et al*. (Gilbert & Hemann, 2010) have identified IL-6 as a mediator in the development of chemotherapeutic-resistant niches, a novel physiological mechanism behind drug-resistant lymphoma and a likely contributor to the development of cancer recurrence. Specifically, IL-6 along with metallopeptidase inhibitor 1 (Timp-1) were released from the thymus in response to chemotherapy-induced DNA damage, creating a ''chemo-resistant niche''. The niche helps promote the survival of undetectable residual disease, serving as a reservoir for eventual tumor relapse. Currently it is not known if chemo-resistant niches are present in other cancers or if tamoxifen can elicit the development of such niches.

Notwithstanding, in breast cancer cells, tumor necrosis factor alpha (TNFα) can induce the expression of IL-6 along with IL-11, promoting osteoclast formation and mediating osteolysis at the site of breast cancer bone metastases (Suarez-Cuervo *et al.*, 2003). IL-6 has also been implicated in mediating oncogene-induced cellular senescence (Kuilman *et al.*, 2008). Cellular senescence, where cells continue to be metabolically active but generally lose their ability to divide may act as a safeguard against a variety of cellular insults, including anti-estrogen therapy. Interestingly, Young *et al*. (2009) identified autophagy as a new effector mechanism of senescence by allowing for rapid protein turnover and cellular re-modeling that occurs when a cell transitions form a proliferative to a senescent state. How knockdown of IL-6 confers resistance to tamoxifen therapy is unclear. The pleiotropic actions of IL-6 complicate our understanding of its role in anti-estrogen resistance and highlight a limitation of RNAi based screens, in that one gene may confer multiple phenotypes.

4.3 Raptor, Vps15, and autophagy

Several gene targets identified in our RNA interference screen for tamoxifen resistance were found to be involved directly in macroautophagy, including Raptor and Vps15. Macroautophagy, hereafter referred to as autophagy, involves the delivery of cytoplasmic cargo, such as long-lived proteins and organelles, sequestered inside double-membrane vesicles, termed autophagosomes, to the lysosomes for degradation. Digested components are recycled back to the cell; therefore, autophagy is a cellular process essential for tissue development and homeostasis, and as an adaptive response to stress and nutrient deprivation (Levine & Kroemer, 2008).

The mTOR, a central regulator of cell growth, is activated through signaling by receptor tyrosine kinase-mediated PI3K activation (Efeyan & Sabatini, 2009). Two distinct protein complexes of mTOR mediate its effects on controlling the rate of cell growth and timing of cell cycle progression including, mTORC1 and mTORC2, respectively (Efeyan & Sabatini, 2009). The mTORC1 complex consists of mTOR, Raptor, and mLST8 and activation leads to increased protein translation, ribosome biogenesis and inhibition of autophagy, the combined effects resulting in increased cell growth (Efeyan & Sabatini, 2009). Raptor participates in mTORC1 complex as a key scaffolding protein to bind mTOR substrates such as eukaryotic initiation factor 4E binding protein 1 (4EBP1) and p70 S6 kinase α (p70α), facilitating phosphorylation and activation by mTOR (Hara *et al.*, 2002; Kim *et al.*, 2002).

Under nutrient-replete conditions mTOR-Raptor association is weak but is stabilized by nutrient deprivation resulting in inhibition of mTOR kinase activity (Hara *et al.*, 2002; Kim *et al.*, 2002). Raptor-mediated inhibition of mTORC1 is particularly relevant for its role in autophagy as Raptor inhibition of mTOR phosphorylation of ULK1/2 kinase complex leads to ULK1/2 dephosphorylation and activation to initiate autophagosome formation by Atg family of proteins (Kroemer *et al.*, 2010).

Recent attention to development and application of mTOR inhibitors in cancer therapy, including breast cancer, highlights the significance of our finding that loss of Raptor contributes to tamoxifen resistance (Dancey, 2010). In the context of autophagy, loss of Raptor may lead to mTOR phosphorylation of ULK1/2 and autophagy inactivation. In breast cancer, inhibition of mTOR activity has been found to restore tamoxifen sensitivity in breast cancer (deGraffenried *et al.*, 2004).

Vps15 is a putative serine/threonine protein kinase (Stack & Emr, 1994), required for Vps34 activity, a Class III PI3K that produces phosphatidylinositol-3-phophate (Backer, 2008; Schu *et al.*, 1993; Volinia *et al.*, 1995). Vps34 lipid kinase activity has recognized critical functions in early endosome fusion (Christoforidis *et al.*, 1999), maturation (Futter *et al.*, 2001), vesicular trafficking (Stack & Emr, 1994) and starvation-induced autophagy (Takahashi *et al.*, 2007). *In vitro*, Vps15 enhances Vps34 lipid kinase activity (Panaretou *et al.*, 1997); however, Vps34 does not appear to be a substrate of Vps15. Further, Vps15 deletion leads to loss of phosphatidylinositol-3-phosphate production and vesicular trafficking (Stack & Emr, 1994). Lastly, regulation of Vps34 activity by autophagy-related proteins or nutrients requires Vps15 (Yan *et al.*, 2009). The Beclin-1 complex containing Beclin 1, Vps34, Vps15, UVRAG, Atg14L and Rubicon orchestrates demarcation of sites for formation of double membrane organelles associated with Atg5 and Atg12, termed autophagosomes (Zhong *et al.*, 2009). Vps15 regulation of Vp34 activity is necessary for marking sites of autophagosome formation and eventual autophagic removal of protein aggregates (Lindmo *et al.*, 2008).

While little is known about Vps15 in breast cancer, Vps34 function appears to have a pro-survival function mediated through promotion of autophagy. First, increased Vps34 expression levels and tyrosine phosphorylation by pp60c-Scr contributes to enhanced tumorigenic activity in breast cancer cells (Hirsch *et al.*, 2010). Second, in MCF-7 cells, knockdown of Vps34 by RNA interference reduced cytoprotective autophagy mediated by Bcl-2 homology domain 3 (BH3) mimetic gossypol and potentiated apoptosis induction (Gao *et al.*, 2010). Lastly, Vps34 has tumor suppressor function in MCF-7 mouse xenograft tumors mediated through distinct Beclin-1 binding and enhancement of starvation-induced autophagy (Furuya *et al.*, 2005). These studies corroborate lines of evidence demonstrating that PI3K/AKT/mTOR pathway, implicated in cell survival, contributes to anti-estrogen resistance (Musgrove & Sutherland, 2009).

In contrast, our studies have identified loss of Vps15 and Raptor as candidate gene target for mediating breast cancer tamoxifen resistance *in vitro*, suggesting that Vps15- or Raptor-mediated autophagy promotes cell death mediated by tamoxifen. In support of this, inhibitors of each of PI3K, Akt, and mTOR are in clinical trials (Dancey, 2010) and inhibition of mTOR activity is thought to restore tamoxifen sensitivity in breast cancer (deGraffenried *et al.*, 2004). Also, others have also demonstrated that tamoxifen and anti-estrogen-binding site ligands induce autophagic cell death in human breast cancer cells (de Medina *et al.*, 2009; Payre *et al.*, 2008). Tamoxifen-induced cell death through autophagy was associated with cholesterol accumulation. Interestingly, our laboratory's RNA interference screen also identified several genes involved in cholesterol metabolism including Niemann-Pick C 2

(NPC2) protein, reportedly involved NPC disease, in which cells accumulate sterols in multilamellar bodies resulting in onset of autophagy (Pacheco *et al.*, 2007). Furthermore, an ectopic over-expression screen identified a small heat-shock protein family member, which limited autophagic cell death mediated by tamoxifen (Gonzalez-Malerva *et al.*, 2011).

Controversy remains as to whether autophagy limits or promotes tumor malignancy, including drug resistance. Genetic inactivation of autophagy is known to promote tumorigenesis and constitutes a new category of tumor suppressors including Beclin 1. And while oncogenes including PI3K/AKT/mTOR and Bcl-2 inhibit autophagy and hence the ability of tumor cells to proliferate, other oncogenes including Ras and myc stimulate autophagy (Kondo *et al.*, 2005). These variegated effects could represent the significance of autophagy at different stages of tumor progression. Further investigation into the impact of autophagy inactivation on clinical tamoxifen resistance is warranted, specifically on the candidate targets Vps15 and Raptor.

5. Final remarks

Over the last several decades the therapeutic use of tamoxifen has undoubtedly saved countless lives. However, predicting responsiveness and understanding resistance is critical to advances in treatment. Although this chapter has focused on tamoxifen, future studies investigating the mechanisms of resistance to aromatase inhibitors and other anti-estrogens will likely reveal similar mechanisms as the clinical data and experience with these drugs matures. Progress towards better treatment and understanding resistance may be made by further examining the role of IGF signaling and crosstalk with the ER and the role of autophagy.

6. References

Ahn, B. Y., Elwi, A. N., Lee, B., Trinh, D. L., Klimowicz, A. C., Yau, A., et al. (2010). Genetic screen identifies insulin-like growth factor binding protein 5 as a modulator of tamoxifen resistance in breast cancer. *Cancer Res*, 70, 3013-3019.

Akkiprik, M., Feng, Y., Wang, H., Chen, K., Hu, L., Sahin, A., et al. (2008). Multifunctional roles of insulin-like growth factor binding protein 5 in breast cancer. *Breast Cancer Res*, 10, 212.

Ali, S., & Coombes, R. C. (2002). Endocrine-responsive breast cancer and strategies for combating resistance. *Nat Rev Cancer*, 2, 101-112.

Backer, J. M. (2008). The regulation and function of class iii pi3ks: Novel roles for vps34. *Biochem J*, 410, 1-17.

Bartucci, M., Morelli, C., Mauro, L., Ando, S., & Surmacz, E. (2001). Differential insulin-like growth factor i receptor signaling and function in estrogen receptor (er)-positive mcf-7 and er-negative mda-mb-231 breast cancer cells. *Cancer Res*, 61, 6747-6754.

Bauer, J. A., Ye, F., Marshall, C. B., Lehmann, B. D., Pendleton, C. S., Shyr, Y., et al. (2010). Rna interference (rnai) screening approach identifies agents that enhance paclitaxel activity in breast cancer cells. *Breast Cancer Res*, 12, R41.

Beatson, G. (1896). On the treatment of inoperable cases of carcinoma of the mamma: Suggestions for a new method of treatment, with illustrative cases. *The Lancet*, 148, 162-165.

Beattie, J., Allan, G. J., Lochrie, J. D., & Flint, D. J. (2006). Insulin-like growth factor-binding protein-5 (igfbp-5): A critical member of the igf axis. *Biochem J*, 395, 1-19.

Becker, M., Sommer, A., Kratzschmar, J. R., Seidel, H., Pohlenz, H. D., & Fichtner, I. (2005). Distinct gene expression patterns in a tamoxifen-sensitive human mammary carcinoma xenograft and its tamoxifen-resistant subline maca 3366/tam. *Mol Cancer Ther*, 4, 151-168.

Behrens, D., Gill, J. H., & Fichtner, I. (2007). Loss of tumourigenicity of stably erbeta-transfected mcf-7 breast cancer cells. *Mol Cell Endocrinol*, 274, 19-29.

Berns, K., Horlings, H. M., Hennessy, B. T., Madiredjo, M., Hijmans, E. M., Beelen, K., et al. (2007). A functional genetic approach identifies the pi3k pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell*, 12, 395-402.

Brockdorff, B. L., Heiberg, I., & Lykkesfeldt, A. E. (2003). Resistance to different antiestrogens is caused by different multi-factorial changes and is associated with reduced expression of igf receptor ialpha. *Endocr Relat Cancer*, 10, 579-590.

Brummelkamp, T. R., Bernards, R., & Agami, R. (2002). A system for stable expression of short interfering rnas in mammalian cells. *Science*, 296, 550-553.

Bunone, G., Briand, P. A., Miksicek, R. J., & Picard, D. (1996). Activation of the unliganded estrogen receptor by egf involves the map kinase pathway and direct phosphorylation. *EMBO J*, 15, 2174-2183.

Christoforidis, S., Miaczynska, M., Ashman, K., Wilm, M., Zhao, L., Yip, S. C., et al. (1999). Phosphatidylinositol-3-oh kinases are rab5 effectors. *Nat Cell Biol*, 1, 249-252.

Clarke, R., Leonessa, F., Welch, J. N., & Skaar, T. C. (2001). Cellular and molecular pharmacology of antiestrogen action and resistance. *Pharmacol Rev*, 53, 25-71.

Clarke, R., Liu, M. C., Bouker, K. B., Gu, Z., Lee, R. Y., Zhu, Y., et al. (2003). Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. *Oncogene*, 22, 7316-7339.

Cuzick, J., Powles, T., Veronesi, U., Forbes, J., Edwards, R., Ashley, S., et al. (2003). Overview of the main outcomes in breast-cancer prevention trials. *Lancet*, 361, 296-300.

Dahlman-Wright, K., Cavailles, V., Fuqua, S. A., Jordan, V. C., Katzenellenbogen, J. A., Korach, K. S., et al. (2006). International union of pharmacology. Lxiv. Estrogen receptors. *Pharmacol Rev*, 58, 773-781.

Dancey, J. (2010). Mtor signaling and drug development in cancer. *Nat Rev Clin Oncol*, 7, 209-219.

de Medina, P., Payre, B., Boubekeur, N., Bertrand-Michel, J., Terce, F., Silvente-Poirot, S., et al. (2009). Ligands of the antiestrogen-binding site induce active cell death and autophagy in human breast cancer cells through the modulation of cholesterol metabolism. *Cell Death Differ*, 16, 1372-1384.

deGraffenried, L. A., Friedrichs, W. E., Russell, D. H., Donzis, E. J., Middleton, A. K., Silva, J. M., et al. (2004). Inhibition of mtor activity restores tamoxifen response in breast cancer cells with aberrant akt activity. *Clin Cancer Res*, 10, 8059-8067.

Early Breast Cancer Trialists' Collaborative, G. (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: An overview of the randomised trials. *Lancet*, 365, 1687-1717.

Efeyan, A., & Sabatini, D. M. (2009). Mtor and cancer: Many loops in one pathway. *Curr Opin Cell Biol*, 22, 169-176.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., & Tuschl, T. (2001). Duplexes of 21-nucleotide rnas mediate rna interference in cultured mammalian cells. *Nature*, 411, 494-498.

Fagan, D. H., & Yee, D. (2008). Crosstalk between igf1r and estrogen receptor signaling in breast cancer. *J Mammary Gland Biol Neoplasia*, 13, 423-429.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded rna in caenorhabditis elegans. *Nature*, 391, 806-811.

Furuya, N., Yu, J., Byfield, M., Pattingre, S., & Levine, B. (2005). The evolutionarily conserved domain of beclin 1 is required for vps34 binding, autophagy and tumor suppressor function. *Autophagy*, 1, 46-52.

Futter, C. E., Collinson, L. M., Backer, J. M., & Hopkins, C. R. (2001). Human vps34 is required for internal vesicle formation within multivesicular endosomes. *J Cell Biol*, 155, 1251-1264.

Gao, P., Bauvy, C., Souquere, S., Tonelli, G., Liu, L., Zhu, Y., et al. (2010). The bcl-2 homology domain 3 mimetic gossypol induces both beclin 1-dependent and beclin 1-independent cytoprotective autophagy in cancer cells. *J Biol Chem*, 285, 25570-25581.

Garcia, M., Derocq, D., Freiss, G., & Rochefort, H. (1992). Activation of estrogen receptor transfected into a receptor-negative breast cancer cell line decreases the metastatic and invasive potential of the cells. *Proc Natl Acad Sci U S A*, 89, 11538-11542.

Gee, J. M., Robertson, J. F., Gutteridge, E., Ellis, I. O., Pinder, S. E., Rubini, M., et al. (2005). Epidermal growth factor receptor/her2/insulin-like growth factor receptor signalling and oestrogen receptor activity in clinical breast cancer. *Endocr Relat Cancer*, 12 Suppl 1, S99-S111.

Giacinti, L., Claudio, P. P., Lopez, M., & Giordano, A. (2006). Epigenetic information and estrogen receptor alpha expression in breast cancer. *Oncologist*, 11, 1-8.

Gilbert, L. A., & Hemann, M. T. (2010). DNA damage-mediated induction of a chemoresistant niche. *Cell*, 143, 355-366.

Gluckman, P., Klempt, N., Guan, J., Mallard, C., Sirimanne, E., Dragunow, M., et al. (1992). A role for igf-1 in the rescue of cns neurons following hypoxic-ischemic injury. *Biochem Biophys Res Commun*, 182, 593-599.

Gonzalez-Malerva, L., Park, J., Zou, L., Hu, Y., Moradpour, Z., Pearlberg, J., et al. (2011). High-throughput ectopic expression screen for tamoxifen resistance identifies an atypical kinase that blocks autophagy. *Proc Natl Acad Sci U S A*, 108, 2058-2063.

Gutierrez, M. C., Detre, S., Johnston, S., Mohsin, S. K., Shou, J., Allred, D. C., et al. (2005). Molecular changes in tamoxifen-resistant breast cancer: Relationship between estrogen receptor, her-2, and p38 mitogen-activated protein kinase. *J Clin Oncol*, 23, 2469-2476.

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., et al. (2002). Raptor, a binding partner of target of rapamycin (tor), mediates tor action. *Cell*, 110, 177-189.

Harvey, J. M., Clark, G. M., Osborne, C. K., & Allred, D. C. (1999). Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol*, 17, 1474-1481.

Herynk, M. H., & Fuqua, S. A. (2004). Estrogen receptor mutations in human disease. *Endocr Rev*, 25, 869-898.

Hirsch, D. S., Shen, Y., Dokmanovic, M., & Wu, W. J. (2010). Pp60c-src phosphorylates and activates vacuolar protein sorting 34 to mediate cellular transformation. *Cancer Res*, 70, 5974-5983.

Hopp, T. A., Weiss, H. L., Parra, I. S., Cui, Y., Osborne, C. K., & Fuqua, S. A. (2004). Low levels of estrogen receptor beta protein predict resistance to tamoxifen therapy in breast cancer. *Clin Cancer Res*, 10, 7490-7499.

Hoskins, J. M., Carey, L. A., & McLeod, H. L. (2009). Cyp2d6 and tamoxifen: DNA matters in breast cancer. *Nat Rev Cancer*, 9, 576-586.

Huynh, H., Yang, X. F., & Pollak, M. (1996). A role for insulin-like growth factor binding protein 5 in the antiproliferative action of the antiestrogen ici 182780. *Cell Growth Differ*, 7, 1501-1506.

Jensen, E. V., Block, G. E., Smith, S., Kyser, K., & DeSombre, E. R. (1971). Estrogen receptors and breast cancer response to adrenalectomy. *Natl Cancer Inst Monogr*, 34, 55-70.

Jordan, V. C. (1976). Effect of tamoxifen (ici 46,474) on initiation and growth of dmba-induced rat mammary carcinomata. *Eur J Cancer*, 12, 419-424.

Jordan, V. C. (1994). The development of tamoxfen for breast cancer therapy. In V. C. Jordan (Ed) *Long-term tamoxifen treatment for breast cancer* pp. 3-26). Madison: University of Wisconsin Press.

Jordan, V. C. (2006). Tamoxifen (ici46,474) as a targeted therapy to treat and prevent breast cancer. *Br J Pharmacol*, 147 Suppl 1, S269-276.

Jordan, V. C., & Dowse, L. J. (1976). Tamoxifen as an anti-tumour agent: Effect on oestrogen binding. *J Endocrinol*, 68, 297-303.

Jordan, V. C., & Koerner, S. (1975). Tamoxifen (ici 46,474) and the human carcinoma 8s oestrogen receptor. *Eur J Cancer*, 11, 205-206.

Juul, N., Szallasi, Z., Eklund, A. C., Li, Q., Burrell, R. A., Gerlinger, M., et al. (2010). Assessment of an rna interference screen-derived mitotic and ceramide pathway metagene as a predictor of response to neoadjuvant paclitaxel for primary triple-negative breast cancer: A retrospective analysis of five clinical trials. *Lancet Oncol*, 11, 358-365.

Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., et al. (1995). Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science*, 270, 1491-1494.

Kennedy, B. J. (1965). Hormone therapy for advanced breast cancer. *Cancer*, 18, 1551-1557.

Kim, D. H., Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., et al. (2002). Mtor interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell*, 110, 163-175.

Knowlden, J. M., Hutcheson, I. R., Barrow, D., Gee, J. M., & Nicholson, R. I. (2005). Insulin-like growth factor-i receptor signaling in tamoxifen-resistant breast cancer: A supporting role to the epidermal growth factor receptor. *Endocrinology*, 146, 4609-4618.

Knupfer, H., & Preiss, R. (2007). Significance of interleukin-6 (il-6) in breast cancer (review). *Breast Cancer Res Treat*, 102, 129-135.

Kondo, Y., Kanzawa, T., Sawaya, R., & Kondo, S. (2005). The role of autophagy in cancer development and response to therapy. *Nat Rev Cancer*, 5, 726-734.

Kroemer, G., Marino, G., & Levine, B. (2010). Autophagy and the integrated stress response. *Mol Cell*, 40, 280-293.

Kuilman, T., Michaloglou, C., Vredeveld, L. C., Douma, S., van Doorn, R., Desmet, C. J., et al. (2008). Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell*, 133, 1019-1031.

Kushner, P. J., Agard, D. A., Greene, G. L., Scanlan, T. S., Shiau, A. K., Uht, R. M., et al. (2000). Estrogen receptor pathways to ap-1. *J Steroid Biochem Mol Biol*, 74, 311-317.

Lee, A. V., Weng, C. N., Jackson, J. G., & Yee, D. (1997). Activation of estrogen receptor-mediated gene transcription by igf-i in human breast cancer cells. *J Endocrinol*, 152, 39-47.

Levine, B., & Kroemer, G. (2008). Autophagy in the pathogenesis of disease. *Cell*, 132, 27-42.

Lewis, J. S., & Jordan, V. C. (2005). Selective estrogen receptor modulators (serms): Mechanisms of anticarcinogenesis and drug resistance. *Mutat Res*, 591, 247-263.

Lindmo, K., Brech, A., Finley, K. D., Gaumer, S., Contamine, D., Rusten, T. E., et al. (2008). The pi 3-kinase regulator vps15 is required for autophagic clearance of protein aggregates. *Autophagy*, 4, 500-506.

McDonnell, D. P., Clemm, D. L., Hermann, T., Goldman, M. E., & Pike, J. W. (1995). Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens. *Mol Endocrinol*, 9, 659-669.

Michiels, F., van Es, H., van Rompaey, L., Merchiers, P., Francken, B., Pittois, K., et al. (2002). Arrayed adenoviral expression libraries for functional screening. *Nat Biotechnol*, 20, 1154-1157.

Mokbel, K. (2002). The evolving role of aromatase inhibitors in breast cancer. *Int J Clin Oncol*, 7, 279-283.

Musgrove, E. A., & Sutherland, R. L. (2009). Biological determinants of endocrine resistance in breast cancer. *Nat Rev Cancer*, 9, 631-643.

Ngo, V. N., Davis, R. E., Lamy, L., Yu, X., Zhao, H., Lenz, G., et al. (2006). A loss-of-function rna interference screen for molecular targets in cancer. *Nature*, 441, 106-110.

Nicholson, R. I., & Golder, M. P. (1975). The effect of synthetic anti-oestrogens on the growth and biochemistry of rat mammary tumours. *Eur J Cancer*, 11, 571-579.

Osborne, C. K. (1998). Tamoxifen in the treatment of breast cancer. *N Engl J Med*, 339, 1609-1618.

Pacheco, C. D., Kunkel, R., & Lieberman, A. P. (2007). Autophagy in niemann-pick c disease is dependent upon beclin-1 and responsive to lipid trafficking defects. *Hum Mol Genet*, 16, 1495-1503.

Panaretou, C., Domin, J., Cockcroft, S., & Waterfield, M. D. (1997). Characterization of p150, an adaptor protein for the human phosphatidylinositol (ptdins) 3-kinase. Substrate presentation by phosphatidylinositol transfer protein to the p150.Ptdins 3-kinase complex. *J Biol Chem*, 272, 2477-2485.

Parisot, J. P., Leeding, K. S., Hu, X. F., DeLuise, M., Zalcberg, J. R., & Bach, L. A. (1999). Induction of insulin-like growth factor binding protein expression by ici 182,780 in a tamoxifen-resistant human breast cancer cell line. *Breast Cancer Res Treat*, 55, 231-242.

Paruthiyil, S., Parmar, H., Kerekatte, V., Cunha, G. R., Firestone, G. L., & Leitman, D. C. (2004). Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a g2 cell cycle arrest. *Cancer Res*, 64, 423-428.

Payre, B., de Medina, P., Boubekeur, N., Mhamdi, L., Bertrand-Michel, J., Terce, F., et al. (2008). Microsomal antiestrogen-binding site ligands induce growth control and

differentiation of human breast cancer cells through the modulation of cholesterol metabolism. *Mol Cancer Ther*, 7, 3707-3718.

Perez, E. A. (2007). Safety profiles of tamoxifen and the aromatase inhibitors in adjuvant therapy of hormone-responsive early breast cancer. *Ann Oncol*, 18 Suppl 8, viii26-35.

Pollak, M. (2008). Insulin and insulin-like growth factor signalling in neoplasia. *Nat Rev Cancer*, 8, 915-928.

Pollak, M., Schernhammer, E. S., & Hankinson, S. E. (2004). Insulin-like growth factors and neoplasia. *Nat Rev Cancer*, 4, 505-518.

Pratt, S. E., & Pollak, M. N. (1993). Estrogen and antiestrogen modulation of mcf7 human breast cancer cell proliferation is associated with specific alterations in accumulation of insulin-like growth factor-binding proteins in conditioned media. *Cancer Res*, 53, 5193-5198.

Riggins, R. B., Schrecengost, R. S., Guerrero, M. S., & Bouton, A. H. (2007). Pathways to tamoxifen resistance. *Cancer Lett*, 256, 1-24.

Ring, A., & Dowsett, M. (2004). Mechanisms of tamoxifen resistance. *Endocr Relat Cancer*, 11, 643-658.

Saji, S., Hirose, M., & Toi, M. (2005). Clinical significance of estrogen receptor beta in breast cancer. *Cancer Chemother Pharmacol*, 56 Suppl 1, 21-26.

Salerno, M., Sisci, D., Mauro, L., Guvakova, M. A., Ando, S., & Surmacz, E. (1999). Insulin receptor substrate 1 is a target for the pure antiestrogen ici 182,780 in breast cancer cells. *Int J Cancer*, 81, 299-304.

Schiff, R., Massarweh, S., Shou, J., & Osborne, C. K. (2003). Breast cancer endocrine resistance: How growth factor signaling and estrogen receptor coregulators modulate response. *Clin Cancer Res*, 9, 447S-454S.

Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D., & Emr, S. D. (1993). Phosphatidylinositol 3-kinase encoded by yeast vps34 gene essential for protein sorting. *Science*, 260, 88-91.

Sharma, D., Saxena, N. K., Davidson, N. E., & Vertino, P. M. (2006). Restoration of tamoxifen sensitivity in estrogen receptor-negative breast cancer cells: Tamoxifen-bound reactivated er recruits distinctive corepressor complexes. *Cancer Res*, 66, 6370-6378.

Stack, J. H., & Emr, S. D. (1994). Vps34p required for yeast vacuolar protein sorting is a multiple specificity kinase that exhibits both protein kinase and phosphatidylinositol-specific pi 3-kinase activities. *J Biol Chem*, 269, 31552-31562.

Suarez-Cuervo, C., Harris, K. W., Kallman, L., Vaananen, H. K., & Selander, K. S. (2003). Tumor necrosis factor-alpha induces interleukin-6 production via extracellular-regulated kinase 1 activation in breast cancer cells. *Breast Cancer Res Treat*, 80, 71-78.

Sugiura, H., Toyama, T., Hara, Y., Zhang, Z., Kobayashi, S., Fujii, Y., et al. (2007). Expression of estrogen receptor beta wild-type and its variant erbetacx/beta2 is correlated with better prognosis in breast cancer. *Jpn J Clin Oncol*, 37, 820-828.

Swanton, C., Marani, M., Pardo, O., Warne, P. H., Kelly, G., Sahai, E., et al. (2007). Regulators of mitotic arrest and ceramide metabolism are determinants of sensitivity to paclitaxel and other chemotherapeutic drugs. *Cancer Cell*, 11, 498-512.

Takahashi, Y., Coppola, D., Matsushita, N., Cualing, H. D., Sun, M., Sato, Y., et al. (2007). Bif-1 interacts with beclin 1 through uvrag and regulates autophagy and tumorigenesis. *Nat Cell Biol*, 9, 1142-1151.

Tzukerman, M. T., Esty, A., Santiso-Mere, D., Danielian, P., Parker, M. G., Stein, R. B., et al. (1994). Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. *Mol Endocrinol*, 8, 21-30.

Valentinis, B., & Baserga, R. (2001). Igf-i receptor signalling in transformation and differentiation. *Mol Pathol*, 54, 133-137.

Volinia, S., Dhand, R., Vanhaesebroeck, B., MacDougall, L. K., Stein, R., Zvelebil, M. J., et al. (1995). A human phosphatidylinositol 3-kinase complex related to the yeast vps34p-vps15p protein sorting system. *EMBO J*, 14, 3339-3348.

Waters, E. A., Cronin, K. A., Graubard, B. I., Han, P. K., & Freedman, A. N. (2010). Prevalence of tamoxifen use for breast cancer chemoprevention among u.S. Women. *Cancer Epidemiol Biomarkers Prev*, 19, 443-446.

Yan, Y., Flinn, R. J., Wu, H., Schnur, R. S., & Backer, J. M. (2009). Hvps15, but not ca2+/cam, is required for the activity and regulation of hvps34 in mammalian cells. *Biochem J*, 417, 747-755.

Young, A. R., Narita, M., Ferreira, M., Kirschner, K., Sadaie, M., Darot, J. F., et al. (2009). Autophagy mediates the mitotic senescence transition. *Genes Dev*, 23, 798-803.

Zhong, Y., Wang, Q. J., Li, X., Yan, Y., Backer, J. M., Chait, B. T., et al. (2009). Distinct regulation of autophagic activity by atg14l and rubicon associated with beclin 1-phosphatidylinositol-3-kinase complex. *Nat Cell Biol*, 11, 468-476.

Edited by Esra Gunduz and Mehmet Gunduz

Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various therapeutic modalities from signaling pathways through various anti-tumor compounds as well as herbal medicine for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

ISBN 978-953-51-6557-6

Photo by ancroft / iStock

IntechOpen