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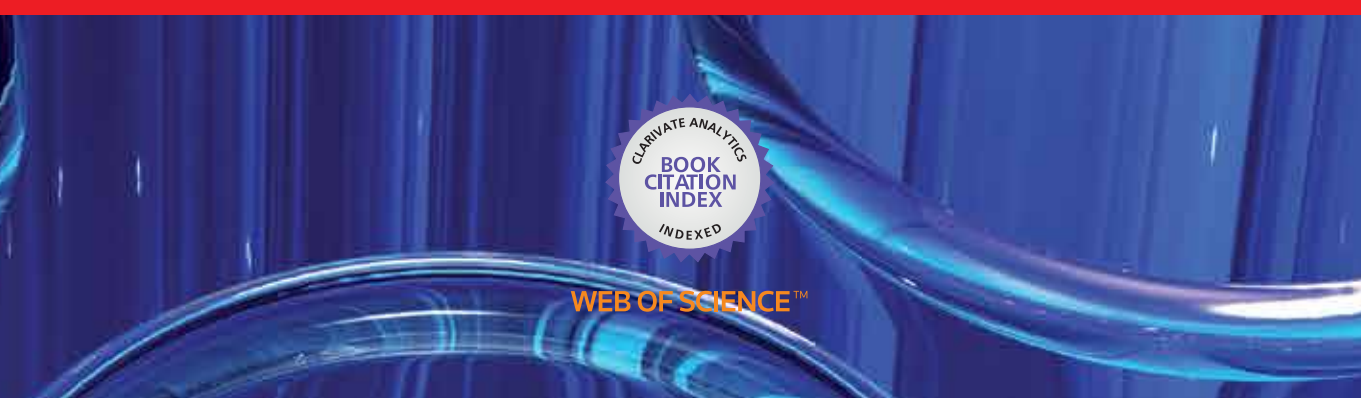
# Using Old Solutions to New Problems

## Natural Drug Discovery in the 21st Century

*Edited by Marianna Kulka*



# USING OLD SOLUTIONS TO NEW PROBLEMS - NATURAL DRUG DISCOVERY IN THE 21ST CENTURY

Edited by **Marianna Kulka**

**Using Old Solutions to New Problems - Natural Drug Discovery in the 21st Century**
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# Meet the editor

Dr. Marianna Kulka obtained her PhD from the University of Alberta in Edmonton, Alberta, Canada where she studied the role of ion channels in mast cell activation. Dr. Kulka completed her research fellowship at the National Institutes of Health in Bethesda, Maryland, USA where she became interested in human mast cell differentiation and expression of pathogen recognition receptors. Dr. Kulka was a Research Assistant Professor at Northwestern University and investigated mast cells and their role in allergic inflammation until she accepted a research position at the National Research Council (NRC) Canada in 2007. At the NRC, Dr. Kulka has established an immunology research group that is interested in skin microenvironments, human mast cell biology and the use of nanotechnology to improve human health.

# Contents

# Preface

Modern medicine has been, and will continue to be, extremely dependent upon the discovery of new drugs. As our modern civilization continues to develop, we will be challenged with new and more difficult-to-treat diseases. In large part, these diseases will be the consequence of our rapidly changing lifestyles to which we have not had time to evolve appropriate biological coping mechanisms – obesity and our sedentary lifestyle being just one obvious example. The rise in obesity-related disease, allergic inflammation and certain types of cancers has challenged us to create a new arsenal of drugs that are capable of treating or even preventing these complex diseases. If our search for new medicines is to be successful, we must consider naturally-sourced bioactives as a good starting point. After all, many of our modern drugs are derivatives of natural products and this historical pharmacopeia probably contains a great deal more compounds that have beneficial biologic activity.

The word "drug" is not always associated with herbs and naturally-derived medicine. In fact, herbal and naturopathic remedies have been dissociated from one another in our modern medical lexicon. In essence, however, herbal and folk medicines were the first drugs in the truest sense of the term. The word "drug" is etymologically derived from the Middle Dutch or German word droog or droge meaning "dry barrels" or droge waere, meaning "dry wares" but specifically drugs and spices. The word drogue was later used in Old French in the 14th century meaning "supply, stock or provision" again probably referring to the supply of dried herbs and plants used for medicinal purposes. The word drug was associated with poisons in the 16th century and was later applied to narcotics and opiates in the late 19th century.

Today, the word drug can be generally defined as any chemical or biological agent that affects biologic processes. Drugs can have medicinal, enhancing or intoxicating effects on any living organism, including humans. Drugs can be chemical compounds used in the diagnosis, treatment or prevention of disease but drugs can also be biological products (such as blood products or hormones) used to enhance an athlete's performance in a sport, for example.

Drugs are defined according to their specific ability to affect or modify an abnormal biological condition such as disease or illness. However, medicine has always incorporated a variety of strategies to achieve health – not just drugs – and our modern understanding of healthy diets and enriched foods has given rise to a number of new terms that represent these various approaches to modern health. Therefore, it is useful to define some of these terms like "functional foods" and "nutraceuticals" in the context of what constitutes a drug. "Natural Health Products" or NPH are defined in the US as .....

Nutritional foods consumed for healthy and normal biological functions are not considered drugs, but if the same foods are consumed, often in a more pure form, with the specific pur-

pose of treating a pathological condition, they may be classified as a drug. Foods that have been infused with biological compounds that give them more healthful characteristics (vitamin-enriched water, for example) have been classified as "functional foods" because they function to either prevent disease or alleviate symptoms associated with a particular illness. Still yet are nutraceuticals which refers to products that range from isolated nutrients, dietary supplements and herbal products and is a term that was first coined by Dr. Stephen L. DeFelice, founder and chairman of the Foundation of Innovation Medicine in Crawford, New Jersey. In the US, the term nutraceutical has no legal meaning and most "nutraceutical" products are regulated as drugs, dietary supplements, food ingredients or food. In Canada, the term is defined by Health Canada as "a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease." The term nutriceutical is new, but the idea is not. Hippocrates, considered by many to be the father of modern medicine, wrote in his corpus wrote "let food by thy medicine" and health practitioners have promoted certain foods and drinks as fundamentally "healthy" for centuries. It is from these origins that drug discovery undoubtedly began.

The ancient Egyptians, Indians and Chinese all had extensive treatises of herbs or mixtures to alleviate and treat disease. The Ebers Papyrus, dating back to 1550 BC, for example, lists about 800 ancient Egyptian prescriptions for over 700 remedies. Most of the Egyptian drugs have been identified with drugs and active compounds that have been described in current formulations and recipes. The Indian Ayurveda, dating back to 900BC, has been used in India for thousands of years and describes the use of plants for ailments such as hypertension and mental disease. Greek and Latin medicine was heavily influenced by Egyptian medicine and reached a peak during Dioscorides in the first century AD. Claudius Galen was a physician and pharmacist and his approach to therapy often involved complex mixtures called "galenicals" from which he believed the body would select the appropriate medicinal ingredient that it needed.

With the fall of the Roman Empire, the use of medicinal plants came to be associated with witchcraft and sorcery and ancient science was, for the most part, neglected by western society. However, some scholars preserved and transmitted some of this vast information and Islamic schools and monastic abbeys played an important part in this process. By the Renaissance, some of this ancient information was re-discovered and revised. Valerius Cordus was a German botanist who in 1542 wrote a botanic treatise in which he introduced "laudanum," a formulation that contained opium extract and was used widely by European and North American doctors until the mid-19th century.

Modern drug discovery began with the breakthroughs in chemical and biological sciences in the 19th century wherein the isolation, chemical characterization and analytical determination of bioactive molecules could be done. It was at this point that the chemical identity of these bioactive compounds could finally be defined and this knowledge was used to create more effective, potent and targeted drugs that were also more stable and had fewer side-effects. This new field of drug discovery is beautifully examined in the first chapter of this book.

As drug discovery progressed, we made new leaps in our understanding of molecular structures and how they can be manipulated to make bioactive compounds much more potent or more specific. This allowed us to not only create "designer" drugs but gave us the opportunity to go back to the ancient pharmacopeia to find similar compounds.

As our population ages, our need for new and more dynamic medications will increase. The diverse set of professionals that are now involved in drug discover must all work together to achieve this goal.

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Section 1

# Agronomy and Molecular Characterization

Chapter 1

# Discovery, Development, and Regulation of Natural Products

Juergen Krause and Gailene Tobin

Additional information is available at the end of the chapter

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## 1. Introduction

Natural products have historically been an extremely productive source for new medicines in all cultures and continue to deliver a great variety of structural templates for drug discovery and development. Although products derived from natural sources may not necessarily represent active ingredients in their final form, the majority of all drugs in the market have their origin in nature [1, 2]. Therefore, the foremost emphasis in this chapter is given to aspects concerning the identification, properties, and development of potential drug candidates from natural products. It is the intention to give a high-level overview of the current status and developments in the field. Many important aspects in the arena of natural therapeutics including natural product sources, discovery, characterization, development and uses have been addressed and covered in depth in excellent recent reviews by extremely competent authors referenced in this contribution.

### 1.1. Definition of a natural product

The extent to which the term natural product has been characterized is both limited and debatable. Therefore, a common definition that is accepted by all involved disciplines will remain a moving target, but likely will evolve as researchers unveil the vast amount of compounds projected to be discovered in this field [3]. In the simplest of terms, a natural product is a small molecule that is produced by a biological source [3]. As a central theme of exploration bordering chemistry and biology, natural products research focuses on the chemical properties, biosynthesis and biological functions of secondary metabolites [3]. In this context, the task of defining "natural" is more straight forward and encompasses isolation

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from a native organism, synthesis in a laboratory, biosynthesis *in vitro*, or isolation from a metabolically engineered organism whereby the chemical structure has been determined and the resultant compound is chemically equivalent to the original natural product [3]. Thus, in summary, and for the purposes of this chapter, one can still agree with the refuted definition that a natural product is a pharmacologically or biologically active chemical compound or substance, which is found in nature and produced by a living organism and can even be considered as such if it can be prepared by a totally synthetic approach [4]. Albeit, we realize this definition can be challenged as many biosynthetic enzymes are nonspecific and may result in the production of multiple analogs combined with the fact that identifying the entirety of natural products is in the infant stage [5].

Generally the term "natural product" is regarded as being synonymous with "secondary metabolite" [6]. Secondary metabolites are organic compounds in the correct chiral configuration to exert biological activity, but have no "primary" function directly involved in the normal growth, development or reproduction of an organism [7]. Natural products are usually relatively small molecules with a molecular weight below 3,000 Daltons and exhibit considerable structural diversity [6]. The product categories in which natural compounds can be found as active ingredients include prescription and non-prescription drugs (pharmaceuticals), cosmetic ingredients (cosmeceuticals) and dietary supplements and natural health product ingredients (nutriceuticals) [8].

The respective studies leading to the identification, isolation, and characterization of natural products constitute an important part of the scientific field of pharmacognosy. The American Society of Pharmacognosy defines pharmacognosy as "*the study of natural product molecules (typically secondary metabolites) that are useful for their medicinal, ecological, gustatory, or other functional properties. The natural species that are the source of the compounds under study span all biological kingdoms, most notably marine invertebrates, plants, fungi, and bacteria*" [9]. Amongst the various assortments and exciting capacities that are being explored within the arena of pharmacognosy, this chapter will mostly address the study of health relevant medicinal properties of natural compounds for drug discovery and development.

### 1.2. History

Natural substances have evolved over a very long selection process to form optimal interactions with biological macromolecules [10] which have activity on a biological system that is relevant to the target disease. They have historically been the most productive source of active compounds and chemical lead structures for the discovery and development of new medicines [11]. Since ancient times, civilizations used plants and plant extracts to ameliorate diseases and foster healing. Early historic examples for medical treatments from natural sources include the discovery of the beneficial effects of *cardiotonic digitalis* extracts from foxglove for treating some manifestations of heart disease in the 18$^{th}$ century, the use of the bark of the willow and cinchona trees in treating fever and the effectiveness of poppy extracts in the treatment of

dysenteries [12]. Morphine, largely reproducing the analgesic and sedative effect of opium, was isolated from opium obtained from the seed pots of the poppy plant in 1804 [12]. Throughout the century, purified bioactive natural products were extracted from the Peruvian bark cinchoa (quinine), from cocoa (cocaine), and from many other plants [12]. By 1829, scientists discovered that the compound salicin, in willow trees, was responsible for pain relief and in 1838 salicylic acid was isolated [13]. The problem was that salicylic acid was harsh on the stomach and in the second half of the 19th century acetylsalicylic acid was synthesized which served as a less-irritating replacement for standard common salicylate medicines [13]. A number of additional plants served as sources of natural product derived agents that are still used in current routine medical practice [14].

The discovery of valuable therapeutic agents from natural sources continued into the 20th century. Inspired by the discovery and benefits of penicillin, pharmaceutical research expanded after the Second World War into intensive screening of microorganisms for new antibiotics [12]. The study of new bacterial and fungal strains resulted in the expansion of the antibacterial arsenal with additional agents such as cephalosporins, tetracyclines, aminoglycosides, rifamycins, chloramphenicol, and lipopeptides [15, 16]. In the 1950's, two nucleosides isolated from Caribbean marine sponges paved the way for the synthesis of vidarabine, and the related compound cytarabine, which eventually received approval as therapeutics for clinical use in viral diseases and cancer, respectively [17]. A more recent example is the cancer therapeutic paclitaxel (Taxol®) derived from the Yew tree, which was discovered in the 1970s, but due to difficulties in obtaining commercial compound quantities only reached the market in late 1992 [18-20]. Overall, only 244 prototypic chemical structures (over 80% came from animal, plant, microbial or mineral origin) have been used as templates to produce medicines up to 1995, and relatively few new scaffolds have appeared since [21,22]. About half of the marketed agents in today's arsenal of drugs are derived from biological sources with the large majority being based on terrestrial natural product scaffolds [23]. Approximately 50% of the new drugs introduced since 1994 were either natural products or derivatives thereof [21, 23, 24].

## 2. Discovery and development

### 2.1. Discovery

Drug discovery involves the identification of new chemical entities (NCEs) of potential therapeutic value, which can be obtained through isolation from natural sources, through chemical synthesis or a combination of both. The field of natural products drug discovery, despite the success stories of penicillin, paclitaxel, etc., also had aspects that made it less attractive. In the traditional approach, drug targets were exposed to crude extracts, and in case of evidence of pharmacological activity the extract was fractionated and the active compound isolated and identified. This method was slow, labor intensive, inefficient, and provided no guarantee that a lead from the screening process would be chemically workable or even patentable [25, 26]. As natural products usually are molecules with more complex structures,

it was more difficult to extract, purify or synthesize sufficient quantities of a NCE of interest for discovery and development activities [25]. Enriched or pure material is needed for the initial characterization of the chemical and biological properties as well as the elucidation of structure-activity relationships in drug discovery studies; furthermore, even larger quantities need to be supplied for potential later development activities and ultimately, the market [24, 27].

The pharmaceutical industry's interest in natural products diminished with the advent of such promising new technologies like combinatorial chemistry (CC) and high throughput screening (HTS) [28]. The prospect of such disciplines, aimed at accelerating drug discovery efforts for NCEs, led some companies to dismiss their natural product programs [28]. Combinatorial chemistry employs parallel synthesis techniques allowing the creation of libraries containing hundreds of thousands of compounds, whereas HTS allows rapid testing of large numbers of compounds [28]. High-throughput screening grew out of automated clinical analyzer technologies and miniaturization in the late 1980's, as drug companies focused on methods aiming to increase the pace of testing and lower the cost per sample [12]. As a result, large libraries of synthetic molecules could be exploited very quickly. These new synthetic libraries were also given preference because of the lack of compatibility of traditional natural product extract libraries with HTS assays [28-30]. Compounds obtained from commercial libraries, in-house collections of pharmaceutical companies containing hundreds of thousands of compounds and new libraries generated through CC could be now screened rapidly [21]. Although the initial hopes for such advances were high, they were not fulfilled by either of the improved technologies. To be successful, HTS needed appropriate therapeutic targets matched to collections of NCEs that are highly diverse in their structural and physicochemical properties. The approach to exclusively bank on synthetic compounds did not meet the initial expectations, as the newly created compound libraries had limited structural diversity and did not provide enough quality hits to be of value. For CC, the most valuable role of parallel synthesis therefore appears to be in expanding on an existing lead, rather than creating new screening libraries [12]. Consequently, the interest in natural sources experienced some renaissance; however, even if natural product extracts were tested first, the pace of their isolation made it difficult to keep up with the demand for testing candidates in high-throughput models [25, 26, 29]. Therefore, natural products, and derivatives thereof, are still under-represented in the typical screening decks of the pharmaceutical and biopharmaceutical industry [31]. Specifically, it has been noted that major pharmaceutical companies in the United States continue to favor approaches that do not enable the integration of natural products of marine origin into their screening libraries [32]. More risk friendly institutions like academic laboratories, research institutes and small biotech companies venturing in the natural products arena have now a greater role in drug discovery and feed candidates into the development pipelines of big pharmaceutical companies[32].

Overall, there are limited systematic approaches to exploring traditionally used natural products for compounds that could serve as drug leads. Additionally, the pharmaceutical industry has decreased their emphasis on natural product discovery from sources in various countries. Both of these facts may be based on possible uncertainties and concerns over expectations about benefits sharing resulting from the United Nations Convention on Biolog-

ical Diversity (CBD) [21, 33, 34]. Countries are increasingly protective of their natural assets in flora and fauna and may not authorize the collection of sample species without prior approval [35]. In this context, potential handicaps may arise for companies as they develop and market new products from natural sources in the form of very difficult to negotiate agreements as well as significant intellectual property and royalty issues [25, 26, 28, 35].

Nonetheless, natural products continue to provide a valuable and rich pool for the discovery of templates and drug candidates and are suitable for further optimization by synthetic means because the chemical novelty associated with natural products is higher than that of structures from any other source [10]. This fact is of particular importance when seeking out lead molecules against newly discovered targets where no small molecule lead exists or in mechanistic and pathway studies when searching for chemical probes [24]. It is assumed that. in many cases, structures devised by nature and evolution are far superior to even the best synthetic moieties in terms of diversity, specificity, binding efficiency, and propensity to interact with biological targets [24]. In comparing a large number of natural products to compounds from CC and synthetic drugs derived from natural substances, it has become evident that drugs and products obtained from natural sources exhibited more diverse and chemically complex structures [36]. In fact, only a moderate structural overlap was found when comparing natural product scaffolds to drug collections with the natural product database containing a significantly larger number of scaffolds and exhibiting higher structural novelty [37]. The structural diversity of these naturally sourced compounds supports the belief that the assortment of natural products represents a greater variety and better exemplifies the 'chemical space' of drug-like scaffolds than those of synthetic origin [30, 38, 39]. As Newman and Cragg (2012) have stated, and demonstrated in their reviews for the 30-year period of 1981 to 2010, natural products do play a dominant role in the discovery of lead structures for the development of drugs for the treatment of human diseases [1]. We agree with these authors in their assumption that it is highly probable that in the near future totally synthetic variations of even complex natural products will be part of the arsenal of physicians [1].

In general, there is growing awareness of the limited structural diversity in existing compound collections. The historic focus of the pharmaceutical industry on a relatively small set of 'druggable' targets has resulted in the exploration of a very narrow chemical space appropriate for these targets [40]. The 207 human targets described for small-molecule drugs correspond to only about 1% of the human genome and half of all drugs target only four protein classes [41]. So called 'undruggable' targets, such as protein-protein interactions and phosphatases, still await the identification of lead structures with the required qualities for lead or development candidates [40]. Although the expectations in natural products for the future are still high, an analysis of the distinct biological network between the targets of natural products and disease genes revealed that natural products, as a group, may still not contain enough versatility to yield suitable treatments for all heritable human diseases [42]. Nevertheless, the importance of natural product related compound collections, as the most promising avenue to explore new bioactive chemical space for drug discovery, continues to be emphasized; consequently, efforts have been made over the last decade to generate CC libraries inspired by natural product scaffolds [31, 43, 44]. Those scaffolds, which have presumably undergone

evolutionary selection over time, might possess favorable properties in terms of bioactivity and selectivity and therefore provide biologically validated valuable starting points for the design and generation of new combinatorial libraries [25, 26, 45, 46]. Thomas and Johannes state that the production of relatively small natural product like libraries have revealed biologically active compounds, while modification of natural products identified activity that is entirely unrelated to the parent molecules [31].

Libraries of small molecules of natural origin have already served as templates for the majority of approved therapeutics including important compounds for the treatment of life-threatening conditions. Moreover, these small molecule libraries are constantly growing through products extracted from various natural sources. Harvey *et al.* reviewed the current approaches for expansion of natural product based compound libraries and CBD compliant collections exist at the U.S. National Cancer Institute, academic institutions and commercial companies [11]. However, large collections of pure natural products are rare and the quantities of individual compounds that are isolated are typically small. A more recent strategy has been to use natural product scaffolds as templates for creating libraries of semi-synthetic and synthetic analogues [21, 28, 47]. Rosen *et al.* identified several hundred unique natural products which could serve as starting points in the search for novel leads with particular properties [48]. Based on the continuous efforts of researchers in the field of marine drug discovery, more potent bioactive lead structures are expected with new or unknown mechanisms of action [23, 48]. The progress made in the areas of cellular biology, genomics, and molecular mechanisms increased the number of druggable targets, allowing screening for candidates of natural compound libraries against an ever increasing number of potential molecular sites for therapeutic intervention. This increase in defined molecular targets combined with more automatization, more sensitive detection instruments, and faster data processing allows for high throughput assays, which can rapidly screen large existing libraries of new and specific biological targets.

In the last decade there has also been a major shift to technologically advanced and more complex screening assays conducted in cells, including those in which biological function is directly measured. These more complex approaches provide higher stringency which can mean lower hit rates. However, the specificity of such hits results in an increase in the quality of leads with more desired biological properties [12]. In this context, bioassays based on zebrafish embryos are noteworthy, as they can be used in 96-well plates and allow for *in vivo* bioactivity screening of crude extracts and natural substances at the microgram scale [49-52]. A further improvement, potentially leading to new secondary metabolites of interest for drug discovery, is based on the development of refined analytical and spectroscopic methods. This involves rapid identification and structural elucidation (dereplication) of natural products in complex mixtures (such as crude or pre-fractionated extracts) in parallel with profiling their bioactivity in information-rich bioassays [53]. In addition, stress can be applied to stimulate the number and levels of bioactive compounds in organisms. Wolfender and Queiroz presented examples of dynamic responses resulting from stress, which induced chemical defenses in elicitation experiments in both plants and microorganisms [30]. A significantly increased number of hits, including antibacterial, antifungal and anticancer agents were described for extracts from elicited plants [30]. New groups of microorganisms obtained through small scale,

high-through-put cultivation methods and employing nutrient deficient media, specific nutrients and long cultivation times constitute another approach potentially leading to new secondary metabolites of interest for drug discovery [54]. Genome mining, the analyses of plant and microbial genome sequences for genes and gene clusters encoding proteins, is a further recent approach which has allowed the discovery of numerous novel natural products and also revealed gene clusters and novel pathways for the biosynthesis of several known natural compounds [55, 56].

Although plants are still the major source for many natural products and remedies, microbes and marine organisms also constitute promising, abundant, and valuable sources for bioactive natural compounds [57]. Like it is true for plants, also for these, only a very small fraction of structures of potential therapeutic relevance have been chemically analyzed or examined in a broad panel of screening models or bioassays. But even if discovered and identified, active substances from natural sources may not be readily available for further investigations, development or introduction to the market. A number of biologically relevant natural products can only be isolated in small amounts, consequently adding to efforts, timelines and costs by forcing the development of an economically viable synthesis [31].

### 2.2. Development

The time required to develop a pharmaceutical can range from only a few to as many as 20 years. For natural products, an additional challenge can be the provision of sufficient quantities from natural sources for development and consequently commercial market supplies. Early *in vitro* tests may only require microgram to milligram amounts but the demand for compound quantities will increase quickly when *in vivo* animal models, safety and toxicology studies, formulation development and ultimately clinical trials are initiated. As mentioned earlier, one of the more recent respective examples is the cancer drug paclitaxel (Taxol®), which was discovered in 1967 as the cytotoxic active ingredient in extracts of *Taxus brevifolia* but only approved for the market in 1992 [20]. From 1967 to 1993, almost all paclitaxel produced was derived from bark from the Pacific yew tree [18]. Harvesting of the bark kills the tree in the process, however, this production method was replaced by a more sustainable approach using a precursor of Taxol® isolated from the leaves and needles of cultivated yew tree species [18, 20].

The compounds in development today target a variety of indications, mainly cancer and infectious diseases (bacterial, viral, fungal, and parasitic), but also address other therapeutic areas such as cardiovascular diseases, neurological illnesses and depression, metabolic diseases (like diabetes and cholesterol management), and inflammatory diseases (like arthritis) [1, 15, 16, 25, 26]. The cytotoxic properties of many secondary metabolites from marine organisms and bacteria are of particular interest for the development of new anticancer treatments [58]. For infectious diseases, natural products are effective because most of these compounds evolved from microbial warfare and show activity against other microorganisms at low concentrations [25, 26, 29]. The renewed interest in natural drugs is determined by the urgent need to find and develop effective means to fight infections caused by viruses, like HIV (Human Immunodeficiency Virus) and so called “superbugs” (bacteria with multiple resist-

ance against antibiotics) currently in use [29]. Pathogens having only limited and rather expensive treatment options include penicillin-resistant *Streptococcus pneumonia*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), *Clostridium difficile*, and *Mycobacterium tuberculosis* [29]. However, some new structures identified from marine fungi exhibited activity against bacteria like MRSA [59].

Before the advent of high throughput screening and the post-genomic era, more than 80% of drug substances or active ingredients were natural products, semisynthetic analogs thereof, or were obtained using structures of natural compounds as templates for synthetic modification [60, 61]. Chin reported 23 drugs from natural sources being approved between 2000 and 2005 [2]. Between 1998 and 2007 a total of 34 natural products and natural product-derived drugs were approved in different international markets [15, 62, 63].

According to Brahmachari (2011), 38 natural product-derived drugs were approved in the decade from 2000 to 2010 for various indications including 15 for infectious diseases, 7 each for oncology, neurological diseases and cardiovascular disorders, 4 for metabolic disorders and 1 for diabetes [22]. It is therefore not surprising that by 2008 more than a hundred new drug candidates from natural sources like plants, bacteria, fungi and animals or those obtained semi-synthetically were reported to be in clinical development with a similar number in preclinical development [60]. Of those in clinical development, 91 were described to be plant-derived [63]. Although this was a lower number than in the years before, the interest in natural sources to obtain pharmacologically active compounds has recently been rekindled with improved access to a broader base of sources including those from new microbial and marine origins [23, 64]. Brahmachari (2011) reported 49 plant-derived, 54 microorganism-derived, 14 marine organism derived (including 2 from fish and 1 from a cone snail), and 1 terrestrial animal-derived (bovine neutrophils) drug candidate(s) in various phases of clinical evaluation [22].

Natural products have been the biggest single source of anti-cancer drugs as evidenced by the historical data reviewed by Newman and Cragg [1]. Of the 175 anti-cancer agents developed and approved over the seven decades from 1940 until 2010 in Western countries and Japan, 85 compounds representing 48.6%, were natural products or directly derived from natural products [1]. The four major structural classes of plant derived cancer treatments include Vinca alkaloids, Epipodophyllotoxin lignans, Taxane diterpenoids and Camptotectin quinolone alkaloid derivatives. Approximately 30 plant derived anti-cancer compounds have been reported to be clinically active against various types of tumors and are currently used in clinical trials [65].

A potential development candidate is typically isolated from its natural source only in milligram quantities [6]. Testing *in vitro* occurs in assays such as the U.S National Cancer Institute 60-cell-line panel, followed by human tumor-derived cell lines in primary culture and *in vivo* animal models such as the above mentioned zebrafish embryos, the hollow-fiber human tumor cell assay or human tumor xenografts in rodents [6, 50, 52, 66]. Harvey and Cree have recently reviewed current screening systems for anti-cancer activity suitable for use with collections of natural products. These include quantification of cell growth or cell death in standard cancer cell, three-dimensional and primary cell culture, as well as cell-based reporter

and molecular assays [50]. The quantification of cell growth or cell death in culture using signals like caspase-3 as a marker for apoptosis come with the handicap that the artificial culture environment may not be suitable to predict activity in *in vivo* animal models or cancer patients [50]. Another concern raised is the fact that compounds which kill readily proliferating cancer cells in culture may not eliminate the tumor because of the persistence of cancer stem cells for which suitable screening assays with significant throughput are still lacking [50]. Cancer stem cells are only present in low abundance and remain in a quiescent state until receiving environmental cues such as overexpression of growth factors, cytokines, or chemokines resulting in recurrence of cancer after initially successful treatment and loss of efficacy of the initial treatment agent in the relapsed disease [67].

Dietary sources of compounds assumed to have anti-cancer benefits include fruits, vegetables and spices yielding biologically active components such as curcumin, resveratrol, cucurbitacins, isoflavones, saponins, phytosterols, lycopene, and many others [68]. A number of these are gaining importance as adjuvant anti-cancer agents with curcumin, resveratrol and cucurbitacins having activity reported against cancer stem cells [67]. Bhanot *et al* list 39 natural compounds from marine species, mostly invertebrates, and 10 from microorganisms, mostly from bacteria of the *Streptomyces* genus, as potential new anti-cancer agents [68]. It is assumed that many prokaryotic and eukaryotic natural product sources may still reveal a number of valuable anti-cancer compounds in the future and even ancient animal species have been suggested as a particularly valuable source [69].

Anti-virals constitute another important class of needed therapeutics. The HIV type-1 (HIV-1) is the cause of the Acquired Immune Deficiency Syndrome (AIDS), a major human viral disease with over 34 million people infected worldwide in 2012 and approximately 1.7 million dying per year [70]. Failure of anti-HIV therapy is observed due to the emergence of drug resistance and the significant side effect profile of existing therapies [71]. Hence, the quest for novel prospective drug candidates with fewer side effects and increased efficacy against various HIV strains also relies on natural products. Naturally derived anti-HIV compounds found to be most promising for the treatment HIV infections, with the potential to overcome drug-resistance of mutated HIV strains, were described to be flavonoids, coumarins, terpenoids, tannins, alkaloids, polyphenols, polysaccharides or proteins [72, 73]. Despite the need for affordable, effective, and better tolerated treatments, the vast majority of the potential natural anti-HIV compounds described have so far only been tested as *in vitro*, *ex vivo* or *in silico* approaches to identify activity; the findings have not yet been confirmed in relevant *in vivo* systems. Only a few of the many natural products that have been reported to exhibit anti-HIV activities have reached clinical trials and none of them made it on the list of conventional antiretroviral drugs [71, 72].

Antiviral agents from marine sources which demonstrated activity against HIV were recently reviewed by Vo and Kim (2010). These include phlorotannins from brown algae, sulfated derivatives of chitin from the shells of crabs and shrimps including chitosan (produced commercially by deacetylation of chitin), sulfated polysaccharides from marine algae, lectins or carbohydrate-binding proteins from a variety of different species (ranging from prokaryotes to corals, algae, fungi, plants, invertebrates and vertebrates) as well as bioactive peptides

isolated by enzymatic hydrolysis of marine organisms [73]. Until now, most of the anti-HIV activities of these marine-derived inhibitors were also only observed in *in vitro* assays or in mouse model systems and still await confirmation of their value in clinical trials [73].

## 3. Natural product sources

Historically, the most important sources for biologically active natural products have been terrestrial plants and microorganisms such as fungi and bacteria. Terrestrial and aquatic species of plants and microorganisms, especially those of marine origin, produce unique bioactive substances yielding a large variety of valuable therapeutics and lead structures for potential new drugs. Even though natural products may not have coevolved with human proteins, they have emerged in nature to interact with biomolecules [74]. Natural products interact with a wide variety of proteins and other biological targets, acting also as modulators of cellular processes when they inhibit the difficult to target protein-protein interactions [27, 40].

Since the middle of the last century, marine species and microorganisms have consistently and increasingly raised interest as sources for new agents and scaffolds [75]. In recent years, other less conventional sources like alcoholic and non-alcoholic beverages, spices, animal and human excreta, and many more have generated interest for natural product researchers [75]. The more conventional sources for secondary metabolites like plants, marine organisms and microorganisms will be described in more detail in the following sections.

### 3.1. Plants

A significant number of drugs have been derived from plants that were traditionally employed in ethnomedicine or ethnobotany (the use of plants by humans as medicine as in Ayurvedic or Traditional Chinese Medicine), while others were discovered initially (through random screening of plant extracts in animals) or later, by determining their *in vitro* activity against HIV or cancer cell lines [6, 50, 71-73]. An avenue that may have influenced ethnopharmacology suggests that some traditionally used remedies may have arisen from observations of self-medication by animals [76]. Studies have shown that wild animals often consumed plants and other material for medical rather than nutritional reasons, treating parasitic infections and possible viral and bacterial diseases [11, 60, 76, 77]. For drug discovery, the chemical and pharmacologic investigation of ethnobotanical information offers a viable alternative to high-throughput screening and the body of existing ethnomedical knowledge has led to great developments in health care. It would appear that selection of plants, based on long-term human use in conjunction with appropriate biologic assays that correlate with the ethnobotanical uses, should be most successful [78]. Nevertheless, therapeutic approaches based on active principles from single plant and polyherbal formulations from traditional medicines, like the ones mentioned in Ayurvedic texts, still require scientific validation and sufficient pharmacoepidemiological evidence supporting their safety and efficacy [79]. This is evidenced by the example of aristolochic acid, a constituent of *Aristolochia* vines, which are used in

complementary and alternative therapies. Aristolochic acid is a powerful nephrotoxin and a human carcinogen associated with chronic kidney disease and upper urinary tract urothelial carcinomas (UUC) [80]. These dual toxicities and the target tissues were revealed when a group of otherwise healthy Belgian women developed renal failure and UUC after ingesting *Aristolochia* herbs in conjunction with a weight-loss regime; subsequently, more cases were reported in Taiwan and countries throughout the world [80]. Importantly, the traditional practice of Chinese herbal medicine in Taiwan mirrors that in China and other Asian countries making it likely that these toxicities are also prevalent in these and in other countries where *Aristolochia* herbs have long been used for treatment and prevention of disease, thereby creating an international public health problem of considerable magnitude [80, 81].

In the early 1900's, 80% of all medicines were obtained from roots, barks and leaves and it is estimated is that approximately 25% of all drugs prescribed today still originate from plants [14, 19, 78]. The plant kingdom, with 300,000 to 400,000 higher species (estimated levels reach from 215,000 up to 500,000 [78], was always a key source of new chemical entities (NCEs) for active pharmaceutical ingredients and lead compounds [12]. It is estimated that only 5% to 15% of these terrestrial plants have been chemically and pharmacologically investigated in a systemic fashion [19]. Approximately 10,000 to 15,000 of the world's plants have documented medicinal uses and roughly 150-200 have been incorporated in western medicine [19, 82]. Marine plants like microalgae, macroalgae (seaweeds) and flowering plants (such as mangroves) have been studied to a much lesser extent and are mostly reported in connection with nutritional, supplemental or ethnopharmacological uses [83]. For over 20 years the U.S. National Cancer Institute has collected higher plants for screening, with the current collection composed of ~ 30,000 species [84]. Only a small percentage of these have reportedly been screened for biological or phytochemical activity until a decade ago and large numbers are constantly being tested for their possible pharmacological value today [35, 78]. Based on their research, the authors justify their assumption that the plant kingdom still holds many species containing substances of medicinal value and for potential pharmaceutical applications, which have yet to be discovered. However, such assumptions may be diminished as the loss of valuable natural sources increases due to factors such as deforestation, environmental pollution, and global warming [85].

Saslis-Lagoudakis *et al.* provided evidence through phylogenetic cross-cultural comparisons that related plants from different geographic regions are used to treat medical conditions in the same therapeutic areas [86]. Accordingly, there has been a recent surge in interest in the components of traditional Chinese medicines and Ayurvedic remedies [11]. Limitations of this approach include the fact that both of these ancient traditions use polyherbal preparations (botanicals) for the majority of prescriptions and that plants as biological systems have an inherent potential variability in their chemistry and resulting biological activity [12, 35]. Fabricant and Farnsworth reported that 25% of all plants showing biological activity in their assay system failed to reproduce the activity on sub-sequent recollections [35]. This may be caused by factors coming into play after the collection of a specimen, however, for plants it is common to dry the collected plant parts thoroughly in the field before extraction to assure that the material does not compose before reaching the laboratory [12].

Rout *et al.* describe the approaches for using individual plants as therapeutic agents as follows: (i) to isolate bioactive compounds for direct use as drugs, (ii) to produce bioactive derivatives of known compounds as new structures, (iii) to use substances as pharmacologic agents or tools, and (iv) to use a whole or partial plant as herbal remedy and provide examples for each category [78]. Mixtures of plant-derived products are known as botanicals, and the term is defined by the United States (US) Food and Drug Administration (FDA) to describe finished, labeled products that contain vegetable matter as ingredients which can include plant materials, algae, macroscopic fungi, and combinations thereof [87]. They can fall under the classification of a food (including a dietary supplement), a drug (including a biological drug), a medical device, or a cosmetic [87]. The vast majority of plant-derived treatments are based on synthetic, semisynthetic, or otherwise highly purified or chemically modified drugs [87, 88]. According to the most recent report by BCC Research, the global plant-derived drug market was valued at US$ 22.1 billion in 2012 and sales are projected to grow to US$ 26.6 billion by 2017 at a compound annual growth rate (CAGR) of 3.8% [89]. The botanicals subgroup currently has only one approved drug, Veregen, with an expected revenue increase from US $ 2.8 million in 2010 to 599 million in 2017 [89].

### 3.2. Marine life

Given the fact that oceans cover nearly 70% of the earth's surface and that life originated in the oceans with the first marine organisms evolving more than 3.5 billion years ago, the enormous diversity of organisms in the marine environment is not surprising and largely unexplored [90]. On some coral reefs, their density can reach up to 1,000 species per square meter, which is believed to be a higher biodiversity than observed in tropical rainforests and inspired researchers for decades to search for novel compounds from marine sources [57, 91]. As the greatest biodiversity is found in the oceans, it is estimated that between 250,000 and one million marine species could provide an immense resource to discover NCEs serving as unprecedented novel bioactive structures and scaffolds that have the potential to serve as medical treatments or templates for new therapeutics [23, 92].

The interest in novel chemical structures from marine organisms started in the 1950s as marine animal taxonomy advanced significantly, but progressed at a slow pace for the first two decades before it started to burgeon in the 1970s [91-94]. Since then, approximately 30,000 structurally diverse natural products with a vast array of bioactivities have been discovered from marine organisms including microbes, algae and invertebrates [92, 95]. Invertebrates alone comprise approximately 60% of all marine animals and were described as the source of almost 10,000 new natural products since 1990 with a pronounced increase to about 1,000 compounds per year in more recent years [23, 32, 93].

By the turn of the 21$^{st}$ century larger percentages of bioactive NCEs were reported for marine organisms in comparison to terrestrial organisms, but nevertheless, marine chemical ecology is still several decades behind its terrestrial counterpart with respect to the total number of characterized and documented natural products [93, 96]. Kong *et al.* specifically compared natural products from terrestrial and marine sources. They found that compounds from marine organisms exhibited a higher chemical novelty and that over 2/3 of those scaffolds were

exclusively used by marine species, but alerted readers to concerns of the suitability of the new scaffolds as drug templates because of their unsuitably high hydrophobicity [96]. As is the case for plant derived natural compounds, the U.S. National Cancer Institute also plays an important role for establishing marine organism collections since the 1980s and has a vast National repository of invertebrate derived compounds and extracts from specimens rigorously identified by taxonomic experts [92].

Many marine natural products appear to arise from multi-functional enzymes that are also present in terrestrial systems, exhibiting a cross phylum activity with terrestrial biota [94, 95]. However, a large number of marine derived compounds also possess a substantial amount of functional groups, which were not previously described from terrestrial metabolites [91, 94, 95]. They range from derivatives of amino acids and nucleosides to macrolides, porphyrins, terpenoids, aliphatic cyclic peroxides, and sterols [91]. These secondary metabolites resulted from evolutionary pressure threatening many marine organisms, especially those which are soft bodied and have a sedentary life style, forcing them to develop the ability to synthetize toxic compounds which serve to deter predators, manage competitors or immobilize prey [57, 91, 93, 94]. The search for new drug candidates from marine species has expanded into circumpolar regions for cold-adapted species as well as harsh environments like deep-sea hydrothermal vents; these approaches have been particularly successful with filter-feeders such as sponges, tunicates and bryozoans [23, 97].

The fact that marine invertebrates contain astounding levels of microbial diversity and form highly complex microbial communities led to the assumption, followed by confirmation in recent examples, that microbial symbionts like bacteria are important producers of natural products derived from marine species [58]. In particular, these include polyketides and non-ribosomally synthesized peptides as well as unique biosynthetic enzymes which emerged as potent biocatalysts in medicinal chemistry [58, 97].

By 2010, four drugs of marine origin had obtained approval for the treatment of human disorders [98]. Cytarabine (Cytosar-U®; Upjohn/Pfizer) for the treatment of white blood cell cancers, vidarabine (Vira-A®; discontinued by distributor Monarch Pharmaceuticals) an ophtalmic antiviral, and ziconotide (Prialt®; Elan) for pain management were FDA approved and trabectedin (Yondelis®; Pharmamar), an anticancer compound against soft tissue and ovarian cancer was approved in Europe [98]. Vidarabine and cytarabine originate from marine sponges, ziconotide is the synthetic equivalent of a conopeptide originating from a marine cone snail, and trabectedin, now produced synthetically, originates from a bacterial symbiont of a tunicate or sea squirt [32, 99]. At the same time, 13 marine organism-derived drug candidates were listed to be in clinical development (3 in Phase III, 7 in Phase II, and 3 in Phase I) and hundreds are in pre-clinical testing as ion channel blockers, enzyme inhibitors, DNA-interactive and microtubule-interfering agents, with the majority of the latter compounds being tested for anti-tumor and cytotoxic properties [91, 94, 98]. Natural products of marine origin with biological activity of interest include, but are not limited to, curacin A, eleutherobin, discodermolide, bryostatins, dolostatins, cephalostatins [16].

### 3.3. Microorganisms

Microorganisms were identified early on as sources of valuable natural products as evidenced by the discovery of penicillin by from the fungus *Penicillium rubens* by Alexander Fleming in 1928 [100]. Historically, microorganisms (amongst them mostly bacteria and fungi) have played an important role in providing new structures, like antibiotics for drug discovery and development. The terrestrial microbial populations are immensely diverse which is also reflected in the number of compounds and metabolites isolated from these microorganisms. As mentioned above, the similarity of many compounds from marine invertebrates like sponges, ascidians, soft corals and bryozoans to those isolated from terrestrial microbes led to the hypothesis that associated microorganisms might be responsible for their production. Over time it became more and more evident, that a significant number of marine natural products are actually not produced by the originally assumed invertebrate but rather by microbes living in symbioses with their invertebrate host [92, 101]. In some instances it could indeed be demonstrated early on that the isolated marine microbes are the original source of the new compounds or secondary metabolites discovered and in recent years marine bacteria have emerged more and more as a source of NCEs [58, 91, 94, 95]. Besides bacteria, marine fungi and deep-sea hydrothermal vent microorganisms are reported to produce bioactive compounds and metabolites [91, 94]. Deep-sea vent sites offer harsh conditions in depth below 200 meters with complete absence of light, pressures in excess of 20 atmospheres, temperatures of up to 400°Celius, pH extremes and high sulfide concentrations and are populated by highly dense and unique, biologically diverse communities [91, 94, 102].

Unique microorganisms are abundant on land, in freshwater and all areas of the ocean. However, the enormous biological diversity of free-living and symbiotic marine microbes has so far only been explored to a very limited extent. The estimates extrapolate the number of marine species to at least a million, but for marine microbial species, including fungi and bacteria, the estimated numbers reach as high as tens or even hundreds of millions [23]. Over 74,000 known species of fungi are reported including around 3,000 aquatic species of which only about 465 are described as marine species, but a vast geographical area has not yet been sampled and estimates for the potential total number of species reach from 0.5 to 9.9 million with about 1.5 million considered as most realistic [103, 104]. The overlap is assumed to be relatively high between species in terrestrial and freshwater habitats, but not between these two and the marine habitat [104]. Nevertheless, a large percentage of the over 270 secondary metabolites isolated from marine fungi resembles analogues of compounds previously discovered from terrestrial fungi but some of the new substances identified exhibited potent activities against tumor cells, microbes or bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA) or even antifouling properties [59, 105]. In comparison to bacteria, fungi appear to be rare in marine environments and few marine fungi isolates exist in culture [106]. Marine bacteria are assumed to constitute approximately 10% of the living biomass carbon and inhabit mainly sediments but can also be found in open oceans and in association with marine organisms [90, 91, 94]. Many marine invertebrates are associated with large amounts of epibiotic and endobiotic microorganisms and for many sponges bacteria can make up to 40% of the animal biomass and even resemble new species [97]. In fact it is assumed that almost all

marine organisms host bacteria on their surface and that the vast majority bare epibiotic films of variable density and composition, which can affect the basibiont's physiology and interactions with its environment in beneficial, detrimental, or ambiguous manners [107]. This constitutes a vast pool for the discovery of new structures and scaffolds if the source can be unanimously established.

To identify the source of a compound, the determination of its mere presence in a certain organism is not sufficient, as this could be the result of active or passive accumulation and does not necessarily reflect the true site of its biosynthetic production. As Gulder and Moore further explain: *"An unambiguous assignment of the biosynthetic origin of a natural product derived from a complex assemblage of marine organisms thus has to originate at the genomic level. This is particularly true for bacterial symbionts, which have to date eluded cultivation"* [58]. These microbes generally organize their biosynthetic genes for each secondary metabolite in compact clusters, which will, following identification and sequencing of the cluster responsible for the respective pathways, allow the transfer of the respective bacterial genes into effective heterologous producers, like *Escherichia coli (E. coli)* [55, 58, 97]. In terms of microbial sources, culturing of the respective microorganism may generally be a viable approach to increase quantities. A major difference between microorganisms from terrestrial and marine sources is the fact that marine pelagic bacteria are much more difficult to grow in culture than the soil borne actinomycetes; therefore determining the conditions for replication, growth of sufficient quantities and induction of metabolite production can be a tedious challenge [12, 17]. This again may necessitate the production in a heterologous system like that observed for *E. coli.*

## 4. Challenges

Natural products, although a valuable and precious resource, also come with their fair share of challenges in a variety of aspects. As mentioned before, one of the major issues concerning the use of natural products are the difficulties associated with obtaining sufficient amounts of material pure enough for discovery and development activities. If a compound is derived from a plant growing only in small quantities or remote locations or a marine organism residing in great depth or difficult to access regions, re-supply becomes a problem.

The threat of losing potentially valuable natural sources of pharmacologically active ingredients is constantly increasing due to the threat of extinction by deforestation of large landmasses and environmental pollution in remote areas as well as global warming [85]. It is estimated that about 70% of the supply of herbal raw material for Ayurveda and other homeopathic medicines in India comes from the wild [82, 85]. To meet the increasing demand for raw material, to conserve wild resources, and to reduce the potential variability in the active ingredient content in medicinal plants from different collection areas, it is important to implement more controlled cultivation programs to ensure quality and to protect resources [82, 85].

Tissues of marine invertebrates present unique problems for extraction, because of their high water and salt content, and the promising compounds may be present only in low amounts and/or can be very difficult to isolate. Sponges and their microbial fauna are mostly not suitable for culture, and the compounds of interest need to be extracted and purified from specimens collected in the wild [17, 93]. Marine organisms and microbes constitute a valuable potential source of NCEs and structural templates for drug discovery in the future, but may necessitate tons of raw material to isolate milligram to gram amounts of the compound of interest [97]. This difficulty combined with the challenges for synthetic approaches to obtain significant quantities of potential new drug candidates, based on their often highly complex structures, are obstacles that can hamper their use in discovery and development [58, 97]. In some cases, supply issues could be resolved by semi-synthesis or total synthesis of the compound, the development of synthetic analogs with more manageable properties, or by design of a pharmacophore of reduced complexity, which can then be synthesized [17, 92, 97]. Fragments or synthetic analogs with simplified structures may retain bioactivity or even show improved activity towards the target [12].

Furthermore, environmental aspects can constitute significant hurdles for supplying material for discovery and development as the product may stem from an endangered species or the wild collection of the producing species may be detrimental to its originating terrestrial or marine ecosystem. Additionally, as mentioned above, because of their low abundance many compounds of interest from natural sources need to be extracted and purified from large quantities of specimen collected in the wild, which in turn carries the risks of over-exploitation and habitat destruction [17, 93]. Radjasa *et al.* provide a positive outlook for marine ecosystems and state "*There is optimism for the future because the international marine bioorganic community clearly recognizes that invertebrates must be harvested and studied in an environmentally sustainable manner"* [92]. Although aquaculture of marine species or culture of bacteria seem like logical alternative sources to obtain product, they are not viable avenues in most cases because it proves difficult to impossible to culture the source organisms (especially invertebrates and/or their microbial symbionts like bacteria etc.) or they may not produce the compound of interest under the given culture conditions [12, 17, 95, 97]. Findings indicate, that the bacterial composition on invertebrates is largely independent from sponge taxonomy or locality of collection and the bacteria most likely are contaminants from the ocean water rather than specific symbionts, which further exacerbates the cultivation problem [97, 108].

Another challenge can result from redundant activity determination in assay systems and the mixed composition of natural product extracts. With over 150,000 known small molecules characterized from natural sources, previously known natural products are often re-isolated in the course of bioassay-guided fractionation [84]. While this may be acceptable if the biological activity is new, it is frustrating to waste resources on the *de novo* structure elucidation of known compounds. Furthermore, not all compounds contained in natural product extracts are drug leads and it is extremely desirable to remove "nuisance compounds" like tannins, phorbol esters, saponins, and anionic polysaccharides; the latter, for instance, being highly active in cellular HIV bioassays [35].

Last, but not least, intellectual property rights can pose a significant hurdle that is difficult to manage. In general, patent protection can be obtained if the active principles derived from natural sources have novel structures and relevant biological activity. However, as mentioned before, additional handicaps may arise in the context of developing and marketing products from natural sources in the form of potentially significant intellectual property issues as well as, possibly, very difficult and costly negotiations to obtain agreements to collect and develop natural products from species collected in foreign countries [25, 26, 35].

## 5. Regulatory requirements and risks

The regulatory requirements for different product categories containing natural substances like pharmaceuticals, nutraceuticals, and cosmeceuticals vary from rather stringent to generous to non-existent at an International level. To exemplify regulatory approaches for the three aforementioned categories, an overview of the current situation in North America (U.S. and Canada) and the European Union is summarized. In many areas we have presented exact wording from the respective governing websites, as the phrasing of such represents the official context and any deviation thereof could be misleading.

### 5.1. Natural product-derived pharmaceuticals

Natural products constitute a key source of pharmacologically active ingredients in a variety of novel agents with therapeutic potential in a wide range of diseases. Pharmaceuticals containing natural products or compounds derived from natural product scaffolds or templates have to undergo the same stringent approval process as drugs obtained from purely synthetic origin.

#### *5.1.1. North America*

##### *5.1.1.1. United States of America (U.S.)*

The U.S. Government Office for the Control of Food and Drug Administration (FDA) oversees the regulatory control of pharmaceuticals including new treatments based on natural products [109]. The FDA's Center for Drug Evaluation and Research (CDER) role is to evaluate and approve new drugs before they can be sold ensuring that drugs are safe and effective for intended use and that their health benefits outweigh their known risks [109].

##### *5.1.1.2. Canada*

In Canada, Health Canada fulfills the same role as the US FDA according to the mandate under the authority of the Food and Drugs Act and the Food and Drug Regulations [110].

*5.1.2. European Union*

The European Medicines Agency (EMA) with headquarters in London/England regulates drugs and medicinal products in the European Union (EU) [111]. On April 30th, 2011 the EU entered into force the directive on herbal medicine products called Traditional Herbal Medicinal Products Directive 2004/24/EC, THMPD [111]. The regulation came as a sub-directive for the act on Human Medicinal Products Directive 2001/83/EC claiming a unique set of information on a herbal substance or herbal preparation for all EU Member States. Such could be used when evaluating marketing applications for herbal medicinal products from companies and covers medicinal products containing herbal substances/preparations [111]. To reach the market, these must fall within one of the following three categories, as outlined on the EMA website [111]:

**1.** a product can be classified under traditional medicinal use provisions ('traditional use') accepted on the basis of sufficient safety data and plausible efficacy: the product is granted a traditional use registration (simplified registration procedure) by a Member State,

**2.** a product can be classified under well-established medicinal use provisions ('well-established use'). This is demonstrated with the provision of scientific literature establishing that the active substances of the medicinal products have been in well-established medicinal use within the Union for at least ten years, with recognized efficacy and an acceptable level of safety. As a result the product is granted a marketing authorization usually by a Member State or by the European Medicines Agency. While both classifications have specific requirements, both regulatory paths involve the assessment of mostly bibliographic safety and efficacy data.

**3.** a product can be authorized after evaluation of a marketing authorization application consisting of only safety and efficacy data from the company's own development ('stand alone') or a combination of own studies and bibliographic data ('mixed application'). As a result the product is granted a marketing authorization by a Member State or by the Agency via the centralized procedure if all requirements are met.

In summary, while safety needs to be shown for products, proof of efficacy is not always a requirement and only the traditional indications in specified conditions must be plausible. Nonetheless, and irrespective of the regulatory pathway to access the market, the quality of the herbal medicinal product must always be demonstrated [111].

The Directive provides definitions for herbal medicinal products, herbal preparations and herbal substances, as follows [111]:

- *Herbal medicinal product:* Any medicinal product, exclusively containing as active ingredients one or more herbal substances or one or more herbal preparations, or one or more such herbal substances in combination with one or more such herbal preparations.

- *Herbal substances:* All mainly whole, fragmented or cut plants, plant parts, algae, fungi, lichen in an unprocessed, usually dried, form, but sometimes fresh. Certain exudates that have not been subjected to a specific treatment are also considered to be herbal substances. Herbal

substances are precisely defined by the plant part used and the botanical name according to the binomial system (genus, species, variety and author).

- *Herbal preparations:* Preparations obtained by subjecting herbal substances to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation. These include comminuted or powdered herbal substances, tinctures, extracts, essential oils, expressed juices and processed exudates

Additionally, it has been noted that from a herbal substance (e.g. valerian root) different herbal preparations (e.g. a valerian root extract using 70% ethanol) can be made; in such cases, both can represent the active ingredient in an individual herbal medicinal product [111].

### 5.2. Nutraceuticals — Dietary supplements (U.S.)/Natural health products (Canada)

Even if natural health products (NHPs) or dietary supplements are considered as or expected to be safe, they may still carry potential risks in themselves or through interactions with prescription or Over The Counter (OTC) drugs. This is illustrated by the previously described example of *aristolochic acid*, a powerful nephrotoxin and a human carcinogen associated with chronic kidney disease and upper urinary tract urothelial carcinomas after ingesting *Aristolochia* herbs in conjunction with a weight-loss regime [80, 81]. Furthermore, interactions between NHPs and prescription medicines are of increasing concern and need to be considered by physicians and patients alike [112]. Mills *et al*, in their evaluation of 47 trials which examined drug interactions with 19 different herbal preparations, observed potentially clinically significant drug interactions with St. Johns Wort, garlic, and American ginseng [113].

#### *5.2.1. North America*

##### *5.2.1.1. United States of America*

In the U.S., biologically active food and dietary supplements are regulated by the FDA and are classified as food and nutrition, not drugs [88]. The FDA website provides a detailed overview of their regulatory approach concerning nutraceuticals. The following paragraphs reflect some core points as outlined on the FDA's respective website [88].

- The FDA regulates both finished dietary supplement products and dietary ingredients under a different set of regulations than those covering "conventional" foods and drug products (prescription and OTC). Under the Dietary Supplement Health and Education Act (DSHEA) of 1994, the dietary supplement or dietary ingredient manufacturer is responsible for ensuring that a dietary supplement or ingredient is safe before it is marketed. The FDA is responsible for taking action against any unsafe dietary supplement product after it reaches the market. Generally, manufacturers do not need to register their products with the FDA nor get FDA approval before producing or selling dietary supplements.
- The Federal Food, Drug, and Cosmetic Act requires that manufacturers and distributors who wish to market dietary supplements that contain "new dietary ingredients" notify the Food and Drug Administration about these ingredients, which must include information

that is the basis on which manufacturers/distributors have concluded that a dietary supplement containing a new dietary ingredient will reasonably be expected to be safe under the conditions of use recommended or suggested in the labeling [87].

- The U.S. Congress defined the term "dietary supplement" and both of the terms "dietary ingredient" and "new dietary ingredient" as components of dietary supplements in the DSHEA. A dietary supplement is a product taken by mouth that contains a "dietary ingredient" intended to supplement the diet.
- In order to be a "dietary ingredient," it must be one or any combination of the following substances:
- A "new dietary ingredient" is one that meets the above definition for a "dietary ingredient" and was not sold in the U.S. in a dietary supplement before October 15, 1994.
- Dietary supplements can also be extracts or concentrates, and may be found in many forms such as tablets, capsules, softgels, gelcaps, liquids, or powders [88]. They can also be in other forms, such as a bar, but if they are, information on their label must not represent the product as a conventional food or a sole item of a meal or diet [88]. Whatever their form may be, the DSHEA places dietary supplements in a special category under the general umbrella of "foods," not drugs, and requires that every supplement be labeled a dietary supplement.

#### *5.2.1.2. Canada*

In Canada, the use and sale of natural health products (NHPs) is on the rise [8]. A 2010 Ipsos-Reid survey showed that 73% of Canadians regularly take natural health products (NHPs) like vitamins and minerals, herbal products, and homeopathic medicines [114]. Health Canada defines natural health products under the Natural Health Products Regulations as:

- Vitamins and minerals
- Herbal remedies
- Homeopathic medicines
- Traditional medicines such as traditional Chinese medicines
- Probiotics
- Other products like amino acids and essential fatty acids [115].

Natural Health Products must be safe to use as OTC products and not need a prescription to be sold [115]. Natural products, compounds and active ingredients derived from natural sources or totally synthesized and needing a prescription are regulated as drugs under the Food and Drug Regulations [115].

### *5.2.2. European Union*

Herbal supplements and nutritional supplements are not regulated on a harmonized EU wide basis and remain under the control of the relevant medical institutions of the individual EU member states.

### 5.3. Cosmeceuticals

Although the term is not recognized by the US Food and Drug Administration (FDA) or by the European Medicines Agency (EMA), it has been widely adopted by the cosmetics industry, which is rapidly expanding in spite of global economic woes in recent years [116]. The global cosmeceuticals market references the seven most developed markets including the U.S. and the top five European countries, namely the UK, France, Germany, Italy and Spain; as well as Japan [116]. In 2011, the global cosmeceuticals market was estimated to be worth $30.9 billion (with the aforementioned European countries accounting for approximately 65% of overall revenues) and is expected to reach $42.4 billion by 2018 [116]. Three major categories have been noted in the cosmeceutical industry including skin care, hair care, and others, with the skin care segment accounting for the largest share of the market at 43% [117]. Dominated by anti-aging products, the skin-care market is expected to contribute significantly to future growth based on the aging populations in the top seven aforementioned markets [116].

Cosmeceuticals are topically applied and represent a hybrid of cosmetics and pharmaceuticals usually containing vitamins, herbs, various oils, and botanical extracts or a mixture thereof including antioxidants, growth factors, peptides, anti-inflammatories/botanicals, polysaccharides, and pigment-lightening agents [117, 118]. The combination of cosmetics and foods resulted in products termed nutricosmetics. Nutricosmetics are foods and supplements claiming cosmetic effects with major ingredients like soy isoflavone proteins, lutein, lycopene, vitamins (A, $B_6$, E), omega-3 fatty acids, beta-carotene probiotics, sterol esters, chondrotin and coenzyme Q10 [119, 120]. These compounds act as antioxidants and the respective nutricosmetics containing them are being promoted for their skin care properties as for instance in anti-aging by fighting free radicals generated as a by-product of biochemical reactions through skin exposure to the sun [119].

#### *5.3.1. North America*

##### *5.3.1.1. United States of America*

In the US, products that can be put in both the cosmetics and drugs category, such as cosmetic products with active ingredients which claim therapeutic use, require New Drug Application (NDA) approval or must comply with the appropriate monograph for an (OTC) drug. Moreover, the FDA also has specific guidelines for Good Manufacturing Practice (GMP) for cosmetics [116].

While the *Federal Food, Drug, and Cosmetic Act (FD&C Act)* does not recognize the term "cosmeceutical", the cosmetic industry uses this word to refer to cosmetic products that have medicinal or drug-like benefits [118]. The FD&C Act defines drugs as those products that cure, treat, mitigate or prevent disease or that affect the structure or function of the human body [121]. Under the FD&C Act, cosmetic products and ingredients, with the exception of color additives, do not require FDA approval before they go on the market [121]. Therefore, while drugs are subject to a review and approval process by the FDA, cosmetics are not approved by the FDA prior to sale. However, when a product makes a therapeutic claim (e.g. to prevent or treat disease), it is classified as a drug and therefore requires evaluation by the FDA's Center

for Drug Evaluation and Research (CDER) and a drug identification number (DIN) before it can be sold.

*5.3.1.2. Canada*

In Canada, the term "cosmeceutical" (used to describe a cosmetic product with pharmaceutical-like benefits) is not employed by Health Canada [122]. Therefore cosmeceuticals fall under either cosmetics or drugs (depending on the claims made and/or the composition of the product) and are subject to the provisions of the Food and Drugs Act and its Cosmetic Regulations regarding composition, safety, labeling and advertising and they are subject to the provisions of the Consumer Packaging and Labeling Act and Regulations [122]. The three most significant features of the Canadian cosmetic regulatory system are mandatory notification of all cosmetic products, safety of ingredients and products, and product labeling [122]. According to Health Canada, a "cosmetic" is defined as *"any substance or mixture of substances, manufactured, sold or represented for use in cleansing, improving or altering the complexion, skin, hair or teeth and includes deodorants and perfumes"* [122].

*5.3.2. European Union*

In Europe, EMA guidelines place a clear demarcation between drugs and cosmetics, whereby a cosmetic is a product that is to be applied topically with an intended cosmetic function and products cannot fall under both categories, unlike in the US [116]. On November 30$^{th}$ (2009), the new Cosmetic Products Regulation, EU Regulation 1223/2009 was adopted, replacing the Cosmetics Directive [123]. With the new Cosmetics Regulation, Europe claims to have a robust, internationally recognized regime, which reinforces product safety taking into consideration the latest technological developments [123]. Most of the provisions of this new regulation will be applicable as of July 11$^{th}$, 2013 [123].

## 6. Conclusion

A natural product or secondary metabolite is a pharmacologically or biologically active chemical compound or substance, which is found in nature and produced by a living organism. The lengthy process of natural products evolution has resulted in optimal interactions with biological macromolecules and targets. Historically, natural substances have been the most productive source of active compounds and chemical lead structures. Natural products have traditionally provided a large fraction of the drugs in use today and millions of terrestrial and marine plants, organisms and microorganisms provide an immense resource to discover unprecedented novel bioactive scaffolds. These have the potential to serve as medical treatments or templates for new therapeutics and may be suitable for production via a synthetic routes or in a heterologous system like *E. coli.* About half of the agents in today's arsenal of marketed drugs are derived from biological sources with the large majority being based on terrestrial natural product scaffolds. Approximately 50% of the new drugs introduced since 1994 were either natural products or derived from natural products. As of today, only a very

small fraction of bioactive structures of potential therapeutic relevance from plants, microbes, and marine organisms have been chemically analyzed or examined in a broad panel of screening models or bioassays. The discovery of valuable therapeutic agents from natural sources continues in the 21$^{th}$ century by reaching into new and untapped terrestrial and marine source organisms as the chemical novelty associated with natural products is higher than that of structures from any other source.

There is growing awareness of the limited structural diversity in existing compound collections and the extreme chemical diversity, the high biological potency, and the potential to frequently discover drug-like characteristics in natural products. Therefore, they constitute a valuable platform for the development of new therapeutics for a variety of indications, although they may still not contain enough versatility to yield suitable treatments for all heritable human diseases.

As some major pharmaceutical companies terminated their natural product programs, the future role to discover and feed candidates into the development pipelines will reside increasingly with research institutes and small biotech companies. Over a hundred new drug candidates from natural sources like plants, bacteria, fungi and animals or obtained semi-synthetically are in clinical development with a similar number in preclinical development. They target a variety of indications, mainly cancer and infectious diseases (bacterial, viral, fungal, and parasitic) but also other therapeutic areas such as cardiovascular diseases, neurological illnesses and depression, metabolic diorders and inflammatory diseases.

Natural products, although a valuable and precious resource, also come with their fair share of challenges concerning the provision of sufficient amounts of pure enough material for discovery and development activities. As mentioned earlier, such apprehensions are based on the threat of losing potentially valuable natural sources through extinction resulting from deforestation of large landmasses, environmental pollution in remote areas as well as global warming. Countries are also increasingly protective of their natural assets in flora and fauna and may not authorize the collection of sample species without significant demands and very difficult negotiations. The regulatory requirements for different product categories containing natural substances like pharmaceuticals, nutraceuticals, and cosmeceuticals vary from rather stringent over generous to non-existent at an international level. Even if natural health products or dietary supplements are considered as or expected to be safe, they may still carry potential risks in themselves or through interactions with prescription or OTC drugs. Therefore, the discovery and development of natural products require cientific validation and sufficient pharmacoepidemiological evidence to support their safety and efficacy.

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Chapter 2

# Genetic, Agronomy, and Metabolomics of Prince Edwards Island Wild Rose Collection and Promise for Cultivar Development

Bourlaye Fofana, Kaushik Ghose, Bob Chapman and Kevin Sanderson

Additional information is available at the end of the chapter

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## 1. Introduction

Agriculture – the control of plants for human consumption – is believed to have appeared and developed during the paleolitic/neolitic period, ~ 10,000 years ago [1]. The first agriculture had no single or simple origin since a wide variety of plants and animals have been independently domesticated at different times and different places [1-4]. The origin of agriculture and crops domestication is intertwined. Plant domestication involves changes in the plant's genetic makeup and morphological appearance following successive selections within wild plants and based upon on the variations that are best suitable for humans needs [5]. Domestication is therefore an artificial selection process conducted by humans for the production of plants showing fewer undesirable traits compared to its wild related plants, and making them more dependent on the new artificial environments for their continued survival and development. The concept of selection assumes the existence of a population or group of individuals from which choices can be made. Thus, the diversity of morphotypes or genetic diversity is considered as the backbone for plant domestication and crop improvement. Nonetheless, the way this genetic diversity was probed across time has constantly evolved while being a continuum from the first day. Moreover, while the selection criteria for the desired traits and purposes in the ancient domestication process were certainly exclusively based on morphology (size, color, shape of leaves and fruits, easiness for identification) and to satisfy man's energy supply needs (taste and flavour, satiety potential), today, the required traits and purposes for plant domestication (seen as continuum) have been refined and expanded. Indeed, new technologies have been developed for probing the genetic diversity whereas human needs

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have increased to include health and wellbeing. As a consequence more specific and defined traits such as a targeted and defined ingredient or metabolite are sought. To date, the pace of plant domestication has slowed down mainly due to the loss of biodiversity but also because of our ability to satisfy our current food needs. Nevertheless, few new crops species are still being introduced into farming system to fill the growing gaps in the need of humans and pets. Although domestication, as a concept, is not the main focus of this chapter (reader can refer to [3, 4, 6-9]), this review will look at some aspects of plant domestication in the 21st century as compared with ancient domestication process, the extent of genetic diversity within North American roses, the challenges associated with the domestication and agronomy of Atlantic Canada wild rose species taken as an example, and how the current biotechnology tools can contribute to an economic crop production.

## 2. Domestication as a science

### 2.1. Definition

Domestication was defined by De Wet [8] as "changes in adaptation that insure total fitness in habitats especially prepared by man for his cultigens". Van Raamsdonk [7] refined this definition by taking into account Simmond's [6] observations on plant domestication syndrome because a considerable number of crop plants are dependent on man for establishing new generations due to non-dehiscence, non-shattering, and absence of seed dormancy. Domestication was thus better defined by van Raamsdonk as a process leading to characteristics that are beneficial to humans but generally unprofitable for plants in natural habitats and in the decrease or total lack of capability to disseminate viable offspring [7]. As such defined, the goal for crop domestication appears obvious: setting plant for human's benefits. However, the paths and process followed, and the tools used towards developing a new crop from its wild related plant can greatly vary (Table 1).

### 2.2. Domestication process and goal

An artificial selection results in a phenotypic evolution [10]. In fact, agriculture started ~10,000 years ago by probing the diversity present within wild plant species and by planting the selected specimens, first in the garden and then in the field setting, a process known as domestication. Although all crops and plant varieties known to man today did not undergo through this classic process (case of known semi-domesticates) [3], the vast majority did go through, and thus being fully or super domesticated [3], depending on era, needs and advances in technology. Domestication is generally considered to be the end-point of a continuum that starts with exploring wild plants, continues through cultivation of plants selected from the wild but not yet genetically different from wild plants, and terminates in the fixation (at some extent), through human selection, of morphological and hence genetic differences distinguishing a domesticate from its wild progenitor. Wild and cultivated populations differ statistically in various characters targeted by human selection, although the cultivated plants may be morphologically indistinguishable from the wild plants [3]. Therefore, cultivated populations

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are not genetically fixed for any characters distinguishing them from wild populations, but the frequencies of alleles governing the characters subjected to human selection presumably differ [3]. Casas et al. [11] considered that changes in allele frequencies resulting from human selection constitute at least an incipient domestication, i.e. a nascent domestication. These authors analyzed the morphological variations in wild, managed *in situ*, and cultivated populations of the columnar cactus *Stenocereus stellatus* in central Mexico. They investigated whether morphological divergence has occurred between manipulated and wild populations by the domestication processes. Multivariate statistical analyses showed that individuals grouped according to management options and the fruit characteristics were the most relevant for grouping. Sweet fruits with non-red pulp colors were more frequent in cultivated populations. The fruits were also larger, contained more and bigger seeds, had thinner peel, and fewer spines in cultivated populations than fruits in wild individuals. Phenotypes common in managed *in situ* and cultivated populations generally occur also in the wild but at lower frequencies. However, Gepts [12] considered cultivation as a necessary but insufficient condition for domestication which, at least incipient or semi-domestication, may occur without cultivation by selective removal of undesirable phenotypes and/or enhancement of desirable phenotypes in wild populations [11]. How these different domestication processes and the available tools may apply to wild rosehip is one of the main topics developed in this review.

## 2.3. Domestication tools

### *2.3.1. Ancient tools*

The oldest cultivated garden rose was *R x richardii* grown and depicted in art works by the Minoan civilization in Crete more than 3500 years ago. Roses were extensively cultivated during the Roman era (625 BC- 476 AD). After the demise of the Roman Empire, the less-appreciated wild-growing roses in Europe and Asia, belonging to *Rosa* section *Canina* and known today as Dogroses were maintained in monasteries for their reputed medicinal properties [13]. By the 18$^{th}$ century, five rose species (*R. gallica, R. alba, R. damascena, R. centrifolia, and R. centrifolia moscosa*) sharing a number of features such as double flower, flagrance, flower colour, frost hardiness, spring flowering, resistance to black spot and rust, and susceptibility to mildew had emerged [14]. These five species fall into 5 broad rose classes namely Gallica, Alba, Damask, Centrifolia, and Moss rose, respectively, and referred to as old European roses. These traditional European roses were crossed with roses from China (*R. chinensis*) leading to *Rosa x hybrid*, the modern rose selected for defined traits such as shape, colour and flagrance of the flower bud and flower qualities, stem length, and vase life. During these times, probing the genetic diversity within wild populations and selection of progenies from crosses were solely based on morphology.

#### *2.3.1.1. Probing the genetic diversity*

During ancient times, botanists such as Linnaeus [15] have played a crucial role in probing rose genetic diversity and defining boundaries between species. Linaeus [15] was one of the first botanists to acknowledge the complexity of the genus *Rosa*. In his book "*Species Planta-*

*rum*" Linaeus stated that "the species of the genus *Rosa* are difficult to distinguish and determine, I have the impression that nature combines just for fun a number of them and then forms a new one out of the lot, those who have seen only some distinguish them more easily than those who have examined many". The complexity of the genus has remained enigmatic to taxonomists of the twentieth century [13, 16-19] as the morphological characters are continuous and possibly polygenic making difficult in assigning genotypes that clearly define taxa. Nonetheless, similar to any other plant species, end-uses have been instrumental drivers for probing the genetic diversity and guiding in the selection process.

*2.3.1.2. Process and goal for probing the genetic diversity (food and ornamentals)*

During the Middle Ages, dogroses were cultivated at monasteries as a medicinal plant and, all parts including rosehips, seeds, petals, leaves and roots were virtually used. Later on in the 19$^{th}$ century, dogroses served as rootstocks to graft modern rose cultivars either as frost or soil born disease resistance sources [13]. They have also been used as a rustic and hardly living fence for fields and public spaces. In the twentieth century, roses have become important horticultural and cosmetic crops receiving much attention from geneticists, breeders, and general public. Hybrid Tea varieties of roses (*Rosa hybrida* L.) are among the most economically important cut-flower plants. The first Hybrid Tea rose was introduced in 1867, and since then more than 10,000 varieties have been released.

The Centre for Variety Research, the Netherlands, has submitted more than 2,800, predominantly Hybrid Tea varieties, for Plant Breeders Rights. This number is increasing annually with 80 applications on average each year. This registration and protection process is based on morphological and physiological characteristics as described by the UPOV (Union Internationale pour la Protection des Obtentions Végétales) guidelines [20]. Wild roses, semi-domesticated and commercial varieties, serve as breeding materials for creating new genetic stocks. These breeding materials generally selected as seed or pollen parents, for flowers that are often flagrant, commonly rose-colored flowers although white or more rarely yellow flowers can be observed in some species [21] are used in crosses. Hence, seedlings of interest with differences in flagrance, colour, shapes, disease resistance genes are selected through extensive field trials and advanced in the registration process [22]. Among the many wild rose species, the selection was obviously based on easy availability, attractiveness of characters, seed set potential, but also the plant morphology such as dwarfness and small size of flowers [22]. During these times less emphasis was made on the wild rose fruit characteristics.

*2.3.2. Modern tools*

In modern times, these classical methods become less and less efficient as the number of varieties to be tested increases and the genetic distances between varieties becomes smaller [20]. As well, because the needs, objectives, and challenges associated with the rose industry are now changing both in terms of flower and fruit production, combination of morphological, cytological, conventional breeding and biotechnological methods are being widely used for the determination of *Rosa* species as well as for the development of new rose cultivars [23-28].

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*2.3.2.1. Probing the genetic diversity*

Domestication and crop improvement involve the selection of specific alleles at genes controlling key morphological and agronomic traits, resulting in reduced genetic diversity relative to unselected genes [10]. This artificial selection process that operates also in almost all agro-systems, including agroforestry, favours abundance of the preferred targeted phenotypes, and acts with more intensity in household gardens [29]. In the 20th century, probing for crops and their wild relative's genetic diversity has been the focus of extensive investigations. In roses in particular, morphometric [13, 30-34], cytological characters [25, 35] were the most used in the *Rosa sp* taxonomy and phylogeny. But these methods have been proven not to be sufficient in assigning individual genotypes that clearly defined taxa [13]. The 21st century is characterized by a remarkable explosion of molecular tools, highly polymorphic and with high discrimination power, for deciphering differences based on DNA nucleotide sequences. The development of these tools were achieved mostly with the event of polymerase chain reaction (PCR) in the mid 1980's [36], which has revolutionized the field of biology by inspiring the development of many PCR-based technologies, large DNA sequence databases, and increased computer power by bioinformatics. Despite the success of these powerful tools and its speed in advancing our current knowledge of the *Rosa* phylogeny [16, 17, 19, 37-43], there is still not exist at present a single method or tool for tracing a clear cut relative phylogenetic position between *Rosa* subgenera, sections and species within the genus [16], mainly due to low sequence divergence, natural hybridization between taxa, and polyploidy [44]. Rather, complementary methods (morpho-cytology, ploidy level, and DNA sequences from both chloroplast and nuclear genomes) using extensive data computing, with iterations and bootstrapping, are now the approach commonly sought [16, 17, 39, 40, 44, 45, 46, 47]. Nonetheless, for well-defined *Rosa* species, the DNA sequence analysis for single nucleotide polymorphism [47] and SSR polymorphism [48] are the preferred choice for distinguishing between genotypes and varieties [20]. The current *Rosa* phylogeny relies mainly on Rehder [49] who subdivided the genus into 4 subgeneras: *Hulthemia, Platyrhodon, hesperhodon,* each with 1 or 2 species, and *Rosa*. Likewise, the large *Rosa* subgenus was divided into 10 sections (*Pimpinellifoliae*, *Rosa*, *Caninae*, *Carolinae*, *Cinnamomae*, *Synstylae*, *Indicae*, *Banksianae*, *Laevigatae*, *Bracteatae*). However, recent molecular evidences do not support distinct subgenera status [16, 50] but did support the presence of 2 main clades. One clade includes subgenera *Rosa* species of sections *Carolinae*, *Cinnamomae,* and *Pimpinellifoliae* (clade 1) and the other clade (clade 2) includes all remaining subgenera *Rosa* sections, excluding the section *Banksianae* which comprises *R. Banksiae* (section *Banksianae*), *R. roxburhii* (subgenera *Platyrhodon*), and *R. persica* (subgenera *Hulthemia*), found to be sister to clade 2 [16]. The section *Caninae* DC forms a large and well-defined group of polyploid taxa and known as dogroses. In this section, pentaploids are the most common, but tetraploid and hexaploids also occur [18]. Bruneau et al. [16] also showed that sections *Cinnamomae* and *Carolinae* form a monophyletic group, and should be merged into one section, referred to as sect *Cinnamomae*. Indeed, section *Cinnamomae* comprises more than 40% of the species in the genus *Rosa*.

*2.3.2.2. Process and goal (life quality)*

One of the main current questions is whether the process and goal for probing rose genetic diversity has changed over time. Although crop domestication and improvement process is a continuum, it evolves constantly with the available technologies in order to meet and fulfill

the societal needs. In the present global economy, the scale of demands for any good has increased and the trade has become multidirectional (selling in all part of globe) with multiple layers (one product could be found in many other products as additive or supplement) (Table 1). Thus, probing the genetic diversity of a plant species which end-product would satisfy these new needs both in terms of quality, quantity, sustainability and stability has become the new challenge for plant products developers. Hence, the need for well characterized germplasm with stable and preserved genetic identity is becoming the landmark for todays and tomorrow's natural product designers and developers. Therefore, sophisticated molecular tools [51, 52] as well as mass tissue culture and plant propagation tools are being employed to insure stability and sustainability.

| | **Ancient domestication** | **Domestication in the 21st century** | **References** |
|---|---|---|---|
| Purposes | Food, medicine clothing, energy, sustainability | Food, clothing, energy, health, life quality, sustainability | [28, 53, 54] |
| Screening methods | Morphology, taste, flavour, energy | Morphology, genetic DNA markers, QTLs, taste, flavour, energy, metabolite profiles, | [20, 41, 51, 52] |
| Production paths | Gathering, yards and small farms sowing and harvesting, human and animal force | Experimental tubes, growth chambers, greenhouse and fields, large commercial fields, high throughput management, human and animal force and mechanization | [28, 53] |
| Purity | Composite | Composite, variety | |
| Ecosystem | Complex | Simple | [53] |
| Yield | Low | High | [28, 55, 56] |
| Value chain | Self, local consumption, | Global, processing, distribution and marketing networks | |

**Table 1.** Comparative pathways of ancient and modern plant domestication processes: purposes, tools, and expectations

## 3. Plant domestication in the 21st century: A case study with PEI wild rosehips

One of the most recent and successful domestication of a wild species is that of the North American ginseng [57]. Similar to ginseng, interests in wild rosehip products are increasing worldwide due to its nutraceutical and natural health products properties [13]. With aging

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and changing eating lifestyles, the incidence of chronic diseases is increasing worldwide. Despite success achieved in fighting these diseases, prevention measures have become top priorities for citizens and public health systems. Recently, increasing interest has been expressed in plant natural products as preventative agents. Hence, plant product preparations such as those from rosehip have been used as food and medicine for centuries. The genus *Rosa* contains more than 150 species. They are widespread in North America within the *Cinnamomae* section and are renowned for the vitamin C content [58-61]. Although formulations from *Rosa canina* have been associated with the treatment and symptom reduction of inflammation and arthritis, the vast majority of wild rose species are fully unexplored for their heath potential. To date, most of the reported studies were focused mainly on *Rosa* species within the *Caninae* section which comprises 20 – 30 *Rosa* species known as dogroses [18, 42] and is currently the focus of major domestication research programs for the production and commercialisation of rosehips (fruits) around the world, particularly in Northern Europe, Germany, Turkey, Eastern Europe and Chile [13]. So far, less emphasis has been made on *Rosa* species belonging to *R. carolina* complex within the *Cinnamomae* section and the rosehips production from the eastern North American native wild roses is new and emerging [55, 56]. This section deals with the genetic diversity of PEI wild rosehips, the challenges associated with their domestication as well as the agronomic practices that could ensure an economic production.

### 3.1. Introduction to the genus *Rosa*

The genus *Rosa* (*Rosaceae*) originated in the temperate regions of the northern hemisphere, including North America, Europe, Asia, and the Middle East, with the greatest diversity of species found in western China, where it is endemic, and is now widespread all over the globe [18]. With this wide distribution range and the high number of species (more than 150 shrub species), the delimitation of the species bounbaries remained a challenge for taxonomists and molecular biologists [16, 21, 41, 44].

### 3.2. *Rosa* species phylogeny and biodiversity

#### *3.2.1. Global Rosa species biodiversity and phylogeny*

The taxonomy and breeding system of the genus *Rosa* has been recently reviewed by several authors [13, 16, 21, 38, 49, 62, 63] and the reader is invited to find more details in these treatments. Of particular interests are works reported by Werlemark and Nybom [13] and Macphail and Kevan [21] on one hands, and those by Bruneau et al. [16] and Joly and Bruneau [44] on the other hands, focusing on the European Dogroses from section *Caninae* and the North American *Rosa* species from section *Cinnamomae*, respectively. Wild rose species from these two sections are currently extensively investigated for domestication purposes and commercial rosehip production [13, 55, 64-67]. As our interest lies mainly in the domestication of North American wild roses, the next section of this review will put more emphasis on the biodiversity and phylogeny of wild rose species commonly encountered in this part of the globe and more specifically in Canada, a country as large as the whole Europe (West and East taken together, excluding the former USSR).

*3.2.2. North American Rosa species biodiversity and phylogeny*

Biodiversity of the North American wild roses has been investigated by botanists in the early 1900's. Watson [68], Crepin [69, 70], Erlanson MacFarlane [71, 72] have described and defined 13 - 22 *Rosa* species in North America. This important polymorphism in *Rosa* species, especially in eastern North America, together with hybridization and polyploidy have long been considered as the major causes of taxonomic confusion in the genus [17]. Alfred Rehder (1869-1949) established the first foundation of *Rosa* species taxonomic relationship in a book entitled "*The Manual of Cultivated Trees and Shrubs Hardy in North America Exclusive of the Subtropical and Warmer Temperate Regions*" published in 1940 [49]. Rehder provided concise physical description, time of flowering, region of native habitat, hardiness zone, distinguishing features and pertinent information on North American roses, and subdivided the genus *Rosa* into 4 subgenera and 10 sections, including the *Rosa carolina* L. complex of section *Cinnamomae*. East of the Rocky Mountain, the *Rosa Carolina* complex is composed of five diploid species (*R. blanda*, Ait., *R. foliolosa* Nutt., *R. nitida* Wild., *R. palustris* March., and *R. Woodsii* Lindl.), three tetraploid species (*R. carolina* L., *R. virginiana* Mill., and *R. arkansana* Porter) and one hexaploid/octaploid species (*R. acicularis* Lindl.) which is morphologically distinct from all other species [17]. The taxonomic problems are well known at the diploid level, where some species hybridize and are also morphologically difficult to distinguish (which is particular true for *R. blanda* and *R. woodsii*), but are even more acute at the polyploidy level. *Rosa carolina* which is widespread East of the Mississipi river hybridizes with *R. Arkansana* in the western part of its distribution [71] but also in the East with *R. virginiana*. Moreover, the morphological similarity cuts across ploidy levels and no single morphological character can be used to distinguish one species to another [17]. Thanks to molecular tools (AFLP, SNP), haplotype network analysis using statistical parsimony, genealogical approach, and multivariate analysis of 25 morphological characters including ploidy determination based on stomatal guard cell lengths, Joly et al. [17] and Joly and Bruneau [44] determined four species at the diploid level and that were separated into 2 groups in the east of the Rocky Mountains: one group consists of *R. blanda - R. woodsii* (which were indistinguishable and should be considered as a single species), and the other group is consisted of *R. foliolosa*, *R. nitida*, and *R. palustris*. The authors also determined 3 species at the polyploid level: *R. arkansana*, *R. carolina*, *R. virginiana*, with evidence of hybridization between them. The diploids that are involved in the origins of the polyploid species in that region were also proposed. For Joly et al. [17], only diploids east of the Rocky Mountains are involved in the origins of polyploids. *Rosa arkansana* is derived from the *blanda-woodsii* group, *R. virginiana* originated from the *foliolosa- nitida-palustris* group, and *R. carolina* is derived from a hybrid between the two diploid groups. Thus, for wild rose species domestication and commercial production purposes in the Canadian Maritimes where both North American native wild species of the *R. carolina* complex grow in sympatry and also along with naturalized species such as *R. rugosa* or other members of dogroses (Figure 1), a careful species determination as well as genotypic identification of collected germplasm for propagation are of critical importance to ensure, genetic purity and traceability.

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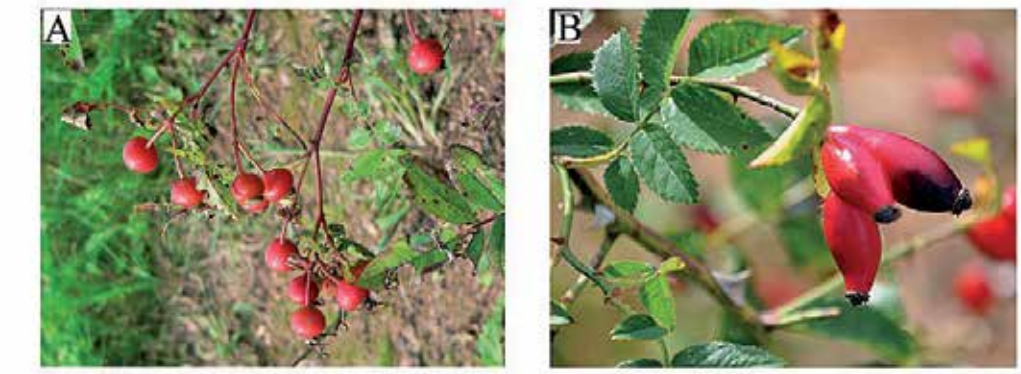


**Figure 1.** Diversity of rosehip morphology in the Atlantic Canada landscape. A, typical morphology PEI grown rosehip. B, morphological feature of a naturalized rosehip to Atlantic Canada.

*3.2.3. Genetic and Metabolite diversity within the Prince Edward Island's field collection*

Using SSR markers [20] and single nucleotide polymorphisms analysis, our group has assessed the genetic diversity within 30 ecotypes under cultivation and identified three major clusters, with cluster 2 and 3 showing 2 and 3 sub-clusters, respectively [65, 73]. The metabolite profiles in the flesh, seed, and fuzz for anthocyanins, flavonols, tilirosides which is a potent antidiabetic compound, tannins and fatty acids were also determined from the 30 ecotypes [65, 73]. The level of anthocyanin was very low in all ecotypes, with only one ecotype showing a level that was 30-40 % higher compared to the average. A large diversity was observed for flavonols and tiliroside among ecotypes. Only 4 ecotypes had a high content for both flavonols and tiliroside in the analyzed tissues (Ghose et al, submitted). One ecotype showed 18:3 level as high as 41.2%. The data suggests that it is possible to select and propagate a given ecotype for its unique metabolite profile for commercial and drug production [65, 73].

**3.3. Domestication and end uses**

Roses have been domesticated by man first for the beauty of their flower and incorporated in many cultural and political practices [74] and are now encountered on all continents, climates, and market places. Nonetheless, the medicinal uses of rose leaves, flowers and fruits were also widespread in human history [13, 54, 75-78].

*3.3.1. Flower roses*

The best known uses for roses are their flowers as ornamental on tables, in home backyards, public gardens and spaces. Historically, only very few wild rose species (at most 5 to 11 species) have been involved as parents in the today flower roses. One example of using native rose species in North America is related to the Parkland Rose series developed at AAFC in Morden, Manitoba. These flower roses are hardy, winter resistant and some of these rose varieties involve in their

pedigree *R. Arkansana* which is encountered east of the Rocky Mountain in Canada. Beside, its ornamental features, rose flowers are valuable for the cosmetic industry [75, 76, 78].

*3.3.2. Wild rosehips*

The fruits of roses, the hips, have been highly regarded as important food and medicinal sources [13, 54, 79]. Rosehip is appreciated as traditional vitamin C rich soup in Sweden where the demand is particularly high [80]. Its flesh and seeds have been used in concoctions and tonics for various ailments, including the use as laxative and diuretic, against common cold, gastroinstestinal disorders, gastric ulcers [77, 81, 82], and anti-inflammatory diseases such as arthritis [83]. A review on the major chemical components of dogrose hips from was recently made by Werlemark [13]. However, a marked variation in chemical composition is associated with species, genotypes, and environments in which the plants evolve. For example, Melville and Pyke [84] found a weak correlation between latitude and vitamin C content of British rosehip populations from Scotland and England. Similarly, Werlemark [13] hypothesised that rosehips produced in a colder climate, especially with colder summer, may have higher vitamin C content compared to those that have been maturing in a warmer climate and also anticipated that local variations in precipitations and temperatures during summer may affect the chemical content of rosehips. It is reasonable to assume that, with different species and cooler summer and fall (Table 2), the Canadian Maritime wild rose species would show different chemical composition, especially in terms of relative amount when compared to their European and South American counterparts. By comparing some rosehip samples from Prince Edward Island, Denmark, Chile and South Africa, our group observed differences between origins, especially with regards to total oil content and fatty acid profiles (Figure 2). Nonetheless, sample preparation (harvesting time and conditioning) can also be a major source of variation. It will be of interest to compare the chemical composition of rosehips collected in each of these regions during the same summer or fall for obtaining factual and conclusive answers to these assumptions.

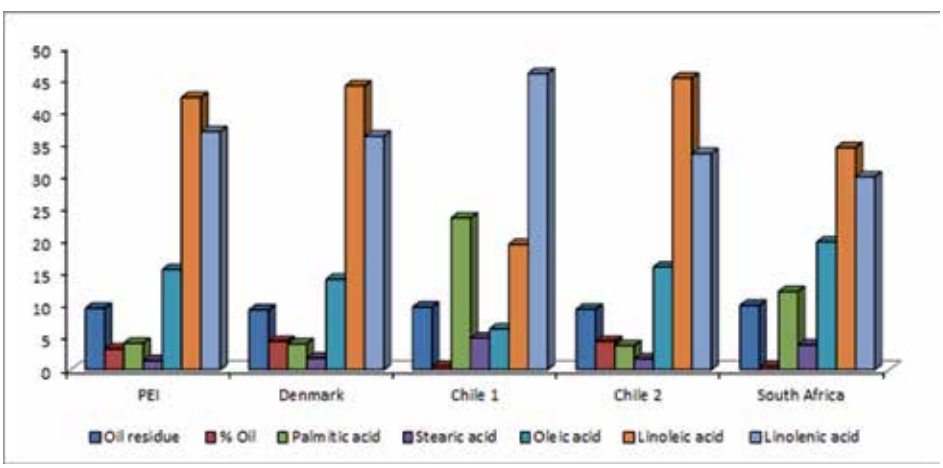


**Figure 2.** Comparative study of rosehip samples from Prince Edward Island, Denmark, Chile, and South Africa.

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Rosehip seed contains pretty well balanced omega-6 (18:2) / omega-3 (18:3) fatty acid ratio and also shows relatively high level of oleic acid as compared to olive and canola oils that are rich in oleic acid but low in both linoleic and linolenic acids (Figure 3). As genetic variability for fatty acid composition has been observed in PEI wild roses (Ghose et al, submitted) and the seed oil content is relatively low, breeding efforts could contribute to increase the oil content.

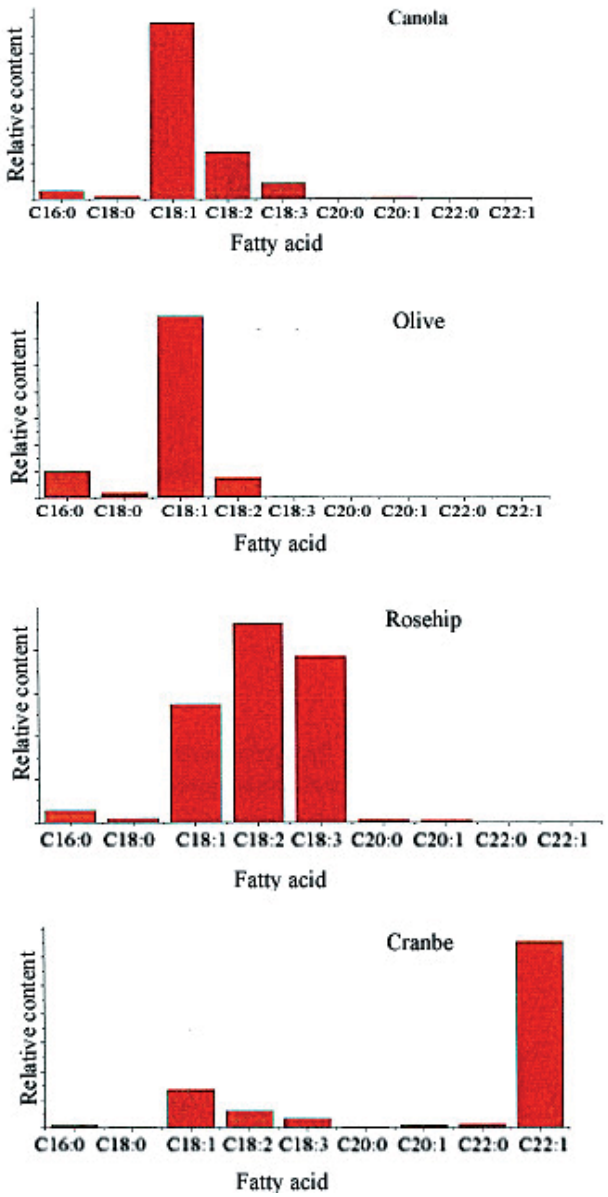


**Figure 3.** Comparative fatty acid profile of rosehip with three oilseed crops.

*3.3.2.1. Agronomy*

Although a high value was recognized to rosehip throughout centuries, it is only recently that the wild roses are being domesticated and cultivated for their fruits and to develop agronomic practices that ensure an economic production of the hips [28, 51, 52, 56, 77, 85]. However, due to the diversity of species, genotypes, soils and climates, different agronomic practices are being implemented and tested in different regions, including Denmark, Turkey, Bulgaria, Chile and Canada. Whereas Chilean started their trials by developing a nursery built on the "Tunnel" greenhouse model with a capacity to accommodate 15.000 cuttings, under an irrigation system with nebulizers to reduce temperature and humidification before a developmental stage in the fields, the Danish, Swedish and Canadian choose to established field trials using wild cutting, spacing, density and nutrient management trials [28, 55]. In Sweden, the germplasm used were mostly concentrated on the Scandinavian *Rosa* species of section *Caninae* especially, *R. dumalis, R. rubiginosa* and their interspecific hybrids [86] whereas Danish rosehips are produced mainly from *R. canina* (www.hyben-vital.com) although it may also involve other Scandinavian species. In Chile, the current production is mainly focused on wild hand-harvested hips from uncharacterized and naturalized species introduced to south America by Spanish and is mostly a mixture of *R. rubiginosa, R. canina, R. moschata* and many other species found in western Europe [66]. In Prince Edwards Island, (Canada), current recent genetic study based on 30 wild ecotypes collected from this province suggested that all accessions currently under field trial are from *R. virginiana* and its natural hybrids with *R. Carolina* (Ghose et al, submitted). At present, very few cultivars have been named and released for commercial fruit production. One cultivar, the cultivar "Mechthilde von Neuerburg" derived from *R. rubiginosa* was reported in Germany. Two cultivars (Sylwia and Sylwana) derived from *R. canina* were reported in Poland, whereas cultivar Plovdiv 1 from *R. canina*, and cultivar Karpatia from *R. villosa* were reported in Bulgaria and Slovakia, respectively [13]. For all of these semi-domesticated wild rosehips, it is not known or reported whether the ongoing domestication process has already impacted on some of the phenotypic traits such fruit size, fruit setting or metabolite profile. By comparing the pomology characteristics of 5 wild rosehip ecotypes growing in the wild or in the field settings, we observed that the field setting contributed to increase the fruits size and delayed the maturity when compared with growing in the wild, suggesting an occurrence of a domestication syndrome for these traits (Fofana, personal observation). However, no significant difference was found between the two environments for the number of seed in each of the ecotype.

*3.3.2.1.1. Soils and climates*

Although originally native to temperate regions of the globe, roses have adapted to warmer regions and grow well now in very diversified habitats and soil types [13, 79]. The soil should be well drained though and not heavy. Species preference for soil type has nonetheless been reported. *R. villosa* was reported to grow better in a dry soil with low calcium content whereas *R. canina* and *R. dumalis* prefer more calcareous soil. *R. rubiginosa* also prefers more calcium and grows well in a relatively heavy soil [13]. *R. palustris* grows in marshes and *R. nitida* in bogs. Similarly, *R. virginiana* likes salt marshes and salty soils (Joly, personal communications).

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In Prince Edwards Island province (Canada), wild rosehips are found in a variety of habitats including hedgerows, wet and dry pastures, thickets, swamps and uplands in dry orthic humo-ferric Podzol sandy soils [55]. In hard winter climates such as Canada, plant survival rate in the field setting can vary from genotype to genotype and for the same genotype, plastic coverage has been shown to increase the winter survival rate (Figure 4).

**Figure 4.** Effect of planting beds coverage with plastic on winter survival.

| | Temperature (°C) | | Precipitations (mm) | | Soil type | Latitude |
|---|---|---|---|---|---|---|
| | **Summer** | **Fall** | **Summer** | **Fall** | | |
| PEI Canada | 16 – 22 | 7 – 18 | 270 | 300 | Orthic humo-ferric Podzol with sandy loam | 46.04 – 46.57 |
| Denmark | 17 | 9 | 170 | 150 | Typic Fragiudalf | 55 – 57.4 |
| Sweden | 13 | 5 | 180 | 120- 140 | Aeric Endoaquept | 55 – 68 N |
| Turkey | 17 – 29 | 6 – 7 | 50 | 70 | Typic Haploxeroll | 36 – 42 N |
| Bulgaria | 25 | 14 | 180 | 120 | pseudopodzolic- podzolic | 41 – 43 N |
| Chile | 17 – 28 | 8 - 20 | 350- 500 | 200 -300 | Andisol - Ultisol | 18 – 58 S |

**Table 2.** Comparison of average temperature and precipitations during summer and fall in major rosehip production countries.

*3.3.2.1.2. Fertilization*

Barry et al [55] described the first time the establishment of field trial for North American wild roses belonging to the *R. carolina* complex, with as an objective to investigate the effects of several field management practices on commercial rosehip production in Atlantic Canada. Treatments were applied at planting in a factorial randomized complete block design in June 2004 and included three in-row mulch (none, bark, and straw) treatments, three in-row fertility

(none, compost, and fertilizer) treatments, and two interrow management (tilled and sod) treatments. The compost consisted of an initial mix of softwood sawdust, lobster waste, and old hay. Prior to planting, compost was applied at 60 t ha−1 (54 kg plot−1) in a 1-m band over the row and was incorporated by hand raking. The fertilizer used was a commercial grade (5N-20P-20K). This fertilizer formulation was chosen for use during the first year to promote root development and plant establishment. During the second year (2005), compost was reapplied as top-dress on 22 June 2005 and the fertilizer used was a commercial grade (10N-10P-10K), which was applied as top-dress on 25 May 2005. A fertilizer with higher nitrogen content was chosen with the aim of improving overall plant health and yield during the second growing season. Fertilizer was applied at a rate of 800 kg ha−1 (648 g plot−1) in a 1-m band over the planting row. In Dogroses, Werlemark and Nybom [13] reported 50 g NPK for each plant at planting and 300 kg/ha of organic-mineral NKP in the subsequent year, with additional calcium amendment depending on soil types and species. In Prince Edwards Island, mulching increased nutrient uptake of N and P and increased plant growth. Fertilizer increased plant growth and yield of rose hips compared to no fertilizer or compost treatments. Tilled interrow treatment increased in shoot lengths, diameters, and plant spreads compared to interrow sod. The study indicated that during the early establishment years of a rose hip plantation in Atlantic Canada, wild roses grow best with the use of mulch, fertilizer, and tillage between the rows [55].

*3.3.2.1.3. Pests and diseases management*

Traditionally, fungal diseases such as black spot caused by *Diplocarpon rosae*, powdery mildew (*Podosphaera pannosa*) rusts (*Phargmidium spp*) and leaf spot *(Sphaceloma rosarum)* have been reported to be problematic in ornamental roses [87-90] and field-grown dogroses [13, 48, 91, 92]. These fungal diseases management is carried through fungicide treatment [93] and selection of genetic resistance [94-96]. Genetic resistance sources within wild rose species within *Caninae* section have been investigated for field rosehip production. Fungal disease tolerance characteristics were identified in *R. rubiginosa* and in interspecific hybrids involving species from *Caninae* and *Cinnamomae* sections [48]. Up to date, no such disease resistance screening has been performed within the *R. carolina* complex for a commercial wild rosehips production in North American. However, our observations in the field showed evidence of these diseases on PEI wild roses (Figure 5). Research in this field should be carried to mitigate the disease incidence in their new field environment. As for any crop, introduction of elite genotypes in cropping systems for rosehips production will lead to a decreased genetic diversity of the cultigens. It is thus anticipated that more susceptibility to major diseases could be observed in the field as compared to the wild populations from which they derive. The preservation of natural habitats hosting the wild populations is of great importance to ensure an availability of genetic stocks to be used in the introgression of disease resistance genes from the wild types to the cultigens.

Insects such aphids (*Aphidina*), grasshoppers (*Orthoptera*), mites (*Tetranychidae*), sawflies (*Tenthredinidae*), gall-making cynipids (*Diplolepis*) as well as the rosehip fly (*Rhagoletis alternate*) have also been reported in dogrose orchards and to cause severe damage in some cases

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[13]. Nematode (*Pratylenchus penetrans*) is causal pests of severe lesions to roots in a wide range of ornamental hosts, including roses, mainly in temperate regions. Peng [97] reported that *R. virginiana* is a good nematode resistance source. Because Prince Edwards Island is world leading potato producing area with prevalence of nematodes in the agricultural landscape, development of rosehips orchards with *R. virginiana* genetic background could be a mean for reducing nematode populations in highly infested fields.


**Figure 5.** Foliar and fruits diseases in wild roses. A, powdery mildew; B, leaf spot; C, lesions on immature rosehips probably caused by *Phargmidium spp* (Rust) or *Sphaceloma rosarum* (leaf spot).

*3.3.2.1.4. Yield and storage*

Rosehip yield vary considerably depending on the plant material, cultivation procedures, age of orchard, and harvesting methods. Werlemark and Nybom [13] reported that up to 8 kg of rosehips per bush could be harvested by hand in commercial planting of dogrose hybrid PiRo 3. Similarly up to 3 t/ha could be obtained from *R. dumalis* and *R. rubiginosa* with mechanical harvesting in Sweden. In these cases however, no mention is made about the age of the orchards as yield increases markedly several years after planting. In contrast, Sanderson and Fillmore [56], reported in 14 rosehip ecotypes of the *R. Carolina* complex grown in field condition an average rosehip yield ranging between 411 and 2000 kg/ha, with a fruit mean weight of 1.01 – 1.62 g, over the first four hand harvesting years. The lowest and highest yielding selections showed 910 and 3634 kg/ha in the fourth years, respectively (Table 3).

Compared with reports by Ercisli and Guleryuz [98], Dogan and Kazankaya [99], Güneş and Dölek [100], the fruit weight reported by Sanderson is lower but showed relatively narrow range of variation between ecotypes, reflecting the relatively narrow genetic diversity among these ecotypes. Joly (personal communication) reported that *R. virginiana* and *R. Arkansana* are the two species with the greatest number of fruits per flowering branches. They have more fruits than *R. carolina* and the height of *R. virginiana* makes it one of the most productive North American roses. To preserve the integrity of rosehip bioactives, the postharvest handling and storage conditions are key factors. Both sun-drying and mechanical dryers are being used at commercial scale and the reader can see more details in Werlemark and Nybom [13].

| | Biological yield | | | | | Mean fruit |
|---|---|---|---|---|---|---|
| **Selection** | **2006** | **2007** | **2008** | **2009** | **Mean** | **weight** |
| | | | **(kg ha$^{-1}$)** | | | **(g)** |
| s26 | 877 | 1413 | 2368 | 3634 | 2000 | 1.62 |
| s30 | 347 | 569 | 1557 | 2676 | 1431 | 1.31 |
| s28 | 498 | 335 | 1136 | 1759 | 946 | 1.57 |
| s22 | 416 | 422 | 831 | 1464 | 783 | 1.29 |
| s67 | 270 | 338 | 910 | 1440 | 740 | 1.39 |
| s25 | 355 | 116 | 562 | 1725 | 719 | 1.29 |
| s57 | 195 | 371 | 941 | 1178 | 675 | 1.03 |
| s33 | 395 | 330 | 654 | 1227 | 657 | 1.33 |
| s55 | 313 | 384 | 679 | 1167 | 638 | 1.42 |
| s36 | 181 | 166 | 862 | 1307 | 622 | 1.01 |
| s140 | 300 | 186 | 576 | 1342 | 610 | 1.21 |
| s142 | 406 | 430 | 464 | 956 | 568 | 1.17 |
| s68 | 284 | 281 | 430 | 1092 | 514 | 1.21 |
| s122 | 246 | 83 | 416 | 910 | 411 | 1.12 |
| Grand mean | 363 | 387 | 885 | 1563 | 808 | 1.28 |

**Table 3.** Yield progression over four years after plantation and mean fruit weight of 14 rosehip ecotypes grown in field (2006-2009)

*3.3.2.2. Biotechnology*

One of the shortcoming issues for the establishment of commercial rosehip production orchard is the availability plant materials for large acreages. So far, all established fields are based on cuttings or seedlings obtained from wild selections. Because of the genetic diversity within the genus *Rosa* and morphological similarities between species, hybrids (interspecific and intraspecific) and their parental species at the collection sites, an accurate identification at the collection site and the traceability of the putative cultivars under development is challenging and not guaranteed. This issue will become major issues in a near future as rosehip provenances will increase and the bioactive metabolites that are associated to each species, provenance, and ecotypes are made available for marketing purposes. Thus, the use of combined

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morphological, cytological, and molecular biology tools for assigning a genetic identity, and the use of regeneration technologies that ensure mass plant production and ensuring the genetic integrity of clones is a research direction that should be undertaken similarly to the ornamental flower industry.

*3.3.2.2.1. Regeneration and propagation*

*3.3.2.2.1.1. Regeneration by seed*

The use of plant regeneration from seed for commercial production has been reported [85, 101]. It ensures the production of higher number of plants for field planting in a relatively short period of time. However, the mating system of *Rosa* species is a major source of genetic variability between plant materials obtained using such an approach, especially when the seed is collected from uncontrolled sources like wild plants.

*3.3.2.2.1.2. Cutting and explants*

Cuttings and explants are currently the materials of choice in commercial wild rose production [64, 86, 101], and most, if not all, of these explants (Figure 6) are derived from wild plants. Wild rose plants grow in the nature as populations that can involve different species, interspecific and intraspecifc hybrids, parental and sibling all growing in a confined area. Collecting cuttings in such an environment, even from the same patch, does not ensure the genetic integrity of the collected material for propagation. Once collected, the material should be well characterized and identified. Now, remains our ability to get enough characterized plant materials for large field planting. We believe that the well characterized plant material should be used as starting point for plant regeneration and mass production in the form of rooted seedling or cuttings. This is the approach we pursue in Canada for commercial wild rose production (Figure 7).

*3.3.2.2.1.3. Tissue culture*

Tissue protocols have been developed and available for flower roses [102-104] and could be applied to rosehip production. Once elite genotypes such as those reported by Sanderson and Fillmore [56] are identified, tissue culture should be able to ensure a sustainable plant production or field planting by growers (Figure 7).

*3.3.2.2.2. Cell culture*

Similar to tissue culture, rose plants can be regenerated by cell culture. Contrary to tissue culture however, the new plants are obtained from callus generated from sterile explants. This method leads to pure line but can also create new lines different from the mother plant from which the explant was obtained because of somaclonal variations that may occur during the induction of callus and regeneration processes. Thus, for the production of mass plant production from a selected elite wild ecotype, tissue culture appears more appropriate as it minimizes the risk of somaclonal variations while showing high rate of plant multiplication.

**Figure 6.** Rose cuttings for multiplication. Sterile rose dormant stems were conditioned to break dormancy. Note the active buds sprouting.

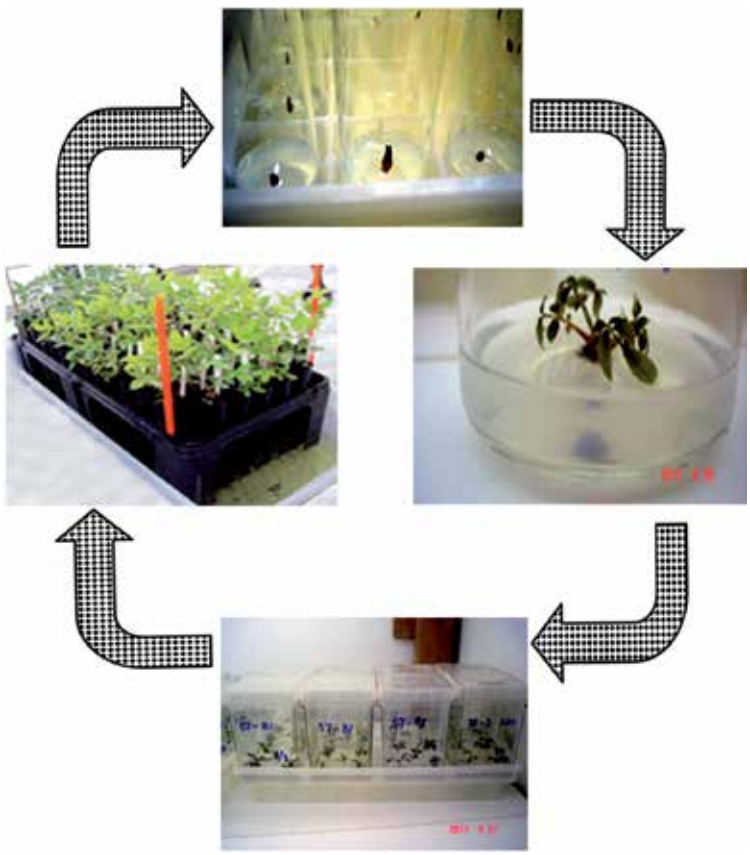

**Figure 7.** Mass rosehip plant regeneration from active buds of well characterized rosehip genotypes. A, active buds in regeneration media; B, regenerated rose plant; C, plant multiplication in rooting media; and D, acclimation in greenhouse.

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## 4. Conclusions

With the increasing demands for natural heath products, plant biodiversity is being thoroughly revisited. The genus *Rosa* has a complex taxonomy that is still being investigated with scrutiny. East of the Rocky Mountain, *Rosa* species belonging to *R. carolina* complex in the *Cinnamomae* section include five diploid species, three tetraploid species and their natural hybrid. Several of these species as well as their interspecific hybrids are encountered on Prince Edwards Islands, Canada. A commercial rosehip production program using wild selected ecotypes has been developed and elite selections with high yielding potential have been identified and agronomic practices set for field management. The collection has been characterised using a combined morphological, cytological and molecular tools and appears to be made of *R. virginina* and its natural hybrids with *R. carolina*. Genetic and metabolite diversity among these wild ecotypes was observed and could be of high potential for large field production and breeding programs. However, the disease resistance status in this complex is unknown. As for any new crop, increased incidence of exiting diseases and recruitment of new diseases is anticipated in the field setting as compared to the wild populations from which they derive. The preservation of natural habitats hosting the wild populations as source of genetic stocks is critical to ensure gene transfer from the wild types to the cultigens through breeding. Future works should also aim at developing mass plant production to ensure sustainable plant material supply from the elite selections.

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Chapter 3

# Magnetic Resonance Technologies: Molecules to Medicine

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Additional information is available at the end of the chapter

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## 1. Introduction

Since the discovery of Electron Paramagnetic Resonance (EPR) spectroscopy in 1944 by Zavoisky[1], and the realization of the Nuclear Magnetic Resonance (NMR) spectroscopic signal in the mid 1940's by Bloch and Purcell[2,3], the capabilities and applications of the technology have continued to advance at an enormous rate particularly after the implementation of Fourier transform NMR in the mid 1960's by Ernst[4]. Magnetic Resonance (MR) spectroscopy was initially utilized to characterize the structure of matter.[1,5] Through the early to mid 1970's, the development of multidimensional (nD) methods and more powerful instruments opened the door for the detailed atomistic characterization of small molecules culminating in structural elucidation of proteins by the mid 1980's.[6] At about the same time, it was proposed that a magnetic field gradient could be applied to obtain a 3-dimensional (3D) image leading to the invention of nuclear Magnetic Resonance Imaging (MRI) with, among other capabilities, the potential to monitor the bio-distribution and bio-accumulation of molecules *in vivo*.[7] Beyond the 1980's MR technologies were mixing with other technologies and evolving to play an integral role for advancing pharmaceuticals and becoming indispensable tools for drug discovery, design and diagnostics.

### 1.1. Capabilities

Early on it was recognized (see Ref. 8 and references therein) that MR techniques can offer a variety of unique advantages over other spectroscopic techniques such as MR is completely non-destructive and non-invasive. Thus, MR technologies can be utilized with inanimate samples or living organisms with no obvious detrimental or destructive effects. In addition, MR techniques can be applied to a variety of states of matter including solution, semi-solids,

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solids and mixtures obtaining comprehensive information of the chemical and physical properties. In addition to the typical static structural information, one can also detail dynamic processes. NMR measurements provide information about dynamic processes with rates in the range from $10^{-2}$ to $10^{-10}$ $sec^{-1}$. Furthermore, many nuclei possess magnetic moments, and with the availability of more sensitive spectrometers, chemists are beginning to take greater advantage of the technique for structure/bonding information for organometallic compounds (for example see Ref. 9).

An important application, although commonly overlooked, is the accurate quantitative information that can be obtained without the need for laborious calibrations. Under quantitative conditions and for all practical purposes with semi-solid or solution state samples, NMR spectroscopy has the unique distinction of having a uniform molar response for all nuclei of the same type *i.e*. all $^{1}H$ nuclei have the same integrated intensity and thus, a single calibrated (internal or more significantly external) standard can be used for accurate quantitation.[10] For the aforementioned reasons NMR is a valuable tool for providing atomistic structural, dynamic and quantitative information on natural products such as small molecules, metabolites, peptides, proteins, complex mixtures, and molecular assemblies such as lipid bilayers or tissues.

Nuclear MRI, on-the-other-hand, can provide 3D images of macroscopic matter, and monitor the bio-accumulation and bio-distribution of MRI tagged natural products *in vivo*. Ultimately MR technologies can be used at almost every stage along the natural product discovery pipeline – from discovery to implementation, from molecules to medicine.

### 1.2. Scope and limitations

MR technologies encompass a range of techniques including electron or nuclear MR spectroscopy, MR time domain, and nuclear or electron MRI. Herein, this chapter focuses on the nuclear MR technologies of spectroscopy and imaging for solution and semi-solid states. We provide a general overview of techniques and methodologies applicable throughout the development pipeline for natural products, as well as some potential impacts the information has for product development. It is well beyond the scope of a chapter (or in fact an entire book) to be a comprehensive description of all applicable MR methodologies. Thus within each section, the reader is directed to review articles, books, *etc*.

## 2. NMR spectroscopy

NMR is a technique that detects electrical currents induced by precessing nuclear magnetic moments within a uniform static magnetic field.[11] Nuclei with non-zero spin moments are MR active and in principle are detectable. Each individual type of spin-active nucleus has a unique precessional frequency dependent upon the strength of the static magnetic field, the magnetic properties of the isotope and the local electronic environment of the nucleus. The general precessional frequency is dependent upon the type of nucleus and thus NMR can readily distinguish among for example $^{13}C$, $^{1}H$, $^{2}H$ or $^{3}H$. The applications and significance

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of NMR has exploded because the exact precessional frequency (*i.e*. the chemical shift) within a group of the same nuclei is influenced by the local electronic environment of the nuclei and thus, NMR can readily distinguish (for example) a $^{1}$H nuclei that is chemically bound to different nuclei (*e.g*. carbon vs. nitrogen, *etc*.), is chemically bound to different oxidation states of the same nuclei (*e.g*. methyl vs. methylene carbon), and/or are in identical bonding environments (*e.g*. methyl $^{1}$H nuclei) but in different electronic environments induced by surrounding functional groups (*e.g*. aromatic vs. carbonyl groups). In addition, if a nucleus is influenced by another spin active nucleus either through a bond connection or in spatial proximity, a correlation exists that may be NMR detectable. In this way atomistic properties (such as the 3D spatial arrangement of nuclei and dynamics) can be determined.[12]

In addition to the distinct nuclear chemical shift, data from MR can be further separated based upon relaxation and/or diffusion properties of a nucleus or molecule.[12,13] Thus, MR technologies can discriminate among large molecules like peptides, proteins and macromolecular assemblies, and small molecules like metabolites or synthetic organic molecules. The relaxation time (influenced by the rotational correlation time and molecular fluctuations) of a molecule plays an important role in distinguishing among small drug molecules and large proteins, or between a single lipid molecule that behaves as a small molecule and an assembly of lipid molecules that, as a collective, behave as large molecules.

One drawback is that under typical conditions MR techniques are sample intensive requiring μM to mM concentrations translating to μg to g quantities of material. For natural product discovery, the sample intensive requirement can be an issue as extracts may only contain nano to micro gram quantities of material.[14] A number of methods have been proposed to overcome the mass demand with the most significant for general applications being the invention of cryogenically helium cooled detection systems that substantially reduce thermal noise ultimately improving the signal-to-noise ratio by up to 10 fold and reducing data acquisition times by up to 100 times.[15,16] A next generation improvement is cryogenically cooled probes that require smaller sample volumes. Currently the combination of the 700 MHz NMR spectrometer and new detection technologies requiring only 35 μl of sample affords the Biomolecular Magnetic Resonance Facility at NRC-Halifax one of the world's most sensitive instruments for mass-limited samples reducing the typical quantities by up to 50 times.[17] The limits of detection for this instrument can be as low as 10 nano-grams for small molecules (IWB, NM, TK and RTS unpublished).

Although to some extent the sample intensive nature of NMR can be addressed, a second drawback for larger proteins and macromolecular assemblies (>40 kDa) is the loss of peak resolution due to spectral overlap, broad line-widths, reduced signal-to-noise ratios and increased spectral complexity[12]. There have been efforts to address this issue however, these efforts are limited in scope and application.[18-20]

### 2.1. Structure elucidation

After a natural product or extract has been verified to be biologically active, an essential component within the discovery pipeline is to identify compound(s) and determine structure(s). Structural elucidation is essential if chemical modifications are to be made, if the

product is for human consumption and/or if a patent application is to be filed as it will distinguish the uniqueness of the compound as well as help identify relationships with pre-existing compounds. Structural characterization is somewhat different between small and large molecules; the distinction between the two regimes is defined by the Nuclear Overhauser Effect (nOe) cross-relaxation rate which is positive or negative depending upon the spectrometer frequency and the overall molecular tumbling time.[12] Generally, "small molecules" are regarded as molecules that do not aggregate and have a molecular mass of <1 000 atomic mass units.

*2.1.1. Small molecules*

The advent of nD experiments propelled NMR to be a leading tool for natural product characterization. Previously natural products were degraded into fragments, chemically derivatized and/or completely synthesised to confirm the structure. It is still valuable for structure elucidation using NMR to obtain information from the aforementioned techniques as well as other techniques such as mass spectrometry (MS; for exact mass, functional groups and connectivities), infra-red spectroscopy (for functional groups), and separation techniques (for classes of compound *e.g.* phenolic, steroid, protein, *etc*.).

The initial NMR spectroscopic assessment as outlined in Scheme 1, typically begins with 1-dimensional (1D) $^{1}$H spectra to determine purity, confirm the compound class, and examine the general appearance of the peaks. A spectrum with sharp well resolved peaks and the anticipated ratio of integrated intensities is indicative of a pure sample dissolved in an appropriate solvent. Broad peaks or peaks that are of fractional ratio could indicate an impure sample, however, they could also be an indication of chemical exchange or limited solubility. The preliminary information gained from other techniques is important when ascertaining if the spectral appearance is appropriate. From 1D data the splitting patterns from J-(*i.e.* scalar)-couplings provide information on the pattern of covalent bonding as well as the torsional angle distributions between spin active nuclei 3-bonds apart.[21]

$^{1}$H detected 2-dimensional (2D) experiments in which magnetic coherence is propagated through J-couplings or magnetization is transferred through dipole-dipole cross-relaxation interactions, reduce the overlap complexity of 1D spectra and provide correlations to other $^{1}$H nuclei or heteronuclei most commonly $^{13}$C or $^{15}$N. Common homonuclear $^{1}$H-$^{1}$H 2D experiments based on J-couplings are TOtal Correlation SpectroscopY (TOCSY)[22], and COrrelation SpectroscopY (COSY)[23]. Both of these experiments provide information on individual spin systems and chemical bonding. COSY experiments are used to connect $^{1}$H nuclei that are within 3-bonds of each other whereas TOCSY experiments can connect all spins belonging to a J-coupled network *e.g.* the entire spin system connected through 3-bond correlations. Analysis of COSY data can provide J-coupling constants which can be related *via* the Karplus curve[24] to torsional angle restraints.[21]

Homonuclear 2D $^{1}$H-$^{1}$H nOe SpectroscopY (NOESY)[25] and Rotating frame Overhauser Effect SpectroscopY (ROESY)[26] experiments are based on dipolar cross-relaxation interactions providing distance information between nuclei that are physically close (up to ≈5 Å) in space. It is noteworthy to mention that for NOESY and ROESY spectra to have correlations,

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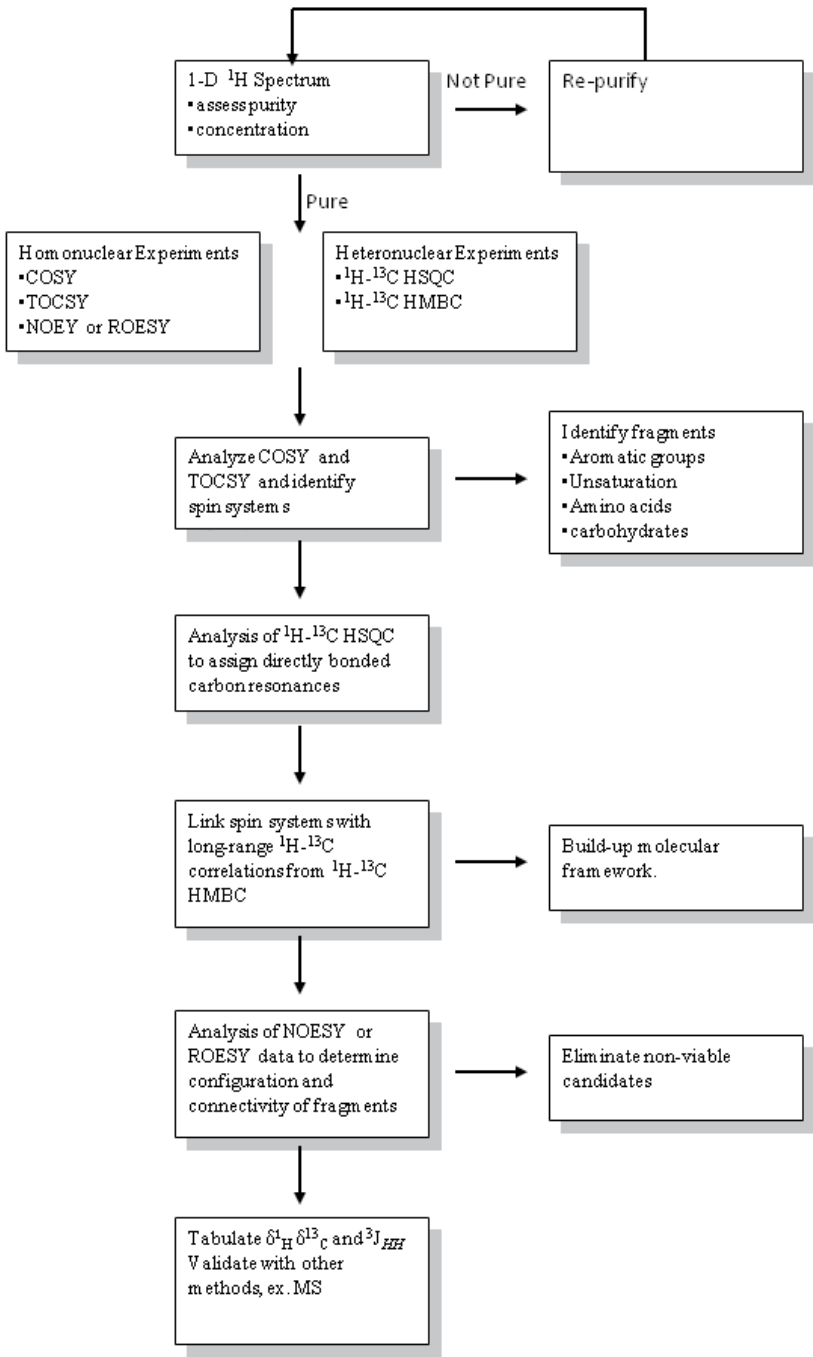
nuclei do not have to be on the same molecule. This aspect of the nOe provides the basis for determining ligand/receptor interaction characteristics (see Section 2.2). The sign and intensity of NOESY cross-peaks are dependent upon the main static magnetic field ($\omega_0 = B_0$) and the rotational correlation time of the molecule ($\tau_c$); for small molecules (<1 kDa) the nOe cross-relaxation rate is positive whereas for larger molecules (>2 kDa) the nOe is negative. ROESY are best suited for medium size molecules of ~1 kDa where for NOESY the nOe becomes zero (*i.e.* $\omega_0\tau_c \approx 1.12$)[26]. Analysis of the NOESY and/or ROESY data is important for determining the configuration/conformation of the compound and for connecting individual spin systems determined from the TOCSY and/or COSY data. Another aspect of nD NMR techniques is the addition of $^{13}C$ editing to the spectra. These heteronuclear experiments are $^{1}H$ detected increasing the sensitivity and indirectly providing $^{13}C$ shifts especially important for mass limited samples. Standard heteronuclear experiments are $^{1}H$-$^{13}C$-HSQC[27-29], $^{1}H$-$^{13}C$-HMBC[30], and $^{1}H$-$^{13}C$-H2BC[31]. Strategies for selecting the proper pulse sequences, acquisition and processing parameters for natural product elucidation has been previously reviewed.[32] Implementing higher-dimensional experiments, for example, HSQC-TOCSY, HSQC-NOESY, provides valuable information for complex natural products on the through bond or space connections by exploiting the heteronuclei chemical shift for further separation.

When assessing the structure of a chemically modified molecule or a molecule for which minor structural changes are suspected, acquiring a complete structural suite of experiments may not be necessary. In such circumstances, a series of edited 1D and 2D experiments have been developed that can isolate the chemical modification of interest and express only correlations to the modification. Isolating a particular peak of interest reduces the time required for data acquisition, simplifies analysis and can help to quickly confirm modifications; valuable tools for isolating information from complex molecules are reviewed in Ref. 33.

A standard approach for small molecule structure elucidation involves identification of the individual fragments or spin systems followed by their assembly.[34] This approach outlined in Scheme 1 uses 1D data to assess purity, classify the compound type and compare with NMR chemical shift databases. Analysis of the 2D homonuclear data (COSY and TOCSY) identifies the individual short spin systems in the compound. Heteronuclear HSQC data provides $^{13}C$ chemical shift information and direct H-C links. HMBC data links distant H-C spin systems that help link molecular fragments. Data from the NOESY and ROESY spectra also aid in linking spins systems, and determining relative configuration and conformation, for example, relative stereochemistry, ring junctions, and double bond regiochemistry. The final step is confirming that proposed shift assignments and structural characteristics agree with coupling constants and splitting patterns among spectra, along with other data collected. There are numerous books detailing the specifics for analyzing NMR data of small molecules, see for example Refs. 35-38.




**Scheme 1.** The flowchart can be utilized as a general scheme for small natural product structural elucidation. Typically, a series of $^{1}$H and $^{13}$C NMR experiments are required in order to fully confirm the structure.

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*2.1.2. Proteins & peptides*

MR allows for structural characterization of moderately sized proteins or peptides.[12,39] Since the line width of the NMR signal depends upon the rotational correlation time $\tau_c$, the resultant signal intensity reduction and decreased spectral resolution typically precludes detailed analysis of large proteins. To date the largest protein to be structurally characterized by NMR is the 82 kDa or 723 amino acid malate synthase G.[40] In contrast, X-ray techniques in principle have no size limit however, not all proteins are amenable to crystallization esp. membrane-associated proteins and crystallization can alter the protein structure[41] making NMR attractive for elucidating structures in "native" solution environments. "Non-native" conditions can also be applicable for protein folding/unfolding or temperature stability. Initial investigation into biomolecular structure elucidation typically requires information from other techniques such as MS, circular dichroism, micro-array for initial determination of the amino acid sequence. A general scheme for the elucidation of a protein 3D structure (Scheme 2) involves initial production of the protein either by synthesis and re-folding, or by recombinant expression, and followed by acquisition and data analysis. From the analysis through bond backbone and side-chain connections, through space nOe connections and additional constraints are utilized as restraints within simulated annealing protocols that calculate superimposable ensembles of lowest-energy structures.[42]

NMR is a sample intensive technique requiring milligrams of the biomolecule. For small peptides, synthesis (mg quantities) is typical and offers the possibility of selective isotopic labelling with the preferred spin active nuclei $^{13}C$ (over the 98% natural abundant spin-inactive $^{12}C$) and/or $^{15}N$ (over the 99% natural abundance quadrupole and difficult to observe $^{14}N$). However for large proteins, synthesis is too onerous and costly. Therefore development of a recombinant expression protocol capable of producing bacterial, mammalian or other proteins with the correct folding and linkages (in the case of lipoproteins or polysaccharide proteins) is required. In addition, expression systems allow for point mutations. For proteins >50 amino acids it is advantageous to label the protein with the isotopes $^{13}C$ and $^{15}N$. Alternatively, structure elucidation of small monomeric peptides of <50 amino acids does not necessarily require labelling and the experiments and strategies outlined in Section 2.1.1 can be utilized. Isotopically labelled proteins can be achieved by growing *E. coli* with the particular expression gene on minimal media supplemented with $^{13}C_6$-glucose and/or $^{15}N$ ammonium chloride. Although *E. coli* is widely used for its low cost, the high productivity yields depend upon the plasmid, host and tags.[43] In addition with prokaryotic systems, protein re-folding can be a potential bottle neck along with failure to express toxic proteins, degradation and the absence of post-modification. Other non-*E. coli* prokaryotic and eukaryotic cell-lines that have been used for labelling, such as insect cells[44], are reviewed in Ref. 45. Eukaroytic *Pichia pastoris* has been widely and successfully used for labelling using minimal media,[46] and cell-free expression allows for high yields with relatively small reaction volumes[47]. With cell-free, any combination of labelled and unlabelled amino acids can be incorporated into the protein without isotopic scrambling.[47] An advantage of the cell-free system is that reagents, that stabilize expression, can be added. For example, protease inhibitors, detergents or membrane mimetics for insoluble or membrane associated proteins.[48]

However, each amino acid is added to the medium which can become costly for uniform $^{15}N$ and/or $^{13}C$ labelling. Overall, the particular expression system used depends upon such factors as post-translational modifications, the labelling scheme, membrane association, total cost and expression efficiency.

Over the past decade specialized isotope labelling strategies have been developed for biomolecular NMR, including full or partial deuteration, specific amino acid labelling and regiospecific labelling.[49] Labelling proteins with deuterium is practical for simplifying complex NMR spectra or for studying protein-substrate complexes. Furthermore, as the molecular weight of the biomolecule increases the spin-spin relaxation time ($T_2$) decreases considerably, inhibiting coherence transfer along amino acid side-chains. Substitution of $^{2}H$ for $^{1}H$ nuclei increases $T_2$ enhancing the coherence transfer. Perdeuteration of the backbone prevents connection to the side-chain $^{1}H$ nuclei, whereas a random, uniform deuteration level between 50% and 90% is most desirable. [50,51] Nevertheless, perdeuteration is beneficial for studying protein-substrate or protein-protein complexes, in which one portion of the complex is "invisible" reducing the overlap and spectral complexity allowing information of conformational changes to be more readily identified. In the pursuit of NMR information of larger proteins, labelling strategies have been developed for selective methyl labelling of alanine, leucine, valine and isoleucine ($H_{\gamma}1$) residues with perdeuteration of the backbone. This labelling scheme allowed for the analysis of relaxation dynamics of a 1 MDa protein complex.[52,53] Site specific information on protein conformational changes upon substrate binding can be obtained from selective amino acid labelling of the backbone. Within the protocol, media is supplemented with isotopically labelled amino acids, isotopic scrambling may result in instances where the supplemented amino acids are precursors to other amino acids. Scrambling of the isotope labels can be overcome by using *E. coli* strains with lesions in their biosynthetic pathways,[54] and recently demonstrated with a prototphic strain.[55] Contrary to selective amino acid labelling it has been proposed that unlabelling specific amino acids against a uniformly $^{13}C/^{15}N$ labelled background is beneficial.[56] Selective unlabelling of the protein still allows for sequential assignment of regions of the protein. Segmental labelling of individual domains or portions of large proteins has been established in which labelled segments of the protein are ligated together.[57,58] Segmental labelling of a large protein is useful for studying domain-domain interactions[59], conformational changes and substrate binding studies.

Preparation of protein samples is straightforward for soluble monomeric proteins at concentrations >1 mM. A wide range of deuterated buffers are available for controlling the pH of the sample. Deuterated or an inorganic buffer are desirable to minimize interference within $^{1}H$ spectra. The pH requires consideration as at pH > 8.0 labile amide $^{1}H$ nuclei can rapidly exchange with water becoming invisible. This can be utilized to reduce spectral complexity; however, it may also affect residues of interest. Approximately one-third of human genes code for membrane-associated proteins and to utilize NMR for studying the structure/function relationship of these proteins requires the protein to be folded in a membrane environment.[60] A range of membrane mimetics are available for solution and solid state NMR.[61]

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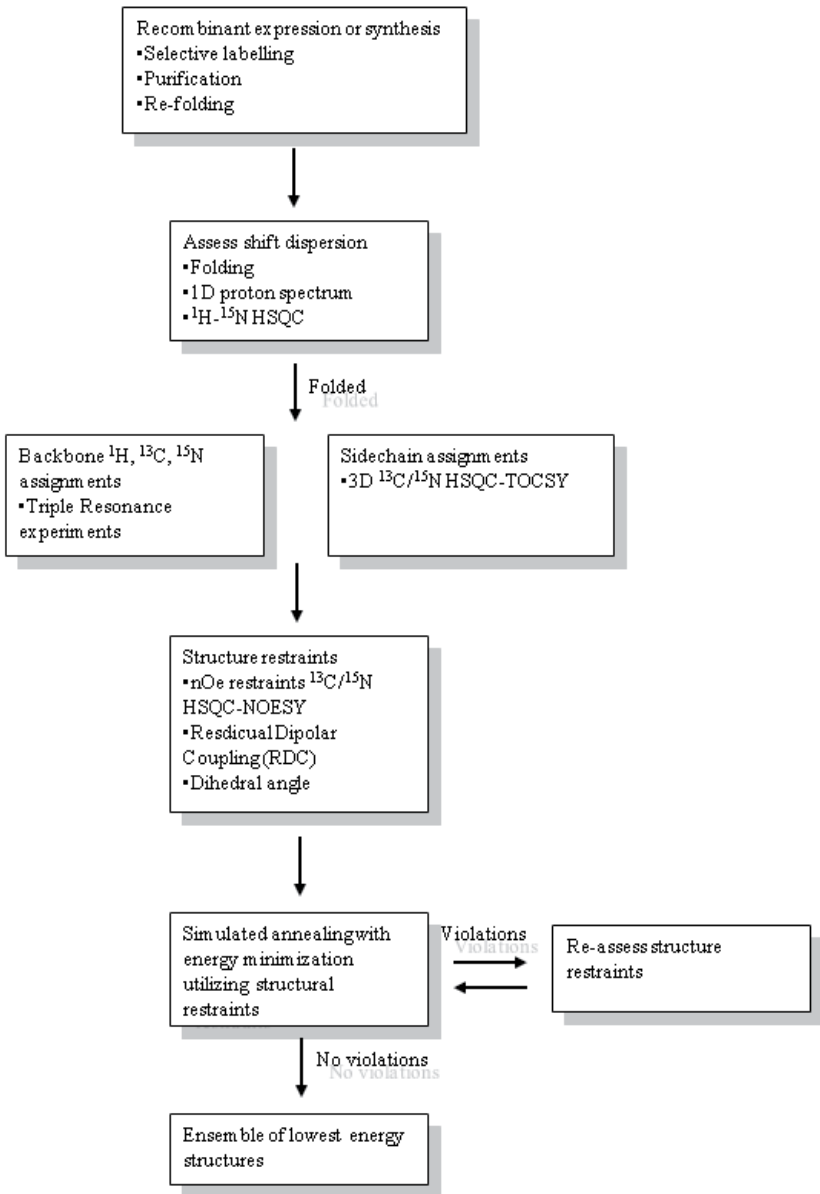


**Scheme 2.** The flowchart can be utilized as a general scheme for protein structural elucidation. Successful structural elucidation for proteins relies on elaborate but well established $^{1}H$, $^{15}N$ and $^{13}C$ NMR experiments.

With the advent of 2D NMR experiments a strategy for the 3D structure determination of small proteins utilizing homonuclear NMR spectra was established.[39] Extension of the 2D NMR experiments to nD experiments along with isotopic labelling allowed for the 3D structure determination of much larger proteins including membrane proteins.[12,62] 3D and 4D

NMR techniques allow NMR data to be filtered by the $^{13}C$ or $^{15}N$ nuclei thus, reducing spectral overlap especially important for large proteins. In the past decade data on larger proteins has been facilitated with the development of Transverse Relaxation Optimized SpectroscopY (TROSY) experiments.[63,64] NMR structural and dynamic analysis of a supra-molecular systems of 1 MDa has been achieved in combination with selective methyl labelling.[65,66]

Typical NMR structure determination involves manual assignment of the backbone and side-chain chemical shifts. Extensive assignment of the $^{1}H$-$^{1}H$ nOe from NOESY experiments yields distance restraints as the volumes of the NOESY peaks are proportional to the average of $1/r^6$ distance between the $^{1}H$ nuclei. More recently routines for automatic resonance and nOe assignments have been developed.[67,68] However, these methods require labelled proteins and high-quality data to be effective. Regardless, protein structures are calculated using a molecular dynamics computer simulation program with predefined *a priori* bond connectivities, lengths and angles, and NMR derived restraints.

NMR restraints are most commonly from nOe experiments but may also include dihedral angles, hydrogen bonding information and residual dipolar couplings.[69] Dihedral angles can be calculated from measured/fitted[70] J-couplings or predicted from backbone chemical shifts of the $^{1}H_{\alpha}$, $^{13}C_{\alpha}$, $^{13}C_{\beta}$, $^{13}CO$ and $^{15}N$ resonances.[42,71] $^{1}H$ nuclei exchange rates are reduced in protein domains which are structured, or can be used to identify binding domains. Residual dipolar couplings are valuable for identifying angular constraints for large domains and structural changes in substrate binding.[72,73]

An ensemble of lowest energy structures that satisfy the NMR derived restraints is calculated. Quality of the calculated structures is based on the consistency of the experimental data compared to the inputted restraints. Agreement of the structures among each other is evaluated with the RMSD to the lowest energy structure. In addition, NMR quality assessment scores, recall, precision and F-measures (RPF scores) have been developed to directly measure the quality of structures compared to the NOESY peak list.[74] Structural calculations are an iterative process as not all restraints will be satisfied during the first simulated annealing calculation. It is typical for misassignments to occur due to spectral overlap or poor volume calculations. In principle the number of correctly assigned and integrated restraints should out-weigh the incorrectly assigned restraints. Thus during the calculations, a set of restraints could be identified as being violated regularly. From the identification, the NMR data is re-examined and the restraints corrected. Calculations are re-run and violations checked iteratively until well defined structures with minimal restraint violations obtained.

Tertiary/quaternary structural aspects can be confirmed with NMR through diffusion experiments. NMR is one of the most accurate and precise methods for determining diffusion constants.[13,75] Diffusion constants are related to the hydrodynamic radius through the Stoke-Einstein equation and thus can be indirectly used to determine the mass of the diffusing species.[13,76] Of particular importance for proteins is determining the aggregation number.[77]

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### 2.2. Pharmacophore identification & binding characterization

Natural products have a diverse range of mechanisms for eliciting a biological response. Some natural products act as free-radical scavengers never directly interacting with the organism, whereas other compounds bind to molecular targets triggering a signaling cascade and altering the physiological state. Determining the mode of action of a small natural product molecule and where necessary the biological target requires extensive micro-biological investigations. NMR can play a role within these investigations in particular by identifying the pharmacophore of the natural product. The pharmacophore is the constituent of the molecule that binds to a biological receptor to modify its biological response.[78] Identifying the pharmacophore is an important aspect for drug discovery and understanding the mechanism of action as it assists with "intelligent" design of drugs through modifications that change binding characteristics (*e.g*. modifying the pharmacophore region) or solubility/ permeability properties (*e.g*. modifying sites distant from the pharmacophore).[79,80]

The difference in the NMR nOe response between a small molecule rapidly tumbling in solution and a small molecule that is bound to a slowly tumbling large protein (see Section 2.1.1) is exploited to isolate and identify the pharmacophore (see chapter 14 of Ref. 81 and Refs. 82,83). In order to clearly define the pharmacophore complete structural analysis of the molecule is required (see Section 2.1.1); in order to sequence identify the active site within the receptor, complete structural analysis of the protein (preferably including $^{15}N$ and $^{13}C$ chemical shifts and connectivities) is required (see Section 2.1.2). A number of these techniques require mg quantities of purified ligand, receptor or both. Purifying compounds can be a detrimental drawback especially if the receptor is a membrane bound protein that is difficult to express and purify. Nevertheless, if the receptor is highly over-expressed within a cell (*e.g*. cancer cell that over-expresses a particular protein) the possibility exists for the experiment to be performed *in vivo*.[84] Essentially 6 fundamental methods are available for pharmacophore/binding characterization:[81,82] chemical-shift perturbations[85], saturation transfer difference (STD)[86], water-logsy (wLogsy)[87,88], transfer-NOESY (tr-NOESY)[89], selective relaxation[90] and diffusion editing[90]. Selective relaxation, diffusion editing and tr-NOESY experiments in principle can be used for nM to mM binding constants ($K_D$) whereas chemical shift perturbations, STD and wLogsy are valuable for pM to mM $K_D$ ranges with the concentration of receptor in the nM range. It is well beyond the scope of this chapter to describe these experiments in detail especially since these experiments can be combined to provide further characterization such as combining diffusion editing with STD to simultaneously determine the pharmacophore and binding constant.[91] There are numerous reviews that provided explicit details (see Refs. 82,92-94).

With these tools both the ligand and receptor can be characterized. Typically chemical shift perturbation or mapping methods helps to characterize the active site of the receptor. A series of $^{1}H$-$^{15}N$ or $^{1}H$-$^{13}C$ HSQC spectra of the labelled receptor are collected as the ligand is titrated. Changes in chemical shifts of the receptor are indicative of $^{1}H$ nuclei that are perturbed during binding, although care must be taken as to not over-interpret data as this could also be indicative of structural alterations distant from the binding site. In cases where the receptor is large (*i.e*. > 30 kDa) extensive resonance overlap may preclude unambiguous in-

terpretation of the HSQC data. Expression techniques to isolate particular amino acids or regions of the receptor are valuable for these experiments (see Section 2.1.2). One disadvantage of this technique is the necessity of a complete resonance assignment of the target or at least the active site.

Diffusion editing is a technique that can be used to determine the $K_D$ by examining the change in diffusion properties of the ligand (typically < 2 kDa) upon titration to the receptor (typically > 100 kDa). Although limited with pharmacophore and binding pocket identification, it is nevertheless a valuable tool to identify binding events from a mixture of possible small ligands or to combine with other techniques.

The most often utilized techniques are the STD, wLogsy, tr-NOESY and selective relaxation. [95] These techniques are used when the target is too large for chemical shift perturbations, is not available with the desired isotopic labelling scheme or the target aggregates/precipitates at high concentrations. For these techniques, non-specific binding events in the nM-μM range may be difficult to rule out unless compared with a known binder or used with a competitive binder.[96] Regardless, these techniques are invaluable for specifically observing resonances of a low-affinity ligands that bind to a receptor. With selective relaxation experiments, differences in relaxation properties of the ligand between a free and bound state help identify interacting $^{1}H$ nuclei that are in close proximity to the receptor. The relaxation properties of the free ligand (*i.e.* no receptor) are compared to the relaxation properties for the ligand at various receptor titers and mixing times. Changes within the relaxation values can distinguish $^{1}H$ nuclei that are in direct contact with the receptor from $^{1}H$ nuclei that show magnetization relay or $^{1}H$ nuclei distant from the receptor. For wLogsy experiments, $^{1}H$ resonances arising from $^{1}H$ nuclei in close proximity to the receptor (*i.e.* are part of the pharmacophore) are opposite sign to $^{1}H$ resonances arising from $^{1}H$ nuclei that are distant from the receptor or are on a ligand that does not bind to the receptor (Fig. 1A). The wLogsy also has the advantage of identifying $^{1}H$ nuclei that are part of salt bridges between the ligand and receptor. For STD experiments, small molecules that do not bind to the receptor show a zero response (Fig. 1B) whereas the $^{1}H$ nuclei of the pharmacophore show a response. Because the wLogsy is a direct observation technique whereas the STD is generated from a difference of spectra, the wLogsy tends to have fewer artifacts and can be more sensitive. The tr-NOESY experiments can provide structural information about the ligand in the bound state as well as potentially information on the type of amino acids on the receptor involved in binding; typically no information regarding the sequence specificity of the amino acids is gleaned. The tr-NOESY is advantageous if the ligand changes conformation upon binding as it can be utilized to determine the bound state structure of the ligand which is a valuable asset for understanding the mode of action. The tr-NOESY is a 2D technique and as such requires mg quantities of ligand and substantially more time to acquire the data. For rapid screening of natural products the STD and wLogsy are the experiments of choice.

Beyond the individual experiments, combinations of these various experiments are possible. For example, to investigate the folding/unfolding properties of a peptide the combination of a $^{1}H$-$^{15}N$ HSQC and wLogsy can be used to monitor amide $^{1}H$ nuclei exchange rates valuable for identifying H-bonding and buried residues.[77,97] This experimental combination

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can also be utilized with/without ligand to identify amide $^{1}H$ nuclei that are involved with ligand binding. Using the wLogsy to saturate the water signal avoids the challenges of the typical methods of monitoring exchange by addition of $D_2O$ such as protein precipitation, conformational changes induced by concentrating/diluting or complete loss of signal due to rapid deuterium exchange.[77,98]

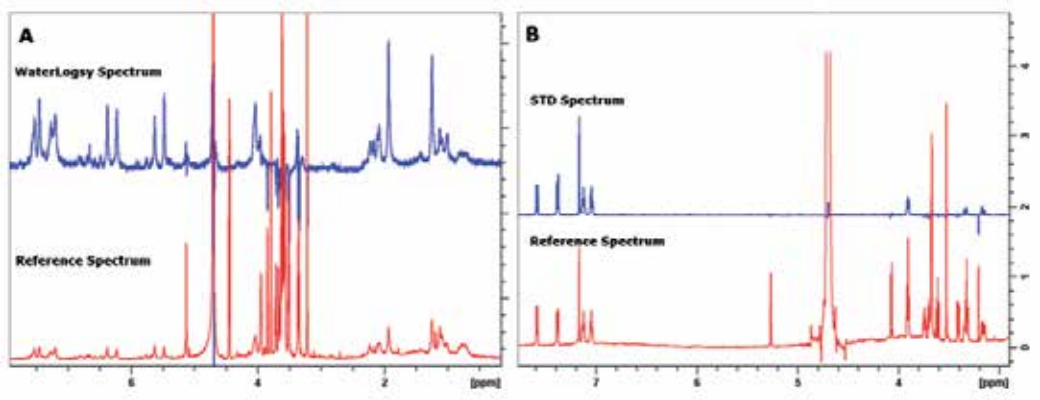


**Figure 1.** STD and wLogsy experiments can be utilized for identifying the pharmacophore of a small natural product molecule. Panel **A** represents a wLogsy experiment (blue spectrum) of a compound that binds (positive peaks) to an enzyme and a compound that does not bind (negative peaks). The positive peaks are of $^{1}H$ nuclei that are adjacent or in proximity to a salt bridge involving water. The negative peaks are from either the binding compound that are not within the binding pocket, or from the control non-binder compound. The red spectrum is the reference spectrum. Panel **B** represents the STD experiment (blue) of human serum albumin, a binder tryptophan and a non-binder sucrose. The positive peaks are from $^{1}H$ nuclei on the tryptophan that are within the binding pocket. Peaks that are not observed compared to the reference spectrum (red) represent either the control compound, or $^{1}H$ nuclei on the tryptophan that are distant to the binding pocket.

### 2.3. Quantitative analysis & QA/QC

Nuclear MR technologies have traditionally been associated with molecular characterization. The quantitative nature has been acknowledged from integration of signals to distinguish between for example methyl and methylene $^{1}H$ nuclei; however, it has rarely been exploited for absolute quantitative analysis (qNMR) or quality assurance/quality control (QA/QC).[10] MR techniques are capable of accurately and precisely determining the concentration of molecules within a purified sample or complex mixture without the need for elaborate calibrations.[99] In addition samples can be in solution or semi-solid states.[100] Relatively simple protocols have been developed that use a single certified external standard to calibrate the instrument. From the calibrated system, other samples can be rapidly quantitated.[10,99]

MR technologies have the unique distinction of having a uniform molar response for all nuclei of the same type, *i.e.*, the NMR signals are proportional to the molar concentration of the nuclei allowing for a direct comparison of the concentration of all compounds within a mixture. Thus for example, for all organic molecules regardless of the concentration, the intensi-

ty of each signal within the NMR spectrum is a direct measure of the number of $^{1}H$ nuclei that contribute to that signal. Furthermore, spectra can be recorded in such a manner as to allow for accurate comparison between different samples within different sample tubes and different solvents; the implications are far reaching for qNMR and QA/QC. For example, concentrations of natural products or impurities can be determined for samples within sealed tubes reducing the handling requirements of toxic or precious samples, or rapid crude or refined product profiling ensuring purity, integrity and consistency with applications to fractionation or end-product QA/QC.[99] Fully automated protocols have been developed that have been coupled with metabolomics investigations providing absolute scaling for temporal data.

### 2.4. Metabolomics analysis

Analysis of metabolites and metabolic flux can help ascertain the effects of a particular natural product or extract on an organism. Metabolic analysis can be utilized while identifying biological activity *in vitro*, or during *in vivo* investigations. Perhaps one of the best practical definitions of metabolomics was offered by Oliver describing it as an approach for simultaneously measuring the complete set of metabolites (low molecular weight intermediates) that are context dependent and which vary according to the physiological, developmental or pathological state of an organism.[101] From the perspective of natural products, such a definition fits in the framework of extracts from plants, fungi and secretions from microorganisms such as bacteria. When metabolomics is viewed from context dependence, only those metabolites that vary according to environmental, biochemical, and/or physiological fluctuations are important. In this regard, there are a vast number of metabolites present in botanical extracts and secretions from organisms that are useful for inducing biological responses in organisms including humans. For instance, ginseng has been argued to induce biochemical changes in humans that lead to anti-tumor, antioxidant, anti-fatigue and anti-stress activities while *Streptomyces coelicolor* secrete therapeutic natural products during their quiescent growth phase.[102,103] The discussion within this section follows the aforementioned definition and is restricted to metabolomics applied to botanical natural products since it is one of the fastest growing subsections established as "plant metabolomics."[104-106] NMR is a suitable method for such analyses since it allows simultaneous detection of a diverse group of both primary metabolites (sugars, organic acids, amino acids *etc*.) and secondary ones (flavonoids, alkaloids tri-terpenes *etc*.) Nevertheless, the applications are extensible to the "animal kingdom" metabolomics since the underlying sample composition is similar and characterized by large heterogeneity exhibiting vast dynamic range in concentration.

The numerous advantages of NMR have been a major driver for developing NMR based metabolomics technologies. NMR is ideal for resolving the complexity of metabolomics samples given that methods exist for compounds with nuclei such as $^{1}H$, $^{13}C$, $^{15}N$ and $^{31}P$ to provide spectral fingerprints with compound specificity and quantitative accuracy even within complex matrices. For example, from the un-annotated 1D $^{1}H$ NMR spectra of seaweed extracts, visible differences in chemical composition are observed (Fig. 2). NMR signals have a uniform molar response (see Section 2.3). This property has particularly been important

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with propelling NMR metabolomics as a technology. NMR is a non-destructive technique ideal suited because many of these samples are difficult to obtain and may be precious. The stability of many NMR instrumentation allows for repeated measures (often years apart) of a sample with accurate reproducibly. This advantage also lends the technology to inter-laboratory studies that are important for establishing the robustness of a given measurement and technique.[52] Advances in technology now allow for high throughput analysis with automated, temperature controlled sample changers such as Bruker's SampleJet®.

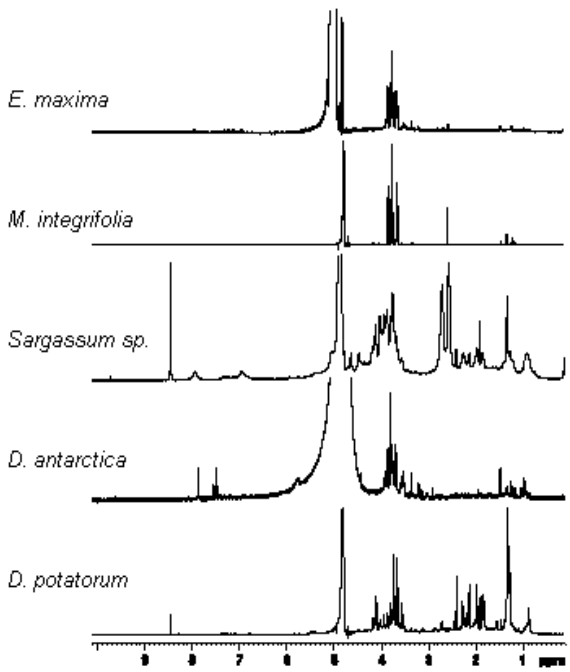


**Figure 2.** NMR spectra of aqueous extracts from various strains of brown seaweeds showing distinct differences within the spectral features.

The most commonly cited disadvantage of NMR for metabolomics is the lack of sensitivity. [106] This is a major hindrance given that in typical sample matrices, the concentration of constituent metabolites often exhibits a large dynamic range in concentration. Low abundance metabolites will invariantly be overlapped by highly abundant ones. Furthermore, in some applications such low level metabolites maybe of high value. For instance a phytochemical preparation may exhibit activity in a biological readout but such activity is induced by the low level metabolites which are undetectable *via* NMR. Approaches for simplifying sample matrices have been developed in order to separate, for instance, lipophilic from hydrophilic metabolites.[107] In addition, recent advances in NMR hardware have significantly improved sensitivity, especially with the advent of cryogenic probes and microprobes.

NMR-based metabolomics applications have been in the literature for over two decades, [108,109] but the technology only realized widespread acceptance and application in the later part of the last decade.[105,110-112] This shift is attributed to the utility of pattern recognition methods for analysis of multiple spectra, allowing the visualization of patterns corresponding to differences among samples and identification of chemical shifts responsible for eliciting such differences.[113] Specifically, principal component analysis (PCA) has been a significant driver as it allows patterns associated with the variability in the relative concentrations of metabolites to be assessed by the human eye, often in two dimensions. PCA analysis assisted with the classification of different extracts of the sea weed *Ascophyllum nodosum* (Fig. 3).[114] In recent years, many other pattern recognition methods (classified either as supervised or unsupervised) have been developed and applied to data. Unsupervised methods do not include *a priori* knowledge of the class memberships of a given sample. Such methods include PCA, SIMCA, independent component analysis (ICA) and the so called machine learning methods such as neural networks and self organizing maps (SOM). On the other hand, supervised methods use information about the samples in order to build models that can later be used to predict the class to which an unknown sample belongs. Such methods include partial least squares discriminant analysis (PLS-DA).

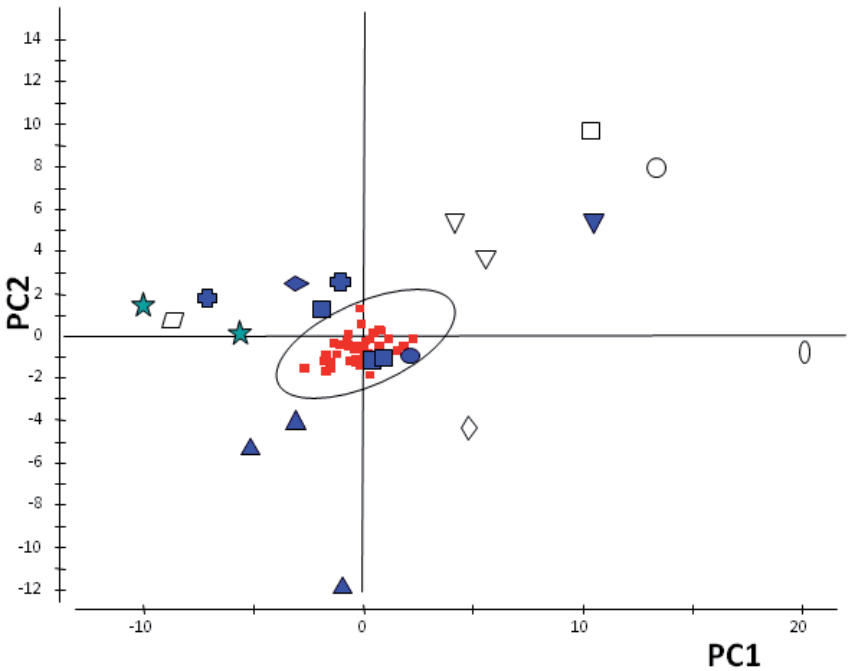


**Figure 3.** A principal component scores plot of a product characterization study showing similar samples from one manufacturer tightly clustered in the center (solid red squares); the ellipse encloses these samples plus two others that are known to incorporate that manufacturer's samples (solid blue squares and circle). Competitors' samples are distributed throughout the plot with each symbol representing products from a single manufacturer. Unfilled symbols represent samples recorded under acidic conditions.

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Ultimately, the impetus for natural product metabolomics analyses is the need for high throughput screening to determine biological activity and the requirements from the nutraceutical industry to purport therapeutic health benefits from unprocessed foods. Traditional approaches with the development of novel drugs are difficult, expensive and time consuming. It is estimated that over $800 million and an average of 14.2 years are spent before a novel drug application is approved. Because natural products are a rich source of lead compounds in the drug discovery pipeline, the metabolomics approach provides an avenue for a systematic characterization of complex mixtures such as phytochemical extracts linking observations made via biological assays without the need for isolation. This promises to complement high throughput screening of compounds in order to shorten the drug discovery process. Indirectly, NMR has been used in metabolomics to measure the fate of consumed natural products and their effects on human physiology. One study has shown that the consumption of dark chocolate, for instance, affects energy homeostasis in humans.[115] In another study, differences in metabolic profiles were observed in human urine following consumption of black compared to green tea specifically increases in urinary hippuric acid and 1,3-dihydroxyphenyl-2-O-sulfate, which are end products of tea flavonoid degradation.[116] Several other studies exist in the literature and have been reviewed for instance within Ref. 117.

Perhaps one of the biggest gaps with metabolomics developments for natural products is the lack of certified reference materials for quantification of analytes. Such reference materials would enhance product characterization and validation of biological observations, especially with studies of bioactivity assessment. Those studies are often inconsistent due to inadequate chemical characterization of complex botanical mixtures, making comparison of results across studies difficult. Fortunately, it is possible to determine the concentration of 'active' compounds using external standards, *via* NMR, as long as both the external standard and the compound of interest are of the same nuclei type (see Section 2.3).[10]

### 2.5. Semi-solid state & macromolecular assemblies

In addition to studying soluble molecules or extracts dissolved in solution state, it can be advantageous and/or necessary to study semi-solids such as intact tissues, cells, raw materials or product formulations; metabolites or components are in their native environment *i.e.* potentially time consuming and disrupting/degrading extractions, or chemical modifications are not required in order to obtain valuable information. Semi-solid materials require specialized NMR probes to overcome sever spectral line-broadening as result of the restricted molecular mobility. To acquire a high-resolution spectrum of a semi-solid, High Resolution Magic Angle Spinning (HR-MAS) was developed in the late 1990's as a hybrid between solid and solution state NMR.[118] Similar to solid state NMR the spinning of the sample at the "magic angle" (54.7°) to the applied magnetic field reduces line broadening effects. Spinning speeds are typically between 3 to 5 kHz and cells remain intact and viable.[119] HR-MAS requires <100 uL of the semi-solid. HR-MAS does not require the high powered pulses that solid state NMR requires and many of the nD experiments that are utilized for structural biology can be applied, although stable iso-

tope labelling is preferred for the less abundant $^{13}C$, and $^{15}N$ nuclei. HR-MAS can be applied for many natural product studies[118,120] commonly used to study metabolic changes in diseased and treated tissues,[121,122] metabolism,[123] combinatorial chemistry[124] and whole cells[125]. Intensities in the HR-MAS NMR spectra are dependent on the environment of the analyte and as such molecules that are in rigid environments with completely restricted mobility are not detected. Macro molecular assemblies on-the-other-hand can be examined for profiling and quantitation of algae lipid content and polysaccharides or metabolites.[100]

## 3. MRI

MRI is a nuclear MR technique applicable for natural product development in particular during *in vivo* testing and diagnostic stages. MRI is capable of producing 3D images that can be used to monitor changes in brain activity in response to application of a natural product *via* fMRI techniques[126-128], indirectly monitor the effects of a natural product on tumours[129], or directly monitor the bio-distribution and bio-accumulation of a natural product by tagging the compound with an MRI contrast agent.[130-133].

Direct monitoring of the natural product is one of the most typical methods for drug development. For direct monitoring however, the molecules should be tagged with a MRI contrast agent which contains a paramagnetic centre causing $^{1}H$ nuclei on water in close proximity to "relax" much faster than $^{1}H$ nuclei distant from the paramagnetic centre.[134] This rapid relaxation is exploited with MRI and is depicted as "dark spots" within the image whereas unaffected water molecules remain as bright areas. In order to "tag" a natural product with a MRI contrast agent such as super-paramagnetic iron oxide (SPIO), the complete structure and pharmacophore identification is valuable (see Section 2.2) as it allows one to pick a functional group distant from the active site that can be chemically coupled to the contrast agent. Many SPIO contrast agents are commercially available with a variety of functional groups amenable to chemical coupling under aqueous conditions.[134]

Once the natural product is conjugated to the paramagnetic particle, it can be administered to an organism and imaged. The bio-distribution and bio-accumulation of the tagged molecule is monitored and compared against a control particle that does not contain the active natural product.[133,135-137] The difference in clearance time is considered as conformation that the molecule is associated with the tissue being examined. As an example the peptide SOR-C27, a 27 amino acid fragment of the paralytic natural product peptide SOR-54 from the Northern Short Tail shrew (*Blarina brevicauda*) was found to bind the calcium ion channel TRPV6 which is highly over-expressed by breast, prostate and ovarian cancers.[138,139] The SOR-C27 peptide was chemically bonded to a maleimide functionalized SPIO particle through the sulfur centre of the Cys-14 residue. From MRI investigations on an ovarian cancer xenograft mouse model, the SPIO-peptide parti-

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cle persisted at the tumour site 24 hours post injection whereas the control SPIO particle rapidly cleared. The persistence of the SPIO-peptide particle at the tumour site is conformation that the peptide is associated with the tumour[139].

## 4. Summary

A comprehensive overview of MR techniques for natural product development is well beyond the scope of a single chapter or book. The fundamental experiments have been briefly outlined and the typical information that is gleaned from the experiments presented. Many other opportunities were not covered such as determining equilibrium dissociating constants, or use of spin labels for lead drug optimization. MRI has tremendous application for later stage *in vivo* applications of the drug development pipeline. Together the MR technologies of NMR and MRI can cover the full range of natural product drug development from discovery through to clinical testing (Fig. 4).

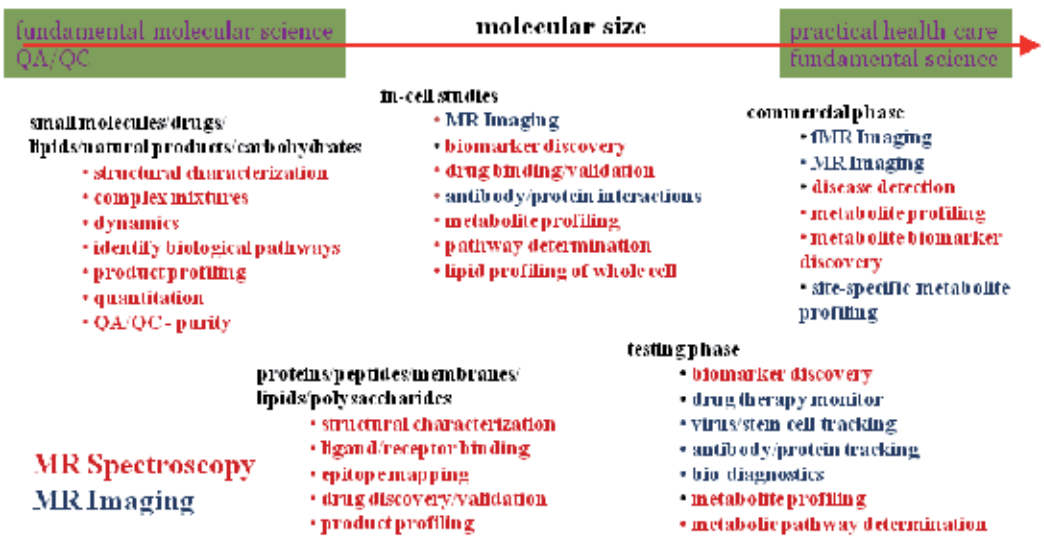


**Figure 4.** Applications for nuclear MR technologies cover the entire range of natural product development from fundamental molecular sciences through to practical health care. Spectroscopic applications (red) are delineated from imaging applications (blue).

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Section 2

# Bioactive Proteins from Natural Sources

Chapter 4

# Marine Natural Products for Protein Misfolding Modulation

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Additional information is available at the end of the chapter

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## 1. Introduction

Proteins are linear covalent chains of amino acids. In most proteins, the chain winds in a specific way to adopt a set fold, or "conformation", that is stable and that allows it to function. A number of proteins remain unfolded in their native state and these are referred to as intrinsically disordered proteins. These unfolded proteins remain soluble and functional and some exhibit specific ligand binding capability, which can cause them to adopt an induced fold. In contrast to these two functional situations of natively folded and disordered proteins, proteins that are normally folded or disordered can misfold, that is, to fold incorrectly.

Proteins may misfold as a result of enzymatic cleavage, post-translational modification, mutation, overabundance or structural destabilization due to alteration in the tissue environment. Accumulation of misfolded proteins can occur more readily when proteostasis mechanisms that foster correct folding of proteins, such as the chaperone system, and that clear the cell of misfolded proteins, such as the ubiquitin-proteasome system, are compromised [1]. A subset of human proteins appears to have an unusual tendency to misfold and this is recognized as a central event in a number of human diseases (reviewed by [2]). Improper folding often leads to problems because the misfolded protein cannot perform its normal role (loss of essential function) and because it assembles into oligomeric forms or larger aggregates that are toxic to the cell (gain of harmful function) [3]. The most recognized misfolded conformation is a stacking of β-sheets forming a crossed-β secondary structure that assembles into a linear multimeric fiber called amyloid (reviewed in [2]).

Several neurodegenerative diseases share protein misfolding as an underlying cause [4, 5]. These diseases can be classified based upon the proteins affected [3] while sharing a common mechanism of emergence. Protein misfolding in neurodegenerative diseases has been the focus of many reviews; excellent examples are in [5-9]. Therefore, a comprehensive re-

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view will not be undertaken here. The canonical neurodegenerative diseases are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, amyotrophic lateral sclerosis and the prion diseases. Although each involves distinct proteins, misfolding has a common role. Huntington's disease is caused by an excessive number of sequential glutamines near the N terminus of the protein huntingtin. An expansion of the natural glutamine stretch exceeding approximately 33 residues results in Huntington's symptoms and the age of onset of the disease declines with increasing residue number [10-12]. A likely reason for this is the increased propensity for aggregation with increasing polyglutamine length [13]. Amyotrophic lateral sclerosis is a disease associated with the death of the upper and lower motor neurons in the spinal cord, brain stem and motor cortex. The affected neurons accumulate aggregate protein inclusions that may be causing the cells to die. Mutations in genes encoding superoxide dismutase-1, TAR DNA binding protein 43 and fused in sarcoma/translation in liposarcoma (FUS/TLS) are implicated in ALS (reviewed in [14]). Prion diseases display several interesting commonalities with these other neurodegenerative diseases. Prion diseases emerge from misfolded prion protein, which serves as a template for subsequent misfolding of native prion protein with ensuing aggregation and neuron loss. The propagation of pathogenic misfolding from one protein molecule to its healthy neighbours by templating was long considered to be unique to prions; however, recent studies have suggested that molecular templating of misfolded proteins occurs with other neurodegenerative diseases as well. Furthermore, templating is inferred by observation of their spatial spread from select foci in the brain [5, 15, 16]. It is notable that prion diseases, which are infectious, may occur in animals or humans of all ages, whereas the apparently non-transmissible protein folding diseases occur mainly with ageing. This implies that a key step in all protein folding disease may be the initial establishment of a misfolded protein in a neuron, whether it is a misfolded protein taken in at any age by infection or misfolding that emerges in a susceptible endogenous protein due to faltering proteostasis with ageing. Therefore, the prevention of misfolding and/or the promotion of disaggregation and refolding of misfolded proteins *in vivo* appear to be the most direct means of dealing with all these diseases.

In this chapter, the focus is on AD and PD, as these are the two most prevalent neurodegenerative diseases, and the proteins that misfold in these diseases are well studied. AD accounts for approximately two thirds of all cases of dementia [17]; it leads to cognitive deficits in reasoning, memory, abstraction and motor skills, with eventual death. It is characterized at the molecular level by extracellular aggregates of a short peptide called amyloid beta (Aβ, and also called beta amyloid), which can form oligomers and larger fibrils with amyloid structure and later emerging intracellular neurofibrillary tangles that are composed of hyperphosphorylated tau protein (reviewed in [18]). In this chapter, Aβ normally refers to $A\beta_{42}$, which is the 42-residue peptide and also appears to be most toxic form. There is increasing evidence that the Aβ oligomers have a key and likely causative role in AD. Most compelling are studies showing mutations in the regulatory region or encoded protein sequence in amyloid precursor gene in families predisposed to AD [19-21]. In contrast to AD, PD has been found to involve the misfolding of α–synuclein (αS) into aggregates found in Lewy bodies and Lewy neurites. PD affects the *substantia nigra*, a region of the brain in-

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volved with reward, addiction and movement. Many of the onset symptoms of PD reflect this, and the diagnosis is based on resting tremor, slowness of movement, rigidity and postural instability [22]. These two proteins are unrelated in sequence and they have distinct structures in their native form, with Aβ a short mainly unstructured peptide and αS a larger 140-residue protein that is post-translationally modified and that can exist in alternative conformations including a largely α-helical form and a disordered state [23, 24]. In spite of these differences, both proteins misfold and aggregate, and the aggregates of each can be detected and measured using assays that will be discussed in the section below.

There is growing interest in the development of protein folding modulators that would offer to prevent or alleviate misfolding and to avoid the damage that occurs in neurodegenerative diseases. There has been progress along several avenues, but no magic bullet to date. Nonetheless, there remains substantial untapped diversity among natural products and notably in marine resources. With a focus on Aβ and αS, which are implicated in AD and PD, respectively, this chapter will examine natural agents that may prevent or ameliorate protein misfolding diseases, with particular attention to the potential of marine resources for possible discovery and development.

## 2. Screening and evaluation for action on protein folding

With the recognition that protein misfolding is a cause of several neurodegenerative diseases and a common mechanism of their progression, promising developments have begun to emerge (reviewed in the next section). These have relied upon the measurement of protein misfolding modulation as an early step in screening and evaluation of new active molecules or as a follow-up step in the evaluation of known and promising candidates because of their suspected effects on the diseases. Identification of a molecule as a folding modulator raises the possibility that it could address the cause of AD or PD, rather than only addressing their symptoms, which makes products identified in this way potentially valuable in prevention as well as in amelioration of existing disease.

### 2.1. *In vitro* analysis of protein misfolding modulation

For both Aβ and αS, the transition from the native state to an amyloid conformation is not direct, but involves steps or stages. These may include unfolding, oligomerization and further aggregation into amyloid fibrils, with the possibility of off-pathway aggregation or reversal of these processes [25, 26]. This is summarized in Fig. 1. Compounds may prevent misfolding and/or templating by inhibiting the unfolding of a natively folded protein, by promoting the folding of an unfolded form, by preventing amyloid formation or by diverting the protein toward an alternative aggregation pathway leading to a less harmful aggregate. A selection of assay approaches to misfolding evaluation will be compared here in terms of information value, throughput and versatility.

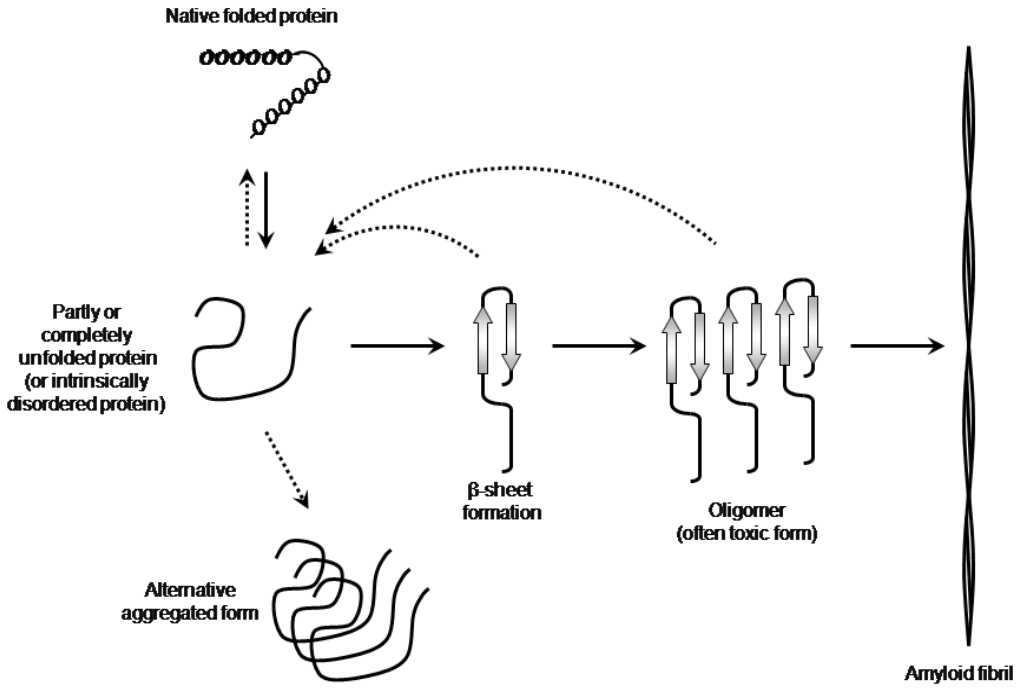


**Figure 1.** Overview of the general trajectory of protein misfolding with amyloid formation. Solid black arrows show pathway toward amyloid formation and stippled arrows show pathways of prevention or reversal of amyloid and related oligomers. This is an original diagram based upon information in reviews [2, 23].

### *2.1.1. Protein unfolding*

For the discovery and development of novel agents against diseases such as AD and PD, a fuller picture of the action of a compound can be obtained by investigating the distinct steps of unfolding, aggregation, misfolding and amyloid formation. Proteins may remain largely folded and still produce amyloid or similar aggregates, provided that a local aggregation-prone sequence is exposed [27]. Nonetheless, for most proteins that readily form amyloid, it appears that that the loss of native folding is a required step [27]. Both Aβ and αS appear to have some unfolded or disordered character, but with regions that can become helical under appropriate conditions [28-30]. Inducing or preserving this helical conformation is a rational approach to preventing amyloid formation [29, 31, 32].

Folded and unfolded proteins differ in several characteristics that can be easily measured and that would allow folding modulators to be detected and evaluated. Some of the most common methods are summarized here. For example, proteins absorb right and left-circularly polarized light differently; this circular dichroism (CD) can be measured over a series of wavelengths to give informative spectra. The CD spectra of unfolded and folded proteins differ and those of proteins with predominantly α-helix and β-sheet secondary structure can be easily distinguished. In more detailed studies, the proportion of each major secondary

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structure in a protein can be calculated from the spectrum using software such as DICHROWEB [33]. Moreover, diagnostic features such as minima at wavelengths of 208 and 222 nm for $\alpha$-helical proteins can be monitored in order to quantify changes in fold in response to temperature, solution components or other factors. To date, CD has been used for small-scale studies on protein folding and protein-ligand interaction because throughput is limited to a single sample at a time. The recent development of a high-speed automated CD spectrometer (ACD, Applied Photophysics) may allow this approach to be employed more broadly in sample screening. An advantage of CD measurement is that it is an intrinsic property of a protein; thus, the spectra require no additive and the protein can be measured directly without accessory molecules or modifications. Another intrinsic feature of proteins that can be measured directly is fluorescence. Proteins with aromatic side chains fluoresce when exposed to UV light and this can undergo a shift in intensity or wavelength of intensity maximum with folding. Fluorescence can also be measured using new devices that allow miniaturized measurements in a parallel manner using a similar rapid-throughput approach (e.g. the Optim 1000, Avacta Innovative Analysis). Likewise, differential binding of dyes such as SYPRO Orange allow protein forms with different exposure of hydrophobic residues to solvent to be distinguished. In the case of SYPRO Orange, the dye is quenched when free in an aqueous solution, but it becomes unquenched when it interacts with the hydrophobic regions that are exposed upon protein unfolding and its fluorescence increases as a consequence. This measurement can be performed efficiently on large numbers of samples using devices designed for quantitative PCR [34]. Measurements by these approaches allow protein folding to be assessed and changes in fold and/or fold stability to be examined. An informative and frequently employed measure of fold stability is the melting temperature (Tm), as proteins with more stable folds normally melt at higher temperatures. The Tm can be determined by any of the above techniques. A compound that stabilizes the folded conformation of a protein would raise its Tm and this change can be easily quantified by the above approaches. A caveat with amyloid-forming proteins is that stabilized folding, as exhibited by a higher Tm, may not necessarily represent the native folding that is assumed to preclude amyloid formation. For this reason, CD is particularly valuable, as it also allows the secondary structure to be determined; a spectrum distinct from that of β-sheet would provide a reasonable indication that the stable form is native and unrelated to amyloid.

### *2.1.2. Amyloid-like structure*

It was through dye binding that amyloid plaques were first discovered. Fibrous aggregates in tissues stained blue when treated with acid followed by iodine and the aggregates were termed amyloid because they were believed to be starch based upon the colour development (reviewed in [35]). Although it is a misnomer, the name amyloid has endured and several different dyes have become invaluable in the study of protein misfolding and amyloid formation *in vitro*.

Thioflavin T (ThT), a benzothiazole dye, and Congo red, a diazobenzidine dye, are both commonly used to quantify the amount of amyloid protein present in a solution. ThT was

first described to have amyloid-binding ability over 50 years ago [36] and it has since been in widespread use in the study of amyloidosis and the search for possible binding molecules. Congo red has also been in use for amyloid detection and quantification for several decades since its first description in that role [37]. Both of these dyes undergo a red shift in their fluorescence emission when they interact with amyloid fibrils. They cannot be used to distinguish between amyloid fibrils and smaller aggregates; however, they allow quantitative analysis and they continue to find wide use in amyloid analysis. Thioflavin S is a mixture of components derived from ThT and it has no spectral shift in absorbance or emission, which makes it suitable only for imaging and not for quantitation in solution because of high background [35]. Derivatives of N-arylaminonaphtalene including ANS and bis-ANS bind primarily to early stage aggregation in amyloid formation [38, 39]. They have not been as widely used as ThT and Congo Red, but they may find greater use with the increasing focus on aggregation pathways (see section 3 below).

Recently, there has been interest in identifying newer binding fluorophores that allow oligomeric aggregates to be distinguished from fibrils. An example is the indole compound tryptophanol. It allows specific detection and quantitation of prefibrillar oligomeric Aβ because its fluorescence is quenched only in the presence of that form of Aβ with no similar quenching in the presence of Aβ fibrils [40]. Another is the carbocyanine dye JC-1 that distinguishes different aggregation states of αS [41]. JC-1 allows real-time tracking of αS aggregation, as different fluorescent signals are emitted for monomer and fibrillar binding and the ratio of the two signals allows for real-time visualization of αS aggregation [41].

Another recent development is the elaboration of antibodies and related agents that recognize proteins exclusively in their amyloid conformation, allowing the amyloid portion of the protein in question to be detected and quantified. A monoclonal antibody raised against αS was shown to recognize the protein in its amyloid form and, although the anticipated use is mainly *in vivo*, it showed effective recognition in an *in vitro* ELISA format [42]. Novel antibodies, termed gammabodies, were engineered by grafting a series of peptide sequences from Aβ into immunoglobulin variable regions [43]. These have found use in distinguishing oligomeric and amyloid forms of Aβ and this technology would be expected to be applicable to other aggregating proteins [43]. Since an amyloid-like structure is relevant to many diseases, a new capture and detection peptoid that behaves in a manner similar to an antibody offers unusual versatility. This peptoid, based upon a 6-amino acid stretch in the human PrP sequence, binds to the amyloid forms of Aβ, αS, amylin and serpin [44].

Model proteins that undergo transition to an amyloid-like conformation may also offer alternatives for amyloid formation-related studies [45]. For example, the 37-residue natively α-helical winter flounder (*Pseudopleuronectes americanus*) antifreeze protein wflAFP-6 (HPLC-6) adopts an amyloid-like structure during the freeze-thaw process [46, 47]. This may offer an interesting model system for the study of the conversion of a protein from its native form into amyloid fibrils [47, 48]. The ability to generate rapid amyloid formation in wflAFP-6 using the freeze-thaw process would offer low background compared with Aβ assays and the consistent α-helical nature of the native AFP may also allow initial unfolding steps to be more

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clearly evidenced than in proteins with less defined native structure. Nonetheless, a molecule found to be an amyloid-relevant folding modulator using AFP amyloid-like transition assays may be universal or nearly so, as in the case of the peptoid detection system described above, or it may be specific to the wflAFP-6 and have no effect on other amyloid-forming proteins. Therefore, in spite of its potential advantages, further study of the antifreeze protein model would be required before its adoption in an assay platform.

*2.1.3. Protein interaction during amyloid formation*

As the interaction between locally or entirely misfolded monomers appears critical to non-native protein association, there is interest in identifying molecules that directly interfere with that process. Therefore, assays for binding competition or impedance have been developed. A convenient new microplate assay employs fluorescently labelled Aβ. Unlabelled Aβ is coated onto the plate surface and then the labelled Aβ is applied in solution and allowed to bind [49]. After unbound material is washed off, the fluorescence remaining can be used to determine the level of interaction of Aβ [49]. Although the aggregation state of the Aβ in solution or on the plate is not indicated, the assay indicates the interaction between in-solution and on-plate Aβ that is inhibitable, which can be taken to represent either oligomer formation or assembly into larger fibrils. Thus, molecules that affect any part of this process can be readily identified. Nonetheless, with the aggregation state of the binding units unclear except where Aβ fragments are used, this assay does not indicate which step(s) in assembly of the monomer-oligomer-fibril are being affected.

Aβ oligomerization and fibril formation can be challenging to study *in vitro* using dyes and other methods because of spurious background oligomer formation and the difficulty in determining how much oligomer may already have formed in a starting sample [50]. For these reasons, assays that avoid these problems have garnered interest. In one example, a procedure was developed involving a semi-denaturing detergent-agarose gel electrophoresis followed by blotting and immunological detection with an appropriate antibody recognizing the protein in all its forms [51]. The method allows amyloid aggregate size distributions to be determined and the user can distinguish among the monomeric, oligomeric and large aggregated (likely fibril) forms. A drawback is that it involves a vacuum transfer of proteins from the gel to a PVDF membrane. A derived method employing capillary blotting to a nitrocellulose membrane, reminiscent of nucleic acid blotting, has made the assay more precise and scaleable for screening purposes [52]. These methods allow the association state of the starting material to be determined and the effect of any product to be evaluated in comparison. Another approach is to eliminate the possibility of aggregates in the starting solution. An example is an assay based upon the expression of the Aβ sequence in tandem with green fluorescent protein (GFP) as a fusion within a bacterial cell [53]. Aggregation of Aβ precludes GFP folding and so there is no fluorescence, whereas addition of a molecule impeding the aggregation of Aβ results in GFP folding to its native structure with consequent fluorescence. A more directly quantifiable *in vitro* form of this assay was developed using the Venus yellow fluorescent protein (vYFP) instead of GFP, which allows denaturation and refolding under precisely controlled conditions to detect molecules that promote or inhibit aggregation [50].

### 2.2. Cell- and organism-based analyses

Once an active extract is identified by efficient *in vitro* assay and similar assay-guided fractionation has revealed the class of molecules or the molecule that is responsible, determination of effects *in vivo* is of interest. Using culture models such as the PC12 primary neuronal cells or the SH-SY5Y neuroblastoma cell line, effects of active molecules can be determined. In a first approach, cells can be treated by transfection or other means to harbour high levels of the protein of interest and then the effect of active molecules on cell survival can be measured using one of several commercially available assays for dead cells, which measure lactate dehydrogenase leakage or other indicative parameters. However, more precise information can be gleaned by histological analysis using stains such as Congo red or thioflavin S, described above, that reveal the presence of amyloid accumulation. Immunohistochemistry using appropriate antibodies against Aβ or αS can be even more informative, as some allow distinction of oligomeric denatured forms from amyloid fibrils.

Cell-based assays can also provide an indication of the possibility of a molecule crossing the blood-brain barrier. Although Aβ circulates in the serum [54] and αS is expressed in red blood cells [55], it is considered preferable for a compound to have direct access to the brain where the pathology takes place. Therefore, blood-brain barrier models in cell culture, such as the MDR-MCCK monolayer [56], are available to predict the permeability for specific substances. Nonetheless, blood brain barrier physiology is more complex than the models or direct plasma/brain ratio determinations would predict [57]. For that reason, results must be interpreted with care.

Animal models provide critical understanding in terms of the distribution, mode of action and effectiveness of an active molecule. Besides the well-known mouse models for many neurodegenerative diseases, there are valuable invertebrate and fish models. An αS-transgenic *Drosophila* fly model is available for PD [58] and an Aβ-transgenic model for AD [59] The nematode worm, *Caenorhabditis elegans,* can be used to study AD and PD [60, 61]. Zebrafish (*Danio rerio*) have an Aβ protein precursor-encoding gene [62] and synuclein genes have been identified in this species [63]. Zebrafish may also become informative for studies of αS once the encoded proteins in this organism are better understood.

## 3. Protein folding modulators from common terrestrial natural products

A large number of molecules have been shown to influence the aggregation of Aβ [18] and a growing number have shown effects on αS (discussed below). Although no effective therapy for PD or AD has emerged to date, progress on a number of natural products is encouraging. This situation is not surprising, as synthetic and combinatorial chemistry normally rely on a limited number of structural scaffolds and random permutations of structures and synthesizable units [64]. In contrast, natural molecules are highly diverse and they have been subject to evolutionary pressure under distinct conditions in which valuable activities

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have been selected. Therefore, they may offer a combination of variety and biological pre-selection that may make a therapeutic activity more likely [64].

### 3.1. Natural protein folding modulators from terrestrial sources

Several natural molecules have garnered interest because they modulate amyloid formation by Aβ (reviewed in [18, 65, 66]). A number of salient examples involving Aβ and αS are presented here.

#### *3.1.1. Curcumin*

Curcumin is a natural polyphenol found in the traditional Indian spice turmeric. This molecule has been extensively investigated, in part because of its role in traditional Ayurvedic medicine [67]. Numerous studies have shown curcumin to have wide ranging biological activities, including Aβ folding modulation. Curcumin shows promising interaction with Aβ. For example, using an oligomer-specific antibody for immunoblotting, cucurmin was found prevent Aβ oligomerization and toxicity *in vitro*. In the same study, curcumin was shown to bind to amyloid plaques in brain sections of mice transgenic for the human amyloid precursor protein [68]. In addition, mature fibrils of the slightly shorter $A\beta_{40}$ peptide were destabilized by curcumin [68]. Curcumin and other polyphenols, including tannic acid, rosmarinic acid, and myricetin, were shown to inhibit fibril formation and destabilized mature fibrils for both Aβ and αS [69]. As a result, curcumin has been a component of interest in clinical trials and, most recently the exclusive molecule in a trial for prevention of early cognitive decline and abnormal Aβ accumulation in the brain [70]. If an effect is observed, it would be interesting to see if it acts directly upon misfolding at the dosage used in subjects.

#### *3.1.2. Epigallocatechin-3-gallate*

In terms of bioactive molecules in traditional medicine, perhaps the most recognized is (-)-epigallocatechin 3-gallate (EGCG) that is found in unfermented (green) tea (*Camellia sinensis*). EGCG is a pleiotropic polyphenolic molecule with interesting protein folding modulation activity. In *C. elegans* expressing human Aβ in muscle cells, western blotting analysis using an anti-Aβ antibody showed EGCG to inhibit Aβ oligomerization [71]. Studies of Aβ and αS using ThT fluorescence, electron microscopy and other biophysical methods showed interaction with EGCG and ensuing "off-pathway" aggregation into spherical oligomers, which thereby prevented the formation of typical aggregation intermediates and amyloid fibrils by both proteins [25]. Further investigation revealed EGCG to bind directly to the β-sheet structure in the amyloid fibrils formed by Aβ and by αS and to direct their rearrangement into smaller non-toxic protein aggregates [26]. A clinical trial is planned to examine the effect of EGCG on early stage AD [72] and this may have effects based upon the protein folding modulation described here, other properties of the molecule, or a combination thereof if adequate levels are attained in the brain. Other tea compounds may offer similar protection. Theaflavins are formed by oxidation of EGCG during the fermentation process to produce black tea. These derivatives were shown to have similar activity to EGCG in modulating the polymerization of Aβ and αS [73].

*3.1.3. Carotenoids and related molecules*

Carotenoids (pro-vitamin A) and vitamin A are present in many commonly consumed foods. The *in vitro* examination of vitamin A and β-carotene activity on AB fibril formation, as measured using dye binding and electron microscopy suggest protective effects that appear to result from binding to a region in the C-terminal half of the peptide [74, 75]. This is consistent with reports that vitamin A and related molecules inhibit the progression of AD symptoms [76]. Also using dye binding and electron microscopy, vitamin A and β-carotene were shown to inhibit the formation of αS amyloid fibrils and to destabilize existing fibrils [77].

*3.1.4. Scyllo-inositol*

Another natural molecule that has garnered interest is scyllo-inositol, which is abundant in the coconut palm (*Cocos nucifera*) [78]. Scyllo-inositol is a stereoisomer of myo-inositol that stabilizes a non-toxic oligomeric aggregate of Aβ [78-80]. This is in contrast to phosphatidylinositol, which was found to promote fibrillogenesis and membrane insertion of Aβ [78]. Scyllo-inositol also protected against Aβ oligomer-induced inhibition of long-term potentiation [80]. A clinical trial of scyllo-inositol was inconclusive after the highest doses were discontinued [81]. Nonetheless, it remains a compound of interest provided that drawbacks can be addressed.

*3.1.5. Emerging sources*

In addition to the well-studied molecules above, there are growing possibilities for other sources of folding modulators. For example, another source of interesting polyphenols is the wolf berry, *Lycium barbarum*, which is used in traditional Chinese medicine and was shown to protect against Aβ-related cell death [82]. Extracts obtained from other species used in traditional Chinese medicine, including cat's claw (*Uncaria rhynchophylla*) and tree peony (*Paeonia suffruticosa*) have shown relevant activity [83-85]. The tree peony contains 1,2,3,4,6-penta-O-galloyl-beta-D-glucopyranose, which prevented Aβ fiber formation and reversed the process in existing fibers [84]. In addition, other polyphenols procyanidins from apple (*Malus domestica*), silymarin from thistle (*Carduus marianus*) and reservatrol from red grapes (*Vitis sp.*) show interesting activities that may warrant further study [86-88].

**3.2. Challenges with natural folding modulators**

Although much attention has been brought to the natural folding modulators present in various terrestrial species, none have yet translated into meaningful therapies. The best prospect currently appears to be EGCG [89], which nonetheless presents challenges.

*3.2.1. Challenges in delivery of natural products*

EGCG is susceptible to decomposition during storage prior to being consumed, much of it may be destroyed by normal stomach acid during digestion, it is not highly bioavailable and it carries the possibility of hepatotoxicity (reviewed in [90]). Therefore, ideal handling, delivery and dosage would need to be determined before this molecule could be employed successfully

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to manage disease-related misfolding in animals or humans. Nonetheless, studies have suggested that the use of green tea is negatively correlated to the prevalence of cognitive impairment [91] and that tea shows a dose-dependent effect in protection from PD [92]. Therefore, even with regular storage, preparation and consumption of tea as part of a normal diet, it appears that tea components, and likely EGCG or its derived theaflavins, have a measure of benefit. It is not yet clear whether the reported benefits involve the modulation of protein misfolding, and this would be an area for future study. Curcumin presents challenges similar to those of EGCG and derivatives. Dietary ingestion of curcumin appears sufficient to offer some measure of protection from neurodegenerative diseases in populations that consume it (reviewed in [93]), suggesting its bioavailability. Yet, bioavailability was found to be limiting and work has been undertaken toward improving delivery of curcumin by combining it with phosphatidyl choline, olive oil and stearic acid [94]. Similarly, plasma levels of EGCG following oral consumption were higher when the product was encapsulated in chitosan nanoparticles [95]. The promising suggestion is that formulation and delivery may be optimized to enhance availability of these protein folding modulators *in vivo*. Other compounds such as carotenoids are bioavailable and cross the blood-brain barrier as well, possibly making them convenient for the establishment of protein folding modulators [96].

### *3.2.2. Requirement for crossing the blood-brain barrier*

It is widely accepted that molecules must cross the blood-brain barrier in order to have relevant biological effects on neurodegenerative diseases; however, interesting new findings are suggesting that this may not always be the case. In a study using radiolabeled $A\beta_{40}$ administered to the brains of rats, elevated peripheral $A\beta_{40}$ levels reduced protein clearance from the brain [97]. Conversely, a decline of Aβ levels in blood plasma can lead to cognitive improvement, presumably by reducing Aβ levels in the brain [98, 99]. Furthermore, genetic study of the presenilin gene encoding a secretase with a role in Aβ synthesis revealed a heritable expression level in the liver, suggesting a role of liver-expressed protein in brain Aβ levels [100]. Follow-up investigation using ST571, a drug that lowers peripheral Aβ levels but that does not cross the blood-brain barrier, showed reduction in brain Aβ [100]. Although these were studies of complete Aβ levels rather than the different conformations and aggregates, together they do imply that modulation of peripheral oligomerized and fibrillar forms of Aβ, which would facilitate clearance, could affect the brain ratios of differently associated Aβ forms. Therefore, protein folding modulators that do not cross the blood-brain barrier may still be worth considering in the prevention and treatment of disease. These studies have the drawback of assuming a stable and functional blood-brain barrier over the course of these diseases; however, other findings suggest that functioning of the blood-brain barrier may become compromised over the course of AD and other neurodegenerative diseases (reviewed in [101]), which may result in inappropriate dosages of molecules from plasma. Therefore, the prevention phase may be critical to the benefit of any molecule that acts peripherally to modulate Aβ, as that is the point at which normal transfer of molecules such as Aβ across the barrier can occur. Alternatively, the compromised blood-brain-barrier during the disease phase may allow therapeutic molecules to reach the brain more efficiently. These possibilities require further study.

## 4. Marine possibilities for natural protein folding modulators

Natural products from the land include promising as sources of novel protein folding modulators for the reasons outlined earlier. By extension, marine sources may hold proportionally greater opportunity for discovery than their terrestrial counterparts because, until this decade, much of the ocean biota was unexplored. The Census of Marine Life uncovered remarkable and previously unknown plant and animal biodiversity in the oceans (reviewed in [102]). In the Gulf of Maine alone, the current species count identifies at least 652 fish species, 184 species of birds, 733 different species of microscopic plants and algae and 32 mammalian species [103]. Microbial diversity is also extensive worldwide, as inferred from the large number of new protein families uncovered from marine microbes [104].

Marine resources offer largely untapped diversity that may offer new options for natural product development [105]. Only six marine natural products and 14 synthetic compounds based upon the structures of natural marine products are FDA-approved agents or in clinical trial (reviewed in [106]). However, this list is likely to grow. In 2010, 895 new citations were reported on marine-derived compounds [107]. Marine species are already being examined for molecules that inhibit β-secretase 1, an enzyme that processes the amyloid precursor protein to give Aβ and other fragments [108]. Nonetheless, the development of inhibitors has been very difficult elsewhere [108] and there may be other biological consequences to the modulation of this enzyme. Therefore, it is ideal to focus more specifically on protein misfolding, which appears to be the linchpin in most of the neurodegenerative diseases. There is a wide variety of challenging and variable marine environments that would naturally select for traits that include protein folding modulation due to temperature or pressure, chemical defense molecules and other products that may be undersampled or undiscovered because of accessibility. Marine plants and sessile marine animals may produce chemical defenses that are remarkable in comparison to those of species that can move away from danger. In addition, species that inhabit highly variable or extreme environments, such as freezing waters, have adaptations to maintain proteostasis during exposure to extreme temperatures and solution properties [109-111]. For these reasons, they may represent ideal sources of natural molecules to prevent or ameliorate protein misfolding.

The discovery, characterization and production of marine-derived molecules appears promising, but with complexity and risk, nonetheless. A general overview of the process is shown in Fig. 2.

### 4.1. Natural protein folding modulators from marine sources

#### *4.1.1. Marine polyphenols*

Polyphenols are abundant and varied in marine algal species (reviewed in [112]). Like their terrestrial counterparts, algal polyphenols have shown folding modulation of Aβ. The brown seaweed (macroalgae) *Ecklonia cava* is found in the waters around Japan and Korea, where it is used as a herbal remedy. A butanol extract from *E. cava* has been shown to prevent production and aggregation of Aβ and to reduce amyloid plaques [113]. Electron microscopy showed

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the Aβ oligomers to be reduced and dye binding as well, indicating an inhibition of fibril formation. The polyphenolic phlorotannins are considered to be the active compounds responsible for the biological activities of *E. cava* [114, 115]. Nonetheless, other molecules cannot be strictly ruled out [113].

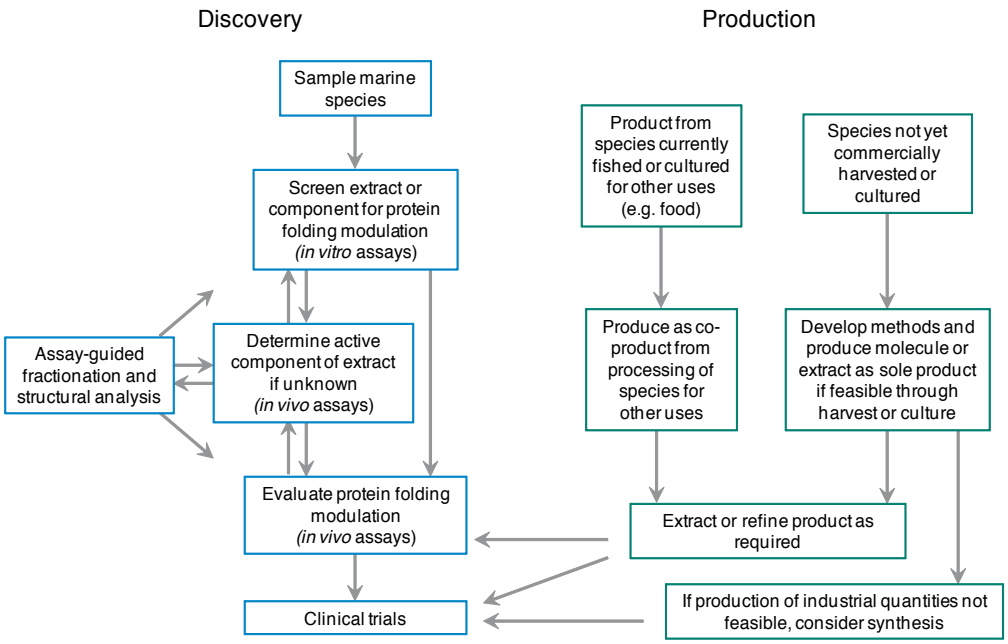


**Figure 2.** Schematic summary of key steps in the identification and development of marine-sourced protein folding modulators. Blue boxes are steps involved in discovery and characterization of protein folding modulation and the molecule responsible. Green boxes are steps involved in production of the molecule (or of an extract containing it). For more detail, see chapter text. Structural elucidation is not included here as it is not covered in this chapter; it is covered in another chapter of this book.

### *4.1.2. Marine carotenoids*

Marine algae and invertebrates harbour large quantities of carotenoids. This is of interest because, as noted above, carotenoids are among the molecules showing promise in modulation of protein misfolding. Algal carotenoids are widely varied (reviewed in [112]). These appear to be particularly accessible through enzyme-assisted extraction (reviewed in [116]) and they may have interesting activities. Shrimp and crab processing wastes are also excellent sources of carotenoids. Crustacean shells left over during processing contain the carotenoid astaxanthin (reviewed in [112]), which gives them their striking colour. This is in addition to the chitin in the shells, and its component glucosamine, which is well known in other contexts. There are no reports of evaluation of marine-derived carotenoids in protein folding modulation, as there are for terrestrial counterparts. With the numerous and varied sources of marine carotenoids, these could be compared with terrestrial versions previously reported to modulate protein folding.

*4.1.3. Marine toxins*

There is a plethora of marine toxins with distinctive neurological effects. These toxins are mainly known for the poisoning risk they carry. Nonetheless, a few of them have valuable biological activities as research chemicals and one has intriguing effects relative to AD. Using cell-based assays, the toxin 13-desmethyl spirolide C from the dinoflagellate *Alexandrium ostenfeldii* was shown to reduce Aβ accumulation in cells by just over 40% [117]. The antibody used (6E10, Covance) detects Aβ without distinguishing between monomeric or oligomeric or fibril forms. It would therefore be valuable to repeat these analyses using fold-specific anti-Aβ antibodies or another fold-sensitive method to see if any effect there could be detected. Otherwise, it may act on Aβ in other beneficial ways that would also be of interest.

*4.1.4. Marine-sourced chemical chaperones and conflicting results*

A variety of chemical chaperones, which are loosely defined as small molecules that promote folding of many proteins, have gained interest in terms of wide-spectrum protection of proteins from misfolding (reviewed in [118]). In marine species faced with protein misfolding risk, the accumulation of one or more chemical chaperones is a common adaptation because these small chaperoning molecules promote general proteostasis [111, 119]. Therefore, at first glance, these molecules would appear to be ideal in terms of proteins such as Aβ and αS. They could be produced naturally or synthetically, depending upon their molecular features, and they may stabilize many different proteins. However, some chemical chaperones may have problematic effects with respect to amyloidotic protein misfolding. Glycerol at supraphysiological concentrations (molar range) and trimethylamine oxide (TMAO) at moderate concentrations were both shown to favour the transition of Aβ from its unfolded conformation to the β-sheet form requisite for fiber formation [120]. Protofibril to fibril conversion was also enhanced [120]. The situation appears more complex for αS, with elevated concentrations (molar range) of TMAO favouring a partially folded form with high propensity for fibril formation and even higher concentrations of TMAO favouring an oligomeric α-helical conformation [121], which may be consistent with a native α-helical form of the protein that resists misfolding [29]. Although these chaperone concentrations used were far in excess of those that would be reached *in vivo*, the effects suggest a possibility to be aware of if a novel folding modulator appears to be non-specific. Stabilization by chemical chaperones may bring an increased risk of aggregation for some proteins [119]. Furthermore, stabilization of a wider range of cell proteins or protein complexes may cause unanticipated problems. Therefore, examination of unusual and off-target effects would be prudent in the evaluation of chemical chaperones.

**4.2. Sources for marine products**

A challenge presented by many rare or remote marine species that may produce pharmaceutical or nutraceutical molecules is the difficulty in obtaining sufficient product in a sustainable manner, from both the environmental and economic standpoints [122]. Options for these products include the development of culture methods for the species or synthesis of the molecule, although in many cases the molecules are too complex to be efficiently synthesized [122, 123]. In contrast, an advantage for nutraceutical or drug development from many

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accessible marine species, both plant and animal, is that they are already supplied as human food sources. Additionally, a large number of marine species are currently farmed, and availability of a standard supply for natural product development would be more straightforward and predictable than it would be for their wild counterparts. Sustainable production or harvesting of species for natural marine products would be a key consideration in production of folding modulators, just as it is for the production of foods.

In the case of some seaweeds, marine harvesting can be carried out. However, on-land cultivation offers the additional benefits of ideal traceability and process control, albeit with substantial costs [124]. For microalgae, this would be the only option. Algal processing wastes are significant and they are of interest for the development of by-products [125].

For commercially fished animal species, processing for food production is a frequent practice. These wild finfish and shellfish species, as well as those grown in aquaculture, can generate substantial waste during processing for food production and this waste often go to landfills or composting. The identification of valuable properties or activities in waste materials allows them to be used to produce co-products and, with green chemistry initiatives, these could be produced in a sustainable fashion from a healthy wild harvesting or aquaculture operation. There is growing interest in co-product development, as highlighted by two contrasting examples. Snow crab (*Chionoecetes opilio*) are fished across the Maritime provinces of Canada. Crab processing waste currently contributes to landfills, but the potential for additional product development with the identification of sufficiently valuable uses is being considered [126] as is the case elsewhere, likely based upon chitin but possibly upon other products as well. Innovative solutions to waste challenges are also being pursued in salmon aquaculture. Integrated multi-trophic aquaculture of Atlantic salmon (*Salmo salar*) with macroalgal species and mussels (*Mytilus edulis*) in the Bay of Fundy, Canada, minimizes waste accumulation by allowing nutrient and resource recovery with diminished impact on the local environment and this process is advancing toward commercial production [127]. The economic viability of this integration initiative will rely upon the development of foods along with other high-value products from the cultured species [128]. A neuroprotective protein folding modulator from such a resource could enhance markets for the food, while offering the possibility of producing sustainable nutraceuticals and eventual pharmaceuticals as co-products if appropriate.

## 5. Conclusions

With protein misfolding identified as a causative event in multiple neurodegenerative diseases and with no definitive means of prevention or cure in place, the progress being made in the discovery and development of natural protein misfolding modulators is of great interest. Although several promising leads from terrestrial sources have been identified, none have been shown to provide a clear result in disease outcome to date. Therefore, while current products are being developed and refined, it would be logical to investigate a wider range of sources for protein folding modulators. In this context, marine species deserve a second (or, in some cases, a first) look.

There has already been extensive work on marine molecules from some sources, but crucial to the proposed endeavour is screening for the most relevant activities. For example, testing marine molecules or extracts for effects in model Aβ or αS cell survival assays may not indicate protein folding modulation. However, the reverse is likely to be true in most cases. In other words, appropriate protein folding modulation activity should lead to enhanced cell survival. It is also important to be mindful of assay subtleties. Testing for anti-fibrillization activity may uncover molecules that cause toxic oligomers to accumulate instead of amyloid fibrils, leading to increased pathology instead of amelioration. Similarly, searching for molecules that bring about a reduction in total Aβ (or αS) may not be prudent, as we have little understanding of the normal roles of these proteins in the brain and therefore drastic reduction in levels may have undesirable consequences [18]. For these reasons, an assay suite that allows determination of toxic oligomers as well as other (non-toxic) oligmers, monomers, amyloid fibrils and other aggregates would be ideal. In order to identify a marine extract or molecule that provides protective protein folding modulation, the spectrum of its effects on protein misfolding and association protein would need to be investigated. Furthermore, because a number of natural molecules appear to affect the misfolding trajectories of both Aβ and αS, these would be of particular interest because of the potential for wider-ranging protection from toxic misfolding.

In closing, examination of marine biota for protein folding modulators using a suitable suite of assays may offer a promising opportunity to identify a safe, effective and sustainable protein folding modulator that addresses the cause of a neurodegenerative disease. The untapped diversity of marine species combined with a rational selection of assays for folding modulator identification based upon examples used in terrestrial investigations and coupled with appropriate downstream *in vivo* analysis would be ideal. For proteins such as Aβ and αS, which form similar amyloids and can even cross-seed in spite of their structural differences [129], a further tantalizing prospect would be the identification of a molecule that would be effective for both AD and PD. Given the paucity of treatments currently available, discovery of an active molecule from a marine resource could bring hope at this time.

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Chapter 5

# Marine Flatfish-Derived Bioactive Peptides: From the Ocean to the Bedside

Susan Douglas

Additional information is available at the end of the chapter

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## 1. Introduction and Relevance

Infectious diseases and cancer are leading causes of mortality and our ability to combat them is compromised due to the emergence of antibiotic-resistant strains of bacteria and chemotherapy-resistant cancer cells. Combined with the scarcity of new classes of antibiotics due to the abandonment of antibacterial research by pharmaceutical companies (Williams and Bax, 2009) and the lengthy development time lines to market (Projan and Bradford, 2007), there is an urgent need for alternative therapeutics. Cationic antimicrobial peptides (CAPs) have emerged as a promising source of novel therapeutics. Not only do they rapidly kill microbes and cancer cells, they also can modulate host innate immunity to augment clearance of microbes and promote tissue healing resulting from inflammation. Furthermore, they are less prone to resistance than traditional antibiotics (McPhee and Hancock, 2005). Synthetic variants of naturally occurring CAPs, have been designed that exhibit greater selectivity and stability. Here I summarize some of the key features of CAPs, directing the reader to recent pertinent reviews, and focus on characteristics of pleurocidin CAPs that make them attractive for clinical applications.

## 2. Physical properties of cationic antimicrobial peptides

### 2.1. Classes

CAPs are small peptides (10-50 amino acids) with a high content of positively charged (net charge +2 to +9) and hydrophobic (up to 50%) amino acids. Currently over 1800 naturally occurring CAPs from almost all forms of life are annotated in the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php). They can be classified into four structural

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groups: linear α-helical, β-sheet, loop, and extended structures enriched in certain amino acids such as Arg, Phe, Pro, Gly, Trp and His (Zasloff, 2002). Linear α-helical CAPs such as magainin, LL-37 and pleurocidin are usually unstructured in aqueous solution but adopt an α-helical conformation upon interaction with lipids, thereby acquiring antimicrobial activity. They often contain a hinge in the middle, due to Gly or Pro residues, which enhances the ability of the peptide to enter the cell (Park et al., 2000). CAPs that form stable β-sheet structures contain cysteine residues that are able to form disulphide bonds (usually 1-4 depending on the type of CAP). These include the protegrins, defensins and drosomycins. Looped CAPs, thanatin and brevinin, contain a C-terminal loop carrying a strong positive charge (Fehlbaum et al., 1996). CAPs such as bactenecin, prophenin and indolicidin are enriched in specific amino acids, usually lack cysteines, and form linear or extended coils. CAPs are usually produced as inactive pre-pro-peptides and proteolytically cleaved to release the mature biologically active peptide.

### 2.2. Pleurocidin

Pleurocidins comprise a family of linear α-helical CAPs consisting of a highly conserved amino-terminal signal sequence and a carboxy-terminal anionic segment flanking the more variable mature peptide of approximately 25 amino acids (Douglas et al., 2001; Patrzykat et al., 2003). This anionic portion may counteract the positively charged mature peptide, thereby keeping it inactive until cleaved. They show some sequence similarity to other fish CAPs, including piscidin, moronecidin and the recently discovered gaduscidin (Browne et al., 2011; Sun et al., 2007). Pleurocidins are encoded as clusters of genes comprised of four exons, and binding sites for transcription factors involved in host defense are located upstream of the promoters (Douglas et al., 2003).

Pleurocidin variant NRC-04 is the best-studied of this family of peptides. Early studies showed that in 25% dodecylphosphatidylcholine (DOPC)/ dodecylphosphatidyl-ethanolamine (DOPE) vesicles, it forms a structure containing between 12% and 24% α-helical content (Yoshida et al., 2001). Supporting this, it was only weakly incorporated into neutral bilayers composed of a 7:3 mixture of DOPC and DOPE; however, it strongly associated with a slightly anionic bilayer of 7:3:1 DOPC, DOPE, and dodecylphosphatidylserine (DOPS), forming well-defined single ion channels (Saint et al., 2002). NMR studies have shown that in 0% DPC, pleurocidin has no identifiable well-defined secondary structure but in 30% TFE and in 10 mM DOPC micelles, it was 25% α-helical and this increased to 95% in 140 mM DOPC (Syvitski et al., 2005). The hydrophobic amino acids residues clustered on approximately 2/3 of the helix and hydrophilic residues on the remaining 1/3 of the helix.

## 3. Distribution and expression of cationic antimicrobial peptides

### 3.1. Organismal distribution

CAPs are ubiquitous in nature and play a crucial role in first-line host defense against pathogens. Fish and marine invertebrates, because of their diversity and reliance on non-

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specific innate immune defenses, have proved to be a particularly good source of novel CAPs with therapeutic potential (Otero-González, 2010; Smith and Fernandes, 2009).

### 3.2. Expression

#### *3.2.1. Tissue specificity*

In mammals, CAPs are expressed in cells of the skin and mucosal surfaces, as well as blood cells such as platelets, monocytes/macropages, neutrophils, and mast cells (Guaní-Guerra et al., 2010). Tissues often express a cocktail of different CAPs with varying activities and the expression of a given CAP variant may be restricted to a specific cell type (see Wiesner and Vilcinskas, 2010). For example, of the six different $\alpha$-defensins, four are produced predominantly in neutrophils whereas two are secreted from Paneth cells of the small intestine.

Fish CAPs such as pleurocidin are expressed in a broad range of tissues and cell types including epithelial, immune and blood cells (Browne et al., 2011). Pleurocidin is produced by epithelial cells of the skin and gut (Cole et al., 1997; Douglas et al., 2001) as well as circulating immune cells (Murray et al., 2007). Interestingly, different pleurocidin variants are produced by different cell types (Douglas et al., 2003), underscoring the importance of screening multiple tissues to uncover the true diversity of CAPs.

#### *3.2.2. Developmental stage*

The presence of CAPs in neonates, who have yet to develop adaptive immunity, provides enteric protection and impacts the composition of the commensal microflora. Milk lactoferrin, from which the CAP lactoferricin is produced by pepsin cleavage in the stomach, plays a major role in maternal and innate immunity (Jenssen and Hancock, 2009). Dermicidin YP-30 is important for maternal and early post-natal protection (Wiesner and Vilcinskas, 2010). Mouse cathelin-related antimicrobial peptide undergoes a developmental switch from constitutive intestinal epithelial expression in neonates to Paneth cell expression in adult intestinal crypts (Menard et al., 2008).

Pleurocidin transcripts were detected early in development of winter flounder, when larvae are most susceptible to pathogen-induced mortality, and showed a progressive increase as development proceeded to adulthood (Douglas et al., 2001). Furthermore, different pleurocidin variants were present at different stages of development, indicating that screening of CAP production from various developmental stages of an organism is an excellent means of discovering novel peptide variants.

#### *3.2.3. Constitutive or inducible*

CAPs may be expressed constitutively or inducibly in response to stress, poor nutrition, inflammation (Wehkamp et al., 2003), injury or microbial infection (Redfern et al., 2011; Zasloff, 2006), and exposure to environmental factors such as zinc (Talukder et al., 2011) or vitamin D (White, 2010). For example, hCAP-18/LL-37 protein and mRNA expression was up-regulated in neutrophils that had migrated to the inflamed gingival tissues of patients

with chronic periodontitis (Turkoglu et al., 2011) and mycoplasma infection induces cathelicidin expression in neutrophils of infected mice (Tani et al., 2011). Interestingly, increased endogenous glucocorticoid levels induced by psychological stress reduce CAP expression in mice, leading to increased susceptibility to infection (Aberg et al., 2007).

Expression of fish CAPs is also regulated in response to stress and disease (Douglas et al., 2003a; Sun et al., 2007), and novel variants are often induced by such stressors. Monitoring levels of CAPs in aquaculture settings provides early warning of immunosuppression due to chronic stress, and conversely induction of CAPs provides protection against anticipated stressful situations such as handling (Noga et al., 2011).

## 4. Mode of action of cationic antimicrobial peptides

### 4.1. Membrane effects

CAPs can cause lysis of biological membranes or cross membranes by spontaneous lipid-assisted translocation. The initial interaction is electrostatic between the cationic residues of the peptide and the negatively charged constituents of the target cell, whether they are in an outer bacterial envelope, a viral envelope or a eukaryotic cell membrane. Because of the high proportion of uncharged zwitterionic lipids and sterols in normal eukaryotic membranes, however, these membranes are not as susceptible as negatively charged bacterial membranes or cancer cell membranes, which contain elevated levels of negatively charged sialylated glycoproteins (van Beek et al., 1973), *O*-glycosylated mucins (Yoon et al., 1996) and phosphatidylserine (Riedl et al., 2011).

Upon binding, the peptide assumes an amphipathic secondary structure that facilitates membrane disruption. A number of mechanisms have been proposed including micelle formation by the carpet model (Shai, 2002) and pore formation by the barrel-stave and toroidal pore models (Brogden, 2005; Hadley and Hancock, 2010). Additional variations on these initial models for bacterial membrane interactions have been proposed including localized membrane disorganization by the aggregate model (Hancock and Rozek, 2002), the formation of disordered toroidal pores, charged lipid clustering, membrane thinning, electroporation, non-lytic membrane depolarization, anion carriers and oxidized lipid targeting (Nguyen et al., 2011).

Pleurocidin has been proposed to act via the toroidal pore model, based on its interactions with model membranes (Saint et al., 2002). This finding is supported by NMR studies that show that in DOPC pleurocidin exists as a very large species, probably composed of 20-25 aggregating molecules (Syvitski et al., 2005), indicating that at high concentrations pleurocidin forms pores within the membrane environment or disrupts the membrane by aggregation. A more recent study proposed that CAPS similar to pleurocidin such as magainin 2 adopt a surface alignment in mixed zwitterionic/anionic membranes and forms disordered toroidal pores (Leontiadou et al., 2006). The interaction of pleurocidin with the anionic lipid phosphatidylglycerol (PG) rather than the zwitterionic lipid phosphatidylethanolamine (PE)

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in mixed membranes, resulting in disruption of membrane integrity, was confirmed using NMR (Mason et al., 2006). Interestingly, the three His residues, which become protonated at acidic pH, play no role in membrane disruption, most likely due to their position along the spine of the helix where they would be unlikely to interact with lipid head groups.

The Gly13 residue in the middle of pleurocidin confers flexibility between a longer $\alpha$-helix at the amino terminus and a shorter $\alpha$-helix at the carboxy terminus allowing it to interact with the negatively charged phospholipid membranes (Yang et al., 2006). Replacing Gly13 with Ala removes this hinge region, resulting in a much higher $\alpha$-helical content and loss of cell selectivity. This analog is hemolytic and interacts with both negatively charged (mimicking bacterial and cancer cell membranes) and zwitterionic (mimicking erythrocyte and normal mammalian cell membranes) phospholipids. Similarly, substitution of both Gly13 and Gly17 by Ala results in a large increase in $\alpha$-helicity and a correspondingly dramatic increase in hemolytic activity, indicating that the hinge region facilitates flexibility and confers bacterial cell selectivity (Lim et al., 2004a). It is interesting that Gly13 is conserved in 15 of the 26 identified pleurocidin variants whereas Gly17 is conserved in only 6, indicating that Gly13 plays the more important role in peptide function.

Sterols are commonly found in eukaryotic but not bacterial cell membranes, and their presence in anionic mixed membranes reduces the ability of pleurocidin to insert even though the peptide maintains its $\alpha$-helical configuration (Mason et al., 2007). Cholesterol, found in membranes of higher eukaryotes, was shown to be more effective than ergosterol, commonly found in those of fungi and certain protozoa, in reducing insertion of CAPs. These differences in membrane sterol content explain the differential sensitivity of bacteria, lower eukaryotes and higher eukaryotes to CAPs such as pleurocidin. Using a fluorescent membrane probe, pleurocidin has been shown to structurally perturb the plasma membrane of the fungal pathogen *Candida albicans* (Jung et al., 2007), confirming earlier killing assay results (Patrzykat et al., 2003).

Structural modelling predicted that only 12 of the 26 described pleurocidin variants formed amphipathic $\alpha$-helices and killing assays showed that these were cytotoxic to HL-60 human leukemia cells, resulting in rapid and complete killing (Morash et al., 2011). Cell death was non-apoptotic and probably occurred by the formation of ion channels that dissipated the membrane potential and led to cell lysis. The active pleurocidin variants were the more highly charged (>+6.5) members of the family; interestingly only one of them was also hemolytic. The anti-cancer activities of two pleurocidin variants (NRC-03 and NRC-07) against breast cancer cells involve binding to negatively charged cell-surface molecules (Hilchie et al., 2011).

### 4.2. Intracellular effects

A number of CAPs, when administered at low doses, are able to penetrate the bacterial membrane and disrupt metabolic processes (see Marcos and Gandia, 2009; Nicolas, 2009). CAPs can assume different structures whilst exerting their inhibitory effects. For example, when bound to DNA, buforin II assumes an extended form whereas magainin and pleurocidin assume an $\alpha$-helical form and bind DNA less effectively (Lan et al., 2010). Combinations

of CAPs that exert their effects on different targets may exhibit synergy, and provide more potent killing activity.

Pleurocidin usually causes membrane disruption at high concentrations whereas at low concentrations, it can translocate into the cytoplasm without causing cell lysis and exert its effects intracellularly, inhibiting DNA, RNA and protein synthesis (Patrzykat et al., 2002). The intracellular effect of pleurocidin NRC-03 has since been probed using zebrafish embryos, and shown to target the mitochondria and generate superoxide (Morash et al., 2011). TUNEL staining indicates that the DNA of some cells becomes degraded, whereas other cells undergo rapid lysis and cell death without DNA fragmentation. Pleurocidin variants NRC-03 and NRC-07 cause mitochondrial membrane damage and production of reactive oxygen species (ROS) in MDA-MB-231 breast cancer cells (Hilchie et al., 2011).

### 4.3. Receptors and binding proteins

In most cases, killing is by nonreceptor-mediated mechanisms since all D-amino acid enantiomers are generally as active as the natural L-amino acid peptides. However, stereospecific receptor-mediated translocation has been described (Nicolas, 2009) and some CAPs transduce their effects via signaling networks upon interaction with receptors. Quite a diversity of receptors have been described, possibly reflecting the diversity in peptide structures. Uptake of apidaecin into Gram negative bacteria is proposed to by an energy-dependent mechanism involving a permease or transporter (Castle et al., 1999). The detection of peptide:receptor complexes is technically very difficult but some receptors have been identified. For example, the outer membrane protein OprI from *P. aeruginosa* (Lin et al., 2010) and the outer membrane lipoprotein Lpp in Enterobacteriaceae (Chang et al., 2012) have been shown to serve as receptors for $\alpha$-helical CAPs. Histatin 5 and some defensins bind Ssa1/2 proteins on the cell surface of *C. albicans* in order to exert their activity, and the potassium transporter TRK1 is also required for histatin5 fungicidal activity (Vylkova et al., 2007). In eukaryotic cells, formyl peptide receptor-like 1 (FPRL1) is used as a receptor for LL-37 to act as a chemoattractant (Yang et al., 2000) and induce angiogenesis (Koczulla et al., 2003). LL-37 has also been shown to mediate keratinocyte migration and cytokine release by transactivation of the epidermal growth factor receptor (Tokumaru et al., 2005) and P2X7 receptor (Elssner et al., 2004), respectively. However, activation of receptors could be via CAP-induced changes in membrane fluidity, ion transport and/or receptor aggregation (Braff et al., 2005) rather than direct binding.

Activation of mast cells by pleurocidin is G protein-dependent and proposed to involve FPRL1 and G protein coupled receptor signaling pathway (Kulka, unpub). The observation that pleurocidin can bind and activate FPRL1 has some important implications for human disease. The FPRL1 receptor subtype is also a receptor for the bacterially-derived peptide fMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine) making it an important innate immune receptor (Selvatici et al., 2006). FPRL1 activates key components of the innate immune system and is responsible for chemotactic responses, superoxide anion production and degranulation by neutrophils, macrophages and mast cells. FPRL function has been shown to be important in chronic obstructive pulmonary disease (COPD) due to cigarette smoking (Car-

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dini et al., 2012). FPRL1 can also transactivate epidermal growth factor receptors (EGFR) making them a potentially important target in lung cancer (Cattaneo et al., 2011).

## 5. Biological activities and therapeutic applications of cationic antimicrobial peptides

### 5.1. Antibacterial

Because of their rapid, broad spectrum bactericidal action, potency, and low host cytotoxicity, CAPs have elicited much excitement as alternatives to current antibiotics, particularly in the fight against antibiotic-resistant pathogens (Nijnik and Hancock, 2009). CAPs can cause bacterial cell death by disrupting the bacterial membrane, by entering the cell and inhibiting intracellular targets, or by stimulating the immune system to eliminate an infection. They can be used alone or together with other antibacterial agents, with which they frequently synergize.

Pleurocidins show broad-spectrum antimicrobial activity at micromolar concentrations and also show synergistic activity with several antibiotics (Cole et al., 2000; Douglas et al., 2003; Patrzykat et al., 2003). In contrast to many CAPs, pleurocidin is insensitive to NaCl concentrations up to 150 mM, and may therefore have application in relatively high-salt bodily fluids and in treatment of lung infections in patients with cystic fibrosis, who have even higher NaCl content (Goldman et al., 1997).

Recent studies have shown that pleurocidin possesses considerable activity against oral microorganisms growing both planktonically and as a biofilm, even in the presence of saliva (Tao et al., 2011). Incorporation of a targeting moiety to pleurocidin NRC-04 has significantly increased its specificity towards *S. mutans*, the main cause of dental caries, relative to other oral bacteria (Mai et al., 2011). Addition of 5 mM EDTA or 10 ppm sodium fluoride, two compounds commonly used in oral treatments, resulted in increased killing of *S. mutans*. Replacement of Ser14 by His improved the activity of the targeted peptide at low pH similar to that found in the oral cavity, showing that this targeted pleurocidin has good potential as an anticaries agent.

Foodborne infections, especially those of seafood are a major health problem, yet only one CAP, nisin, has been approved by the FDA for use as a food preservative and it has only limited activity against Gram-negative bacteria or fungi (Burrowes et al., 2004).

Pleurocidin was tested against Gram-positive and Gram-negative bacteria of interest in food safety and shown to be highly effective against 17 of 18 strains, particularly the fish spoilage bacterium *Vibrio alginolyticus* (Burrowes et al., 2004). In addition, it was active against the yeasts *S. cerevisiae* and *P. pastoris*, and the mold *P. expansum*, but was not detrimental to human red blood cells or intestinal epithelial cells, indicating that it would be safe to use as a food preservative. Furthermore, since pleurocidin is produced naturally by an edible fish, there are fewer concerns over its use in food preservation.

### 5.2. Antifungal

The antifungal properties of CAPs have been recognized for some time (De Lucca and Walsh, 1999); however, it is only recently that the mechanism of action has been elucidated. While some CAPs such as LL-37 kill fungal cells by lysing the cell membrane, others such as histatin and defensins 2 and 3 kill in an energy-dependent and salt-sensitive fashion without cell lysis (den Hertog et al., 2005; Vylkova et al., 2007). Magainin 2 and dermaseptin S3(1-16) also cross the cell membrane and interfere with DNA integrity (Morton et al., 2007a; Morton et al., 2007b). Histatin 5, lactoferrin, arenicin-1 and several other CAPs inhibit mitochondrial respiration and induce the formation of ROS and subsequent apoptosis in *C. albicans* (Andres et al., 2008; Cho and Lee, 2011b; Helmerhorst et al., 2001; Hwang et al., 2011a; Hwang et al., 2011b).

Attempts to improve antifungal activity of CAPs have resulted in structural analogs with increased selectivity towards fungi and less host cytotoxicity. Hydrophobicity was shown to be a crucial factor in the antifungal activity of a series of CAP analogs against zygomycetes vs ascomycetes (Jiang et al., 2008). Synthetic, non-peptidic analogs of naturally-occurring CAPs have shown potent activity as anti-*Candida* agents against biofilms in the oral cavity, and reduced mammalian cytotoxicity (Hua et al., 2010).

Pleurocidin also possesses activity against *C. albicans* (Cole et al., 2000; Douglas et al., 2003; Patrzykat et al., 2003) and various derivatives truncated at the amino or carboxy-termini show reduced hemolytic activity although they are generally less potent (Lee and Lee, 2010). Modification of pleurocidin to decrease hydrophobicity and $\alpha$-helicity also resulted in active peptides with reduced hemolytic activity (Sung and Lee, 2008). A synthetic all-D amino acid entantiomer of pleurocidin has also been designed that showed resistance to trypsin, plasmin and carboxypeptidase B proteases *in vitro* with no loss in antifungal activity (Jung et al., 2007). Pleurocidin induces ROS, oxidative stress, mitochondrial depolarization, caspase activation, and apoptosis in *C. albicans* with concomitant membrane phosphatidylserine externalization, cell shrinkage, DNA fragmentation and nuclear condensation (Cho and Lee, 2011a).

### 5.3. Antiviral

Enveloped viruses are often susceptible to attack by CAPs. Four different linear CAPs were shown to inactivate vaccinia virus by removal of the outer membrane, thereby rendering the inner membrane susceptible to neutralizing antibody against the exposed antigens (Dean et al., 2010). Novel HIV-1-inhibitory peptides have recently been identified by screening the APD Antimicrobial Peptide Database for CAPs with promising antiviral properties. This uncovered 30 candidate CAPs which, when synthesized and tested, resulted in 11 peptides with $EC_{50}$<10 μM against HIV-1 (Wang et al., 2010). Some CAPs are able to exert antiviral effects by blocking cell surface receptors used for viral entry. For example, the polyphemusin analog T22 binds CXCR4 on T cells and prevents T cell line-tropic HIV-1 entry (Murakami et al., 1997).

The antiviral activity of pleurocidin variants has been tested against vaccinia virus grown in HeLa cells and four lead candidates were identified based on viral inhibition without HeLa cytotoxicity at both high and low multiplicity of infection (MOI) (Johnston, pers. comm.). At

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the more biologically relevant low MOI, two peptides were inhibitory with an $IC_{50}$< 1 μg/ml. Further studies are required to determine the mechanism by which pleurocidin exerts its antiviral effect.

### 5.4. Anti-parasitic

The anti-parasitic properties of magainins and cecropins have been known for over twenty years and have subsequently been reported for many other CAPs (Harrington, 2011; Mor, 2009). CAPs are able to traverse the membranes of parasite-infected erythrocytes and are proposed to change the properties of the parasite cell membrane, cause membrane lysis, or become internalized and interfere with biological processes. BMAP-28, BMAP-27 and the less cytotoxic truncated derivative BMAP-18 have recently been shown to possess excellent *in vitro* activity against *Leishmania* and African trypanosomes growing both in mammalian cells and in cells of the insect vector (Haines et al., 2009; Lynn et al., 2011). Importantly, BMAP-18 was also able to inhibit release of leukocyte LPS-induced TNF-α associated with inflammation and cachexia in patients with sleeping sickness. Further *in vivo* studies of naturally-occurring and engineered CAPs are needed to demonstrate their potential in the treatment of serious parasite diseases of humans.

Pleurocidin inhibited the bloodstream form of *Trypanosoma brucei* at low concentration but was not effective against the procyclic culture form or the tsetse symbiont (Haines et al., 2003).

### 5.5. Anticancer

In contrast to conventional chemotherapy drugs that target all actively proliferating cells, CAPs can show selective cytotoxicity towards cancer cells including dormant, slow-growing, and multidrug resistant cells (see Hoskin and Ramamoorthy, 2008)). Both apoptotic (Jin et al., 2010) and non-apoptotic (Ceron et al., 2010; Hilchie et al., 2011; Morash et al., 2011) mechanisms have been proposed. The α-helical CAP, temporin-1CEa, exhibits cytotoxicity towards all of 12 tested human carcinoma cell lines in a concentration-dependent manner, yet no significant cytotoxicity to normal human umbilical vein smooth muscle cells at concentrations that showed potent antitumor activity (Wang et al., 2011). The basis for selectivity is thought to be the presence of phosphatidylserine, which is exposed on non-apoptotic tumor cells including malignant metastatic cells and primary cell cultures. Susceptibility of metastatic cells suggests that CAPs may be useful in treating metastatic as well as primary cancers (Riedl et al., 2011).

In addition to direct killing of cancer cells, CAPs can affect T-cell dependent tumor regulation. Recently, intratumoral administration of a lactoferricin derivative into lymphomas established in mice resulted in tumor necrosis, infiltration of inflammatory cells, and regression of tumors. Transfer of spleen cells from treated mice provided long-term, specific T cell-dependent immunity against the lymphoma, suggesting therapeutic vaccination against cancer using CAPs may be possible (Berge et al., 2010). In contrast, LL-37 was able to

induce apoptosis of T regulatory cells, thus inhibiting their immune suppressor activity and thereby enhancing the anti-tumor response (Mader et al., 2011).

Pleurocidins selectively killed human leukemia cells at low concentrations (<32 μg/mL) (Morash et al., 2011) as well as multiple breast cancer cell lines (Hilchie et al., 2011) by a predominantly membranolytic mechanism. Disruption of the cell membrane also augmented the activity of the chemotherapeutic cisplatin, presumably by enhancing access to the nucleus. Furthermore, when administered intratumorally into breast cancer xenografts in mice, pleurocidin inhibited tumor growth and induced tumor necrosis, while not causing observable adverse effects on the mice (Hilchie et al., 2011). These advantageous properties indicate that pleurocidins should be pursued as novel anticancer agents.

### 5.6. Immunomodulatory

CAPs show multiple activities associated with modulation of the immune system (Jenssen and Hancock, 2010; Yeung et al., 2011) and have been postulated to represent an evolutionary link bridging innate and adaptive immune responses (Selsted and Ouellette, 2005). CAPs are directly chemotactic for immune cells and also stimulate chemokine and cytokine secretion (Lai and Gallo, 2009). Some CAPs are able to boost protein antigens and vaccines that have low immunogenicity by inducing proinflammatory cytokines such as TNF, IFN and IL-1β (Kindrachuk et al., 2009; Mutwiri et al., 2007; Tavano et al., 2011), suggesting that they would be good adjuvants (Huang et al., 2011; Li et al., 2008). CAPs play a critical role in wound healing (Steinstraesser et al., 2008), promoting keratinocyte migration (Aung et al., 2011a), re-epithelialization (Hirsch et al., 2009), deposition of extracellular matrix (Oono et al., 2002), and angiogenesis (Koczulla et al., 2003). Human β-defensins and LL-37 are able to stimulate mast cells, and recently the neuroendocrine CAP catestatin has been reported to induce migration and degranulation of mast cells, release of lipid mediators and production of cytokines and chemokines (Aung et al., 2011b). CAPs also show promise in counteracting sepsis, a major cause of morbidity and mortality in hospitalized patients. For example, intraperitoneal injection of LPS-sensitized mice with S-thanatin reduced serum endotoxin and TNF-α levels, resulting in 100% survival (Wu et al., 2011).

Pleurocidin has a proinflammatory effect in fish cells *in vitro*, inducing expression of IL-1β and COX-2 (Chiou et al., 2006). In addition, pleurocidin variant NRC-08, which has no antibacterial activity, induced the expression of the co-stimulatory marker CD40 as well as IL-12p40, TNF, and TGF from mouse bone marrow-derived immature dendritic cells (Phillips, Lee & Douglas, pers. comm.). The potential immunomodulating effects of this particular pleurocidin variant, which promotes the maturation of DCs, suggest that it could be further explored as a candidate adjuvant for a Th1 response to vaccine antigens.

Pleurocidins activate human mast cells to release granule contents such as histamine and proteases (Kulka, per. comm.) both of which are important in allergic inflammation and tissue hometostasis. Mast cells are critical regulators of the tissue microenvironment capable of responding to many different stimuli including allergens and releasing a huge variety of pro-inflammatory and immunomodulatory mediators. Interestingly, pleurocidin activation of mast cells is unique from that of allergens. Allergens bind to and crosslink surface high

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affinity immunoglobulin E receptors (FcεRI), which activates increases in intracellular calcium and initiates several signaling pathways. Whereas FcεRI activation results in degranulation, arachidonic acid metabolite release and pro-inflammatory mediator production, pleurocidin activation of FPRL1 initiates degranulation and production of relatively small amounts of chemokines. Furthermore, whereas FcεRI engagement does not activate mast cell chemotaxis, pleurocidins modify adhesion via the fibronectin receptor (CD29) and promote mast cell migration. This suggests that pleurocidins activate selective signaling pathways in mast cells and may be useful tools in targeted mast cell-dependent therapy.

### 5.7. Cytotoxic

#### *5.7.1. Hemolysis and host cell lysis*

Although most CAPs are not hemolytic or cytolytic at the concentrations used for killing microbes or cancer cells, some do exhibit that characteristic, melittin being the most notorious (Tosteson et al., 1985). Magainin also forms pores in human cell membranes and enters the cell within minutes, accumulating in the nucleus and mitochondrion (Imura et al., 2008). Bovine myeloid antimicrobial peptides BMAP-27 and BMAP-28 at 10 times minimal inhibitory concentration (MIC) are toxic towards human erythrocytes and polymorphonuclear cells and induce apoptosis in transformed cell lines and *in vitro*-activated lymphocytes at concentrations near MIC (Risso et al., 1998). A recent high-throughput method to assess cytotoxicity of melittin and structural variants differing in hydrophobicity, amphipathicity and helicity, showed it to be more sensitive than standard tests such as erythrocyte lysis and MTT assay and holds promise for identifying CAP variants with non-hemolytic properties (Walsh et al., 2011).

Hemolysis assays of 26 variants of pleurocidin showed that only one, a histidine-rich variant NRC-19, showed any ability to lyse red blood cells up to a concentration of 128 μg/mL, well above the concentrations required to kill bacterial and cancer cells (Morash et al., 2011). The selectivity of these peptides indicates that they would be excellent candidates as antibacterial and anti-cancer agents.

#### *5.7.2. Sepsis*

While many CAPs can reduce sepsis, some actually exacerbate the problem. This is because they are so effective at killing bacterial cells that they cause the release of endotoxin from lysed cells, eliciting an excessive inflammatory response (Risso et al., 1998; Steinstraesser et al., 2003).

Preliminary studies using a mouse cytokine array showed that pleurocidin variants NRC-03 and NRC-08 are able to repress LPS-induced TNF-$\alpha$ and IL-10 secretion from mouse RAW264.7 macrophages, indicating that they may be considered for the control of sepsis (Carroll, Patrzykat & Douglas, pers. comm.).

## 6. Isolation and discovery of cationic antimicrobial peptides

### 6.1. Traditional biochemical purification

Standard biochemical techniques for isolation of small peptides have been used to isolate CAPs from tissues, skin secretions and other biological fluids. Briefly, tissue lysates or fluids are diluted in 0.1% trifluoroacetic acid (TFA) and extracted under ice-cold acid conditions. After centrifugation, the acidic supernatant is loaded on a C18 column and the peptide eluted with increasing acetonitrile (5-80%) in 0.05% TFA. Those fractions with antimicrobial activity are pooled and further purified by reverse-phase HPLC with increasing acetonitrile (5-55%) in 0.05% TFA. The material in absorbance peaks is then pooled, dried under vacuum and reconstituted in MilliQ water for mass and sequence analysis using mass spectrometry and automated Edman degradation.

Pleurocidin was originally isolated from homogenates of the skin and mucus secretions of winter flounder using C18 chromatography followed by size fractionation on Sephadex G-50 (Cole et al., 1997). Biologically active fractions were pooled and subjected to strong cation exchange HPLC and then reversed phase HPLC.

### 6.2.Genomics approaches to antimicrobial peptide discovery

Once even a partial amino acid sequence of a CAP is known, genomic strategies can be employed to identify and isolate DNA sequences encoding CAPs. This can involve screening of cDNA and genomic libraries using oligonucleotide probes or amplification of CAP-encoding sequences from genomic or cDNA by PCR (Iwamuro and Kobayashi, 2010). CAP-encoding sequences can also be identified in genomic and EST sequencing databases by similarity searching using bioinformatics search engines such as BLAST (Browne et al., 2011; Fernandes et al., 2010). Full-length cDNA sequences can then be obtained by 5′- and 3′-RACE. The identification of 28 new human and 43 new mouse β-defensin genes was achieved using a computational search tool to find conserved motifs in draft genome sequences (Schutte et al., 2002).

Pleurocidin was originally cloned from cDNA and genomic libraries using degenerate oligonucleotide probes (Cole et al., 2000; Douglas et al., 2001) or PCR amplicons encoding individual pleurocidin variants (Douglas et al., 2003). Additional pleurocidin variants were amplified by PCR from cDNA of different flatfish species using primers corresponding to the conserved amino-terminal signal peptide and carboxy-terminal anionic propiece (Patrzykat et al., 2003). At least 30 variants have now been cloned (Figure 3).

### 6.3. *In silico* discovery and rational peptide design

Various *in silico* methods have been used to rationally design CAPs and predict their biological activity (Hadley and Hancock, 2010). These include the use of artificial neural networks, support vector machines and quantitative structure activity relationship (QSAR) modeling (Fjell et al., 2011; Torrent et al., 2011). Combinatorial synthesis and high throughput peptide synthesis using SPOT technology (Hilpert et al., 2007) together with a QSAR-based artificial

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neural network system (Fjell et al., 2009) have also been used to design active synthetic CAPs. This approach, coupled with high-throughput screening assays using peptide arrays, is invaluable for inexpensively generating large numbers of potential candidates for clinical assessment (Cherkasov et al., 2009).

## 7. Production of cationic antimicrobial peptides

### 7.1. Peptide synthesis

Isolation of CAPs from the native producer is not feasible on the scale necessary for investigating therapeutic value and pharmaceutical potential. Therefore, CAPs are usually synthesized by standard solid-phase methods using 9-fluorenyl-methoxycarbonyl (F-moc) protecting groups, cleaved from the resin with 95% trifluoroacetic acid, purified by reverse-phase high performance liquid chromatography using an acetonitrile gradient, and the mass is verified by mass spectrometry. Although synthesis of linear $\alpha$-helical peptides is fairly routine, good yields of CAPs that require cysteine oxidation to form the correct disulphide bonding pattern are rarely obtained (Tay et al., 2011). Modifications such as carboxy-terminal amidation, biotinylation, fluorescent labeling, incorporation of D-amino acids, acylation, etc. are available for synthetic peptides.

### 7.2. Fermentation

#### *7.2.1. Bacteria*

Production of CAPs in bacteria using recombinant technology, by definition, is a challenge as they inhibit or kill the host bacteria and bacterial proteases can degrade them. Recombinant technology, particularly of tagged fusion peptides, has been used successfully to produce high yields of pure CAP at relatively low cost (Ingham and Moore, 2007). Factors to be considered include optimization of promoter sequence and codon usage from the source organism to bacterial host, signal peptide, tag, fusion partner, ease and cost of cleavage, and cost of subsequent purification steps. A recent approach involving secretion of the tagged SUMO-CAP hybrid into the medium followed by removal of the mature CAP by sumoase protease has been particularly successful in large-scale production of several CAPs (Bommarius et al., 2010; Li et al., 2009). In general, production of $\alpha$-helical CAPs without disulfide bonds and easily cleavable from their fusion partner has proved to be the most straightforward and cost-effective. A recent report of expression of human $\beta$-defensin 28 in *E. coli* is encouraging, however (Tay et al., 2011). By using a construct with both a His-tag and maltose binding protein and including the site for cleavage by TeV, an inexpensive and efficient protease for releasing the mature CAP, large-scale production of correctly folded defensin was achieved.

Pleurocidin has been successfully expressed as a PurF fusion peptide in inclusion bodies, generating milligram quantities of pure peptide (Bryksa et al., 2006). By incorporating

$(^{15}NH_4)_2$-$SO_4$ as the sole nitrogen source in the growth medium, structural elucidation of the uniformly $^{15}$N-labelled peptide by NMR was greatly facilitated (Syvitski et al., 2005).

*7.2.2. Fungi*

Fungi such as *Saccharomyces cerevisiae* and *Pichia pastoris* represent alternative systems for recombinant expression of CAPs. *P. pastoris* is a methylotrophic yeast that is capable of producing 10-100 times more recombinant protein than *S. cerevisiae.* However, successful production of small proteins less than 10 kDa in *P. pastoris* can be problematic due to low expression, RNA instability or translational blocks. Low amounts (~1 mg/100 mL) of recombinant cecropin were produced in *P. pastoris* (Jin et al., 2006), and recently, high-level production (>200 mg/L) of active recombinant hPAB-β (Chen et al., 2011) and plectasin (Zhang et al., 2011) was achieved in this system.

Production of pleurocidin was attempted using this system; however, no recombinant peptide was recovered, despite correct integration of the pleurocidin sequence into the yeast genome and normal transcription (Burrowes et al., 2005).

*7.2.3. Animal cells*

Production of CAPs in animal cells has the advantage of accurate processing of the recombinant peptide and addition of post-translational modifications. However, there are few reports of CAP production using this approach. The insect cell/baculovirus system has been used to produce recombinant human β-defensins (Feng et al., 2005) and biologically active hepcidin was successfully expressed, processed and secreted in human embryonic kidney cells (Wallace et al., 2006). The complete pleurocidin pre-pro-peptide gene was cloned into a carp cell line under the control of the carp β-actin promoter. The precursor peptide was expressed continuously for over two years and the mature peptide was secreted into the medium (Brocal et al., 2006).

### 7.3. Transgenic organisms

Early attempts were made to express CAPs in the milk of transgenic mice (Yarus et al., 1996), tissues of catfish (Dunham et al., 2002), and leaves of tobacco (Yevtushenko et al., 2005) and rice (Imamura et al., 2010); however, while the recombinant CAPs were able to confer pathogen resistance to the host organism, these systems are not designed for large-scale production of CAPs for human applications.

## 8. Modifications to enhance biological activity and stability

### 8.1. Amino acid substitutions, additions and deletions

Modification of the amino acid sequence of CAPs to change the charge, hydrophobicity and bulkiness can have dramatic effects on their ability to interact with membranes and exert

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their biological activity. Amidation of the carboxy terminus usually results in enhanced activity by increasing the net positive charge but has a negligible effect on protease susceptibility. On the other hand, N-terminal acetylation significantly increases resistance to aminopeptidases but decreases antimicrobial activity (Nguyen et al., 2010). In general, high hydrophobicity confers hemolytic and cytotoxic properties on CAPs (McPhee and Hancock, 2005). The effect of amino acid substitutions on antimicrobial vs hemolytic activity has been comprehensively evaluated using a series of rationally designed CAPs of 20 amino acids that differ in charge, polar angle, hydrophobicity and hydrophobic moment (Chou et al., 2008). Increasing the His content of clavanin resulted in an acid-activated CAP that showed enhanced activity against the caries-producing pathogen, *S. mutans*, at low pH (Li et al., 2010). Substitution of Lys by His in a 15-mer CAP resulted in a peptide that was cationic only in the acidic microenvironment found in tumors, and exhibited enhanced anti-tumor activity and reduced systemic toxicity (Makovitzki et al., 2009). Addition of hydrophobic amino acids to the termini of CAPs can also increase potency, although reduced solubility and increased production cost present important drawbacks (Hadley and Hancock, 2010). Recent reports of a truncated frog gaegurin (Won et al., 2011b) and the naturally short bee anoplin (Won et al., 2011a) CAPs emphasize the enhanced antimicrobial effects of such modifications on bacteria and model membranes.

Naturally occurring pleurocidins have variable amino acid sequences that confer different activities against microbes and cancer cells (Morash et al., 2011; Patrzykat et al., 2003). Alignment of the amino acid sequences of the mature peptides revealed conserved positions that could be of importance in bioactivity. For example, the highly conserved Gly13 residue in the middle hinge region is important for $\alpha$-helicity (Lim et al., 2004b) and the hydrophobic amino acids in the N-terminal region are more crucial for antifungal activity than those in the C-terminal region (Lee and Lee, 2010). Replacement of Ser14 by His improved the activity of a targeted pleurocidin towards *S. mutans* at low pH, which is often found in the oral cavity (Mai et al., 2011).

### 8.2. Non-natural amino acids and enantiomers

Replacement of L-amino acids by their D counterparts generally does not diminish biological activity but does confer protease resistance on the peptide (Lee and Lee, 2008; Park et al., 2010). Retro-inversion, in which amino acid stereochemistry is changed as well as peptide bond direction yielding isomers with similar side chain topology to the native peptide, has generally met with limited success although partially modified retro-inverso peptides show more promise (Fischer, 2003). Incorporation of non-natural amino acid analogs (Knappe et al., 2010; Marsh et al., 2009) is also effective. Recently, a series of CAPs containing Tic-Oic dipeptide analogues was developed to combat 11 potential bio-terrorism and drug-resistant strains of bacteria (Venugopal, 2010). These CAPs were metabolically stable, potent, and showed selectivity for bacterial relative to host cell membranes.

Replacement of L-Lys and L-Arg residues with D-Lys and D-Arg in pleurocidin conferred resistance to digestion by trypsin but also abolished activity, presumably because the $\alpha$-helical structure was disrupted by the inclusion of just a subset of amino acids (Douglas, un-

pub.). In support of this, an all D-amino acid analog of pleurocidin showed proteolytic resistance and double the antifungal (Jung et al., 2007) and antibacterial (Lee and Lee, 2008) potency. Similarly, an all D-amino acid analog of pleurocidin NRC-03, showed improved activity against breast cancer cells compared to the natural L-form peptide (Hilchie, Hoskin, unpub).

### 8.3. Peptidomimetics

Peptidomimetics such as β-peptides and peptoids have been designed that maintain the amphiphilic structure and antimicrobial activity and are resistant to protease degradation; these abiotic structures exhibit *in vivo* stability and enhanced bioavailability (Rotem and Mor, 2009; Scott et al., 2008). Synthetic mimics of antimicrobial peptides (SMAMPs) that adopt amphiphilic secondary structures and possess potent and selective antimicrobial activity have been inexpensively synthesized from small synthetic oligomers (Lienkamp et al., 2008; Scott et al., 2008). Another novel technique is hydrocarbon stapling (Sa'adedin and Bradshaw, 2010) in which an $\alpha$-helical peptide is chemically modified to generate a relatively protease resistant, cell-permeable peptide that binds its target with increased binding affinity.

### 8.4. Acylation

Addition of fatty acid chains to the termini of CAPs can increase their antibacterial activity or endow them with antifungal activity. Linear oligomers consisting of alternating uncharged and cationic Lys residues displayed varying degrees of antibacterial, antifungal and hemolytic activity when they were N-acylated, depending on the length of the acyl group and the different degrees of oligomerization that were induced (Shai et al., 2006). Addition of fatty acids of increasing lengths to magainin increased the extent of oligomerization of the resulting lipopeptide, and concurrently the antifungal activity.

### 8.5. Cyclisation

Cyclisation by, for example, disulfide bridge formation or head-to-tail backbone cyclization, results in a more constrained peptide structure that is less susceptible to protease degradation (Nguyen et al., 2010). Cyclisation of two cationic hexapeptides, including the active portion of lactoferricin, was found to be highly effective for both serum stability and antimicrobial activity. Interestingly, disulfide cyclization resulted in more active peptides while backbone cyclization resulted in more proteolytically stable peptides. The modifications did not result in hemolytic activity, thereby making them attractive therapeutic candidates. Dendrimers of CAPs have enhanced ability to permeabilize membranes and are stabler than monomeric forms (Pieters et al., 2009).

### 8.6. Targeted and hybrid peptides

One of the drawbacks of antibiotic use is the deleterious effect broad spectrum antibiotics have on the normal microflora, which often allows opportunistic pathogens such as *C. albicans* and *S. aureus* to overgrow after treatment. Selectively targeted antimicrobial peptides (STAMPs) can overcome this problem and can selectively kill the target species while leav-

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ing the benign commensals largely unaffected. This effect was first demonstrated using a STAMP consisting of a competence stimulating peptide (CSP) moiety targeting the caries-causing pathogen *Streptococcus mutans* attached to a killing moiety derived from novispirin G10 (Eckert et al., 2006). Incorporation of such a STAMP into an oral rinse resulted in reduction in salivary *S. mutans*, plaque, lactic acid and enamel demineralization, suggesting further clinical evaluation is warranted (Sullivan et al., 2011). Targeting of tumor cells has also been achieved and addition of the tumor-homing peptide bombesin to the amino terminus of magainin 2 resulted in tenfold enhanced killing *in vitro* and decrease in the size of tumor xenografts in mice (Liu et al., 2011).

We have used this approach to combine the minimal targeting portion of CSP with the killing domain of pleurocidin variant NRC-04 to treat *S. mutans* in both planktonic and biofilm conditions. The hybrid peptide was selectively active against *S. mutans* in the presence of saliva and physiological or higher salt concentration and was non-hemolytic (Mai et al., 2011). Furthermore, activity was augmented by a preventative dose of 1 mM NaF, a compound commonly used in oral care. The development of such targeted peptides paves the way for their use as a probiotic treatment to prevent dental caries.

## 9. Advantages and limitations of cationic antimicrobial peptides

### 9.1. Bioactivity

CAPs exert rapid, broad spectrum, bactericidal activity at micromolar concentrations and are valuable weapons in the fight against antibiotic-resistant microbes e.g. MRSA. In addition, targeted CAPs may be used as probiotics to eradicate pathogenic bacteria while leaving normal flora unaffected. Their ability to kill fungi, viruses and parasites is also beneficial clinically. Many CAPs neutralize endotoxin and can be used to counteract sepsis or to enhance host defenses through immunomodulatory effects. Increased innate clearance of microbes results in less bacterial debris (van der Does et al., 2010) and ensuing inflammation (Andra et al., 2006). Their application both in killing tumor cells and in enhancing host anti-tumor responses is gaining momentum (Yeung et al., 2011).

### 9.2. Resistance

CAPs have a low propensity to induce microbial resistance, mainly due to their multiple targets and mechanisms of action. CAPs are usually expressed in high concentrations only at sites of infection; continuous exposure of microbes, which often leads to resistance, is therefore minimized. In addition, cocktails of several different CAPs are often produced simultaneously, leading to increased microbial killing.

Despite this, some reports on the emergence of resistance have appeared. These include reduction in bacterial cell surface negative charge, secretion of exoproteases and induction of transporters (see Nizet, 2006; Peschel and Sahl, 2006), all of which require significant expen-

diture of metabolic energy by the microbe. In most cases, the resistance that arises is very low compared to conventional antibiotics.

In Gram negative organisms such as *Salmonella* and *Pseudomonas*, the PhoP-PhoQ two component sensor system has been shown to control resistance to aminoglycosides, polymyxin B, and cationic antimicrobial peptides (Macfarlane et al., 1999; Macfarlane et al., 2000; Groisman, 2001). In *Pseudomonas*, CAPs and the polymyxins are capable of inducing the pmrA-pmr genes and the putative LPS modification operon, thus increasing resistance to these agents (McPhee et al., 2003). The homologous basR-basS system of *E. coli* is also induced by exposure to sublethal concentrations of the proline-rich CAP, Bac7(1-35), suggesting that it may also mediate resistance (Tomasinsig et al., 2004). Resistance to Gram positive organisms such as *Staphylococcus* is mediated by the Aps three component sensor system, and interestingly, some CAPs are inducers of this system (Li et al., 2007). Recently, additional resistance genes in other bacteria such as *Clostridium difficile* (McBride and Sonenshein, 2011) and *Vibrio* (Shen et al., 2010) have been discovered, emphasizing the importance of this phenomenon.

### 9.3. Cost

Solid phase peptide synthesis is expensive and companies seeking regulatory approval for peptides in their pipelines require suppliers that adhere to good manufacturing practice (GMP) regulations, which also adds to the cost. However, there have been many advances in synthetic chemistry that have significantly reduced the cost of not only peptides but also non-peptide mimics and peptoids, and the lower operating costs of suppliers in Asia has resulted in more affordable peptides (Eckert, 2011).

### 9.4. Stability

Although CAPs represent a promising class of therapeutics, they have several *in vivo* drawbacks such as salt inactivation, protease degradation, and poor bioavailability. As described above, a number of approaches can be used to mitigate these limitations. Serum stability can be overcome by cyclisation or incorporation of amino acid analogs and some CAPs, particularly those such as pleurocidin that originated from marine sources, are active at physiological salt concentrations (Mai et al., 2011; Patrzykat et al., 2003).

### 9.5. Side effects

Some CAPs exhibit *in vivo* toxicity and for others there are unknown toxicity profiles (see section 5.7). Nephrotoxicity has been associated with polymixin cyclic peptides (Mogi and Kita, 2009). Since some CAPs can stimulate growth factor receptors, induction of tumorigenesis must be considered. Similarly, excessive release of histamine from mast cells must be avoided to minimize adverse reactions.

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## 10.Clinical use and commercialization

### 10.1. Companies

A number of CAPs and synthetic CAP analogs such as pexiganan™ (magainin derivative), omiganan™ (indolicidin derivative), novispirin™ (cathelicidin derivative) and iseganan™ (protegrin-1 derivative) are now in commercial development. Several recent reviews describe the companies, their products and their progression through clinical trials (Eckert, 2011; Kindrachuk and Napper, 2010; Yeung et al., 2011; Zhang and Falla, 2010). Some candidates show great promise in the treatment of such disorders as HIV-associated oral candidiasis, acne, wound infections, diabetic foot ulcers, and oral biofilms. However, the inability to demonstrate advantage over existing therapeutics has resulted in failure of a number of CAPs at Phase III clinical trials. As appropriate formulations, bioassays and endpoints are established, the probability that these promising products will receive regulatory approval will improve.

### 10.2. Formulations

Because of systemic toxicity and bioavailability issues as well as the diverse and complex mechanisms of action, the majority of CAPs have been developed as topical formulations e.g. for diabetic foot ulcers (Lipsky et al., 2008) and skin infections (Falla and Zhang, 2010 ). Depending on the application, a number of different ways of formulating CAPs can be envisioned (Eckert, 2011). CAPs have been incorporated into lens preservation and artificial tear solutions (Huang et al., 2005) and aerosols (Lange et al., 2001). Inclusion of CAPs in coatings (Kazemzadeh-Narbat et al., 2010), polymers (Gao et al., 2011), hydrogels (Roy and Das, 2008), liposomes (Lange et al., 2001), micro- and nanoparticles (Bi et al., 2011; Garlapati et al., 2011) and even chewing gum (Faraj et al., 2007) also represent promising formulations. Long-term activity has been achieved by covalent immobilization of gramicidin A on functionalized gold surfaces and resulted in inhibition of Gram-positive and Gram-negative bacteria and the yeast *Candida albicans* for up to 6 months as well as delayed development of bacterial biofilm for 24 hours (Yala et al., 2011). Surface modifications by CAPs will be important in preventing colonization of medical devices such as catheters and bioimplants.

CAPs can also be used in combination therapies with each other or with approved antibiotics. For example, cecropin B enhanced the activities of beta lactams in rat septic shock models (Ghiselli et al., 2004). Cecropin A and magainin 2 administered together showed *in vitro* synergy against *S. aureus* in a mouse sepsis model (Cirioni et al., 2006). Magainin 2 administered with vancomycin showed the highest efficacy in this model.

Pleurocidin showed synergistic activity with inducible histone peptides and lysozyme in a salmonid *in vivo* infection model (Patrzykat et al., 2001). Studies with *S. mutans* showed that the efficacy of a targeted pleurocidin was increased in the presence of EDTA and sodium fluoride, two commonly used components of oral rinses (Mai et al., 2011).

### 10.3. Targeted delivery

CAPs with cell-penetrating properties are under development as vectors for translocation of bioactive cargos with inherently poor membrane-crossing abilities into eukaryotic cells (Splith and Neundorf, 2011). Delivery of CAP-modified liposomes containing therapeutics to bacteria has also been reported, e. g. bacteria-targeted delivery of photodynamic antimicrobial chemotherapy to improve efficiency against MRSA and *P. aeruguinosa* in local infections (Yang et al., 2011).

## 11.Conclusions

CAPs show exciting promise as novel therapeutic agents, particularly in the fight against antibiotic-resistant bacteria and cancer and as immunostimulants. However, translation to clinical use has been hampered by concerns over stability, cost, systemic administration, known toxicity, and unknown long-term toxicity. The applications that show the most promise are those involving topical applications, particularly in combination with established antibiotics. Deeper understanding of the varied mechanisms of action of these diverse peptides and the production of cost-effective, stable, and highly selective CAPs will aid in bringing these molecules closer to the clinic.

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Section 3

# Bioactive Ingredients in Nutrition

Chapter 6

# Anticancer Properties of Phytochemicals Present in Medicinal Plants of North America

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Additional information is available at the end of the chapter

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## 1. Introduction

Cancer is one of the most severe health problems in both developing and developed countries, worldwide. Among the most common (lung, stomach, colorectal, liver, breast) types of cancers, lung cancer has continued to be the most common cancer diagnosed in men and breast cancer is the most common cancer diagnosed in women. An estimated 12.7 million people were diagnosed with cancer across the world in 2008, and 7.6 million people died from the cancer during the same year [1]. Lung cancer, breast cancer, colorectal cancer and stomach cancer accounted for two-fifths of the total cases of cancers diagnosed worldwide [1]. More than 70% of all cancer deaths occurred in low- and middle-income countries. Deaths due to cancer are projected to continuously increase and it has been estimated that there will be 11.5 million deaths in the year 2030 [1] and 27 million new cancer cases and 17.5 million cancer deaths are projected to occur in the world by 2050 [2]. According to Canadian cancer statistics, issued by the Canadian Cancer Society, it is estimated that 186,400 new cases of cancer (excluding 81,300 non-melanoma skin cancers) and 75,700 deaths from cancer will occur in Canada in 2012 [1]. The lowest number of incidences and mortality rate is recorded in British Columbia. Both incidence and mortality rates are higher in Atlantic Canada and Quebec [3].

More than 30% of cancers are caused by modifiable behavioral and environmental risk factors, including tobacco and alcohol use, dietary factors, insufficient regular consumption of fruit and vegetable, overweight and obesity, physical inactivity, chronic infections from *Helicobacter pylori*, hepatitis B virus (HBV), hepatitis C virus (HCV) and some types of human papilloma virus (HPV), environmental and occupational risks including exposure to ionizing and non-ionizing radiation [4].

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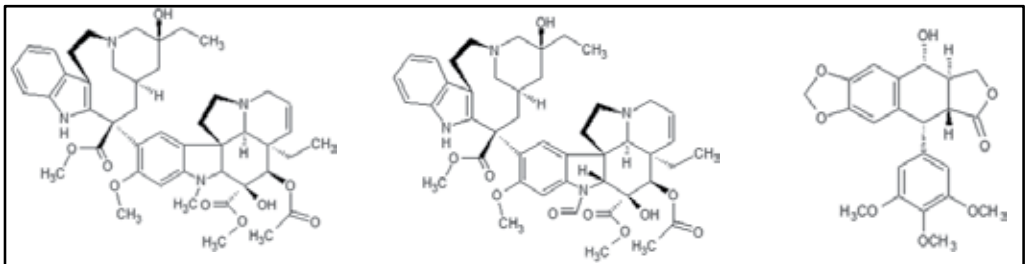
Conventional treatment of cancer includes interventions such as psychosocial support, surgery, radiotherapy and chemotherapy [4]. Currently, the most commonly use cancer chemotherapy includes mainly alkylating agents, antimetabolites, antitumor antibiotics, platinum analogs and natural anticancer agents. However, due to the increasing rate of mortality associated with cancer and adverse or toxic side effects of cancer chemotherapy and radiation therapy, discovery of new anticancer agents derived from nature, especially plants, is currently under investigation. Screening of medicinal plants as a source of anticancer agents was started in the 1950s, with the discovery and development of vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins [5] (Figure 01). The cool temperate climate of North America supports the growth of an enormous number of plant species which are important sources of unique phytochemicals having anticancer properties (Table 01). In this chapter, selected medicinal plants grown in the cool climate of North America (mainly Canada and USA) are discussed. The major bioactive phytochemicals and their mechanisms of action are also reviewed.



**(a) Vinblastine** **(b) Vincristine** **(c) Podophyllotoxins**

| *Metabolite* | *Natural source* | | *Mode of action* | *Ref* |
|---|---|---|---|---|
| | *Botanical name* | *Family* | | |
| Vinblastine | *Catharanthus roseus* (Madagascar periwinkle) | Apocenacea | Inhibition of microtubule assembly | 6, 7 |
| Vincristine | *Catharanthus roseus* (Madagascar periwinkle) | Apocenacea | Inhibition of microtubule assembly | 6, 7 |
| Podophyllotoxins | *Podophyllum hexandrum* (Himalayan mayapple) | Berberidaceae | Inhibition of microtubule assembly | 8, 9 |

(a) Vinblastine – [dimethyl (2β,3β,4β,5α,12β,19α)- 15-[(5S,9S)- 5-ethyl- 5-hydroxy- 9-(methoxycarbonyl)- 1,4,5,6,7,8,9,10-octahydro- 2H- 3,7-methanoazacycloundecino[5,4-b]indol- 9-yl] - 3-hydroxy- 16-methoxy- 1-methyl- 6,7-didehydroaspidospermidine- 3,4-dicarboxylate] (b) Vincristin – [(3aR,3a1R,4R,5S,5aR,10bR)-methyl 4-acetoxy-3a-ethyl-9-((5S,7S,9S)-5-ethyl-5-hydroxy-9-(methoxycarbonyl)-2,4,5,6,7,8,9,10-octahydro-1H-3,7-methano[1]azacycloundecino[5,4-b]indol-9-yl)-6-formyl-5-hydroxy-8-methoxy-3a,3a1,4,5,5a,6,11,12-octahydro-1H-indolizino[8,1-cd]carbazole-5-carboxylate] (c) Podophyllotoxin – [(10*R*,11*R*,15*R*,16*R*)-16-hydroxy-10-(3,4,5-trimethoxyphenyl)-4,6,13-trioxatetracyclohexadeca-1,3(7),8-trien-12-one]

**Figure 1.** Some selected currently used phytochemical-based anticancer agents

http://dx.doi.org/10.5772/55859

| Plant | Family | Parts used | Major bioactive compounds | Growing regions | Ref |
|---|---|---|---|---|---|
| Achyranthes aspera (Devil's Horsewhip) | Amaranthaceae | Leaf | Triterpenoid saponins | USA | 14 |
| Annona glabra (Pond apple) | Annonaceae | Leaf and fruit | Acetogenins | USA | 15 |
| Aralia nudicaulis (Wild sarsaparilla) | Araliacea | Whole plant | Steroids, sarsasapogenin, smilagenin, sitosterol, stigmasterol, pollinastrenol, glycosides, saponins, sarsasaponin parillin, smilasaponin, smilacin, sarsaparilloside, and sitosterol glucoside | Mainly Canada | 16 |
| Aster brachyactis (Rayless aster) | Asteraceae | Aerial parts | Not known | North America | 17 |
| Carduus nutans (Nodding plumeless thistle) | Asteraceae | Aerial parts | Linalool derivatives, aliphatic acids, diacids, aromatic acids, and phenols | North America | 18, 19 |
| Erythronium americanum (Adder's tongue) | Liliaceae | Whole plant | Alpha-methylenebutyrolactone | North America | 20, 21 |
| Eupatorium cannabinum (Bonesets) | Asteraceae | Whole plant | Sesquiterpene lactone, pyrrolizidine alkaloid, and flavonoid | North America | 20, 21,19, 22 |
| Foeniculum vulgare (Wild pepper fennel) | Apiaceae | Seed | α-pinene, anisic aldehyde, cineole, fecchone, limonene, and myrcene | North America | 23 |
| Hydrastis canadensis (Orange root) | Ranunculaceae | Whole plant | Isoquinoline alkaloids (hydrastine, berberine, berberastine, candaline), resin and lactone | Canada, USA | 20, 21 |
| Hypericum perforatum (St. John's wort) | Clusiaceae | Flower | Hypericin and hyperforin | USA, Canada (British Columbia) | 24 |
| Lactuca sativa (Garden lettuce) | Asteraceae | Leaf | Sesquiterpene lactone | USA, Canada | 25 |
| Lantana camara (Wild sage) | Verbenaceae | Whole plant | Alkaloids (camerine, isocamerine, micranine, lantanine, lantadene), phenols, flavonoids, tannins, saponins, and phytosterols | USA | 26, 27, 28 |
| Larrea tridentate (Creosote bush) | Zygophyllaceae | Whole plant | Resins and lignans | Southwestern USA | 18, 29, 30 |
| Linum usitatissimum (Common Flax) | Linaceae | Seed | Enterodiol, enterolactone, lignans, and omega-3 fatty acids | Canada, USA | 31, 32 |

| Plant | Family | Parts used | Major bioactive compounds | Growing regions | Ref |
|---|---|---|---|---|---|
| Olea europrae (Olive) | Oleaceae | Leaf and oil | Oleuropein, hydroxytyrosol, hydroxytyrosol acetate, luteolin-7-O-glucoside, luteolin-4′-O-glucoside and luteolin, oleic acid and polyphenol | USA | 33, 34, 35, 36, 37 |
| Panax quinquefolius (North American Ginseng) | Araliaceae | Root, Leaf | Ginsenosides and saponins | Eastern North America | 20, 21 |
| Plantago lanceolata (Ribwort plantain) | Plantaginaceae | Aerial parts | Phenolics and flavonoids | Canada, USA | 38 |
| Podophyllum peltatum (Mayapple) | Berberidaceae | Rhizome | Podophyllotoxins | Eastern North America | 39 |
| Polygonatum multiflorum (Tuber fleece flower) | Polygonaceae | Whole plant | Saponin and flavonoid and vitamin A | USA | 20, 21, 40 |
| Pyrus malus (Apple) | Rosaceae | Bark and fruit | Quercetin, catechin, flavonoid, coumaric and gallic acids, phloridzin and procyanidin | North America | 21 |
| Rhodiola rosea (Golden root) | Crassulaceae | Rhizome | Monoterpene alcohols and their glycosides, cyanogenic glycosides, aryl glycosides, phenylethanoids, phenylpropanoids and their glycosides, flavonoids, flavonlignans, proanthocyanidins and gallic acid derivatives | Eastern Canada | 41, 42 |
| Saponaria vaccaria (Cowherb) | Caryophyllaceae | Seed | Flavonoids, cyclopeptides, and bisdesmosidic saponins | Western Canada | 43 |
| Silybum marianum (Milk thistle) | Asteraceae | Dried fruit, seed | Silymarin-polyphenoic flavolignans (silybin, isosilybin, silychristin, silydianin and taxifoline) | Canada, USA | 44, 45 |
| Sonchus arvensis (Perennial sow-thistle) | Asteraceae | Whole plant | Alkanes, n-alkenes, n-aldehydes and n-alcohols, shikimate metabolites, carotenoid-derived compounds, terpenoids, steroids, and phenols | Canada | 46, 47 |
| Tanacetum vulgare (Tansy) | Asteraceae | Aerial parts | Monoterpenes, sesqueterpenes, and oxygenated sesqueterpenes | Canada, USA | 48 |
| Taraxacum officinale (Dandelions) | Asteraceae | Root and leaf | Sesquiterpene lactones, triterpenoids, sterols, tannins, alkaloids, inulin, caffeic acid, and flavonoids | North America | 49 |

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| Plant | Family | Parts used | Major bioactive compounds | Growing regions | Ref |
|---|---|---|---|---|---|
| Taxus brevifolia (Pacific yew tree) | Taxaceae | Bark | Taxol (diterpene) | Pacific Northwest | 50 |
| Thuja occidentalis (White cedar) | Cupressaceae | Whole plant | Flavonoid, tannin, and volatile oil | Northeastern USA, Eastern Canada | 13, 51 |
| Xanthium strumarium (Cocklebur) | Asteraceae | Fruit | Sesquiterpene lactones (Xanthatin and Xanthinosin) | Canada | 17 |

**Table 1.** Medicinal plants with potential anticancer properties grown in North America

## 2. Pathophysiology of cancer

Cancer is a population of abnormal cells which divide without control, with the ability to invade other tissues. Cancer and some of the other chronic diseases share common pathogenesis mechanisms, such as DNA damage, oxidative stress, and chronic inflammation [10]. It is understood that both environmental factors and chemical carcinogens play a key role in the initiation and progression of cancer. Among the major environmental factors are asbestos, polluted air near industrial emission sources, exposure to secondary tobacco smoke, indoor air pollution such as radon, drinking water containing arsenic, chlorination by-products, and other pollutants [11]. Chemicals with carcinogenic activity can be classified as DNA reactive (e.g.: nitrogen mustards, chlorambucil, epoxides, aliphatic halides, aromatic amines), epigenetic (e.g.: chlordane, pentachlorophenol, hormones, cyclosporin, purine analogs), dichlorodiphenyltrichloroethane, phenobarbital, minerals (e.g.: asbestos), metals (e.g.: arsenic, beryllium, cadmium) and unclassified carcinogens (e.g.: acrylamide, acrylonitrile, dioxane) [12]. DNA-reactive carcinogens act in the target cells of tissue(s) of their carcinogenicity to form DNA adducts that are the basis for neoplastic transformation [12]. Epigenetic carcinogens lack chemical reactivity and hence, do not form DNA adducts. These carcinogens are produced in the target cells of tissue(s) of their carcinogenicity. Effects of epigenetic carcinogens indirectly lead to neoplastic transformation or enhance the development of tumors from cryptogenically transformed cells [12].

Carcinogenesis is a multi-step process consisting of tumor initiation, promotion and progression [13]. Cancer initiation can be blocked by activating protective mechanisms, either in the extracellular environment or intracellular environment by modifying trans-membrane transport, modulating metabolism, blocking reactive oxygen and nitrogen species, maintaining DNA structure, modulating DNA metabolism and repair, and controlling gene expression [10]. Tumor promotion is the second stage of carcinogenesis and is followed by tumor progression. Both stages can be suppressed by inhibiting genotoxic effects, favoring antioxidant and anti-inflammatory activity, inhibiting proteases and cell proliferation, inducing cell

differentiation, modulating apoptosis and signal transduction pathways and protecting intercellular communications [10]. In addition, tumor progression can also be inhibited by affecting the hormonal status and the immune system in various ways and by inhibiting tumor angiogenesis [10].

## 3. In vitro anticancer activity of phytochemicals and extracts of medicinal plants

Cultured cancer-derived cell lines with comparison to normal healthy cell lines are commonly used to assess the anticancer properties of isolated phytochemicals and extracts of medicinal plants (Table 2). The anticancer properties of ethanolic extract of leaves, pulp and seeds of, *Annona glabra* (L.), commonly known as pond apple, were shown, using human drug-sensitive leukemia (CEM) and its multidrug-resistant-derived (CEM/VLB) cell lines [52]. The most potent anticancer activity was shown in the seed extract of *A. glabra* [52]. Both dried rhizome hexane extract and dried fruit hexane extract, partitioned from total methanol extract, of *Aralia nudicaulis* (L.) caused death of cancer cell lines such as human colon cancer cell (WiDr), human leukemia cell (Molt) and human cervix cancer cell (HeLa) at a lower concentration, than that of required for the death of normal cells [53]. Eupatoriopicrin, a sesquiterpene lactone isolated from *Eupatorium cannabinum* (L.) (Bonesets), indicated anticancer properties on FIO 26 (fibrosarcoma) cells with an $IC_{50}$=1.5 μg/ml [22]. Methanolic extracts of *Hypericum perforatum* (L.) (St. Johns wort) possessed strong antiproliferative activity in the human prostate cell line (PC-3) and the major constituents, hyperforin and hypericin, synergistically contributed to the reduction of the PC-3 cells proliferation [24]. Maslinic acid, (Figure 2(a)) a triterpene from *Olea europaea* (L.) (Olive), has shown to be significantly inhibitory in cell proliferation of the human colorectal adenocarcinoma cell line (HT29) in a dose dependent manner [33]. The major components in the extract were identified to be oleuropein, hydroxytyrosol, hydroxytyrosol acetate, luteolin-7-*O*-glucoside, luteolin-4′-*O*-glucoside and luteolin [34] (Figure 2). All these phytochemicals inhibited the proliferation of cancer and endothelial cells with $IC_{50}$, at the low micromolar range [37]. Methanolic leaf extract of *Plantago lanceolata* (L.) (Ribwort plantain) inhibited the growth of three different cell lines; human renal adenocarcinoma (TK-10), the human breast adenocarcinoma (MCF-7) and the human melanoma (UACC-62) cell lines and the MCF-7 was totally inhibited [54]. Further, the ethanolic extract of *P. lanceolata* (L.), produced by maceration with ethanol : water, showed significant antiproliferative activity on cervix epithelioid carcinoma (HeLa), breast adenocarcinoma (MCF-7), colon adenocarcinoma (HT-29) and human fetal lung carcinoma (MRC-5) [38]. Several chemical constituents (Figure 3) from *Silybum marianum* (Milkthistle) have been isolated and their cytotoxic and anticancer potential has been investigated, *in vitro*, using both cancer and normal healthy cell lines. Silymarin, isolated from seeds of *S. marianum*, is a mixture of series of flavolignans, major constituents being: silybin A and B, (also known as silibinin), isosilybin A and B, silychristin, and silydianin [55, 56].

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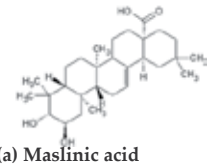
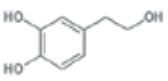
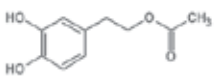


(a) Maslinic acid

| $IC_{50}$ (μg/ml) | MOA | Ref |
|---|---|---|
| 28.8 | Bcl-2 inhibition Bax activation | 33 |

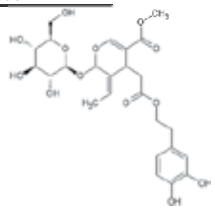


(b) Hydroxytyrosol

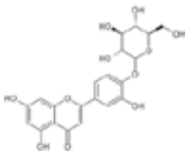


(c) Hydroxytyrosol acetate

| Metabolite | $IC_{50}$ (μg/ml) | | | | | MOA | Ref |
|---|---|---|---|---|---|---|---|
| | BBCE | T24 | MCF-7 | HL60 | HT29 | | |
| Hydroxytyrosol | 2.4 | 12.4 | 24.9 | 50.0 | 750.0 | Cell cycle arrest at $G_0/G_1$ | 34,35,53 |
| Hydroxytyrosol acetate | 9.3 | 23.3 | 28.7 | - | - | | 34 |

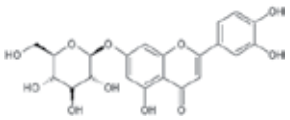


(d) Oleuropein



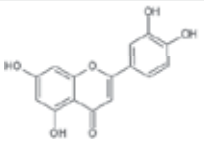
(e) Luteolin-4′-*O*-glucoside

| $IC_{50}$(μg/ml) | | | MOA | Ref |
|---|---|---|---|---|
| BBCE | T24 | MCF-7 | | |
| 2.6 | 7.6 | 12.0 | Induce $G_1$ cell cycle arrest | 35, 37 |



(f) Luteolin-7-*O*-glucoside

| $IC_{50}$ (μg/ml) | | | MOA | Ref |
|---|---|---|---|---|
| BBCE | T24 | MCF-7 | | |
| 30.9 | 31.7 | 10.8 | - | 37 |



(g) Luteolin

| $IC_{50}$ (μg/ml) | | | MOA | Ref |
|---|---|---|---|---|
| BBCE | T24 | MCF-7 | | |
| 3.9 | 11.4 | 40.9 | Inhibition of Topoisomerase - 1 | 37, 55 |

| $IC_{50}$ (μg/ml) | | | MOA | Ref |
|---|---|---|---|---|
| BBCE | T24 | MCF-7 | | |
| 9.7 | 28.2 | 9.1 | Inhibition of Topoisomerase - 1 | 37, 55 |

**$IC_{50}$:** Concentration which inhibited 50% of cell proliferation; **MOA:** Mode of Action; **BBCE:** Bovine Brain Capillary Endothelial cells; **T24:** Human Urinary Bladder Carcinoma cells; **MCF-7:** Human Breast Adenocarcinoma cells; **HL60:** Human Promyelocytic Leukaemia cells; **HT29:** Colon Adenocarcinoma cells

(a) Maslinic acid - [(2a, 3b)-2,3-dihydroxyolean-12-en-28-oic acid]: (b) Oleuropein – [(4S,5E,6S)-4-[2-[2-(3,4-dihydroxyphenyl)ethoxy]-2-oxoethyl]- 5-ethylidene-6-[[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)- 2-tetrahydropyranyl]oxy]-4H-pyran-3-carboxylic acid, methyl ester] : (c) Hydroxytyrosol – [4-(2-Hydroxyethyl)-1,2-benzenediol]: (d) Hydroxytyrosol acetate – [2-(3,4-dihydroxy)Phenyl ethyl acetate;4-[2-(Acetyloxy)ethyl]-1,2-benzenediol]: (e) Luteolin-7-O-glucoside - [2-(3,4-dihydroxyphenyl)-5-hydroxy-4-oxo-4H-chromen-7-yl beta-D-glucopyranoside; 4H-1-benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-7-(beta-D-glucopyranosyloxy)-5-hydroxy-; 2-(3,4-Dihydroxy-phenyl)-5-hydroxy-7-((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy)-chromen-4-one] (f) Luteolin-4′-O-glucoside – [3',5,7-Trihydroxy-4'-(β-D-glucopyranosyloxy)flavone;2-(4-β-D-Glucopyranosyloxy-3-hydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one;2-[3-Hydroxy-4-(β-D-glucopyranosyloxy)phenyl]-5,7-dihydroxy-4H-1-benzopyran-4-one] (g) Luteolin - [2-(3,4-Dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone]

**Figure 2.** Major bio-active compounds present in *Olea europaea* (a,b,c,d,e,f and g) and *Plantago lanceolata* (f and g)

Silybin possessed a dose-dependent growth inhibitory effect on parental ovarian cancer cells (OVCA 433), drug-resistant ovarian cancer cells (A2780 WT) and doxorubicin (DOX)-resistant breast cancer cells (MCF-7) [55]. Both L and D diastereoisomers of silybin inhibited A2780 WT cell growth at low $IC_{50}$ reported with L-diastereoisomer [55]. Furthermore, silybin potentiated the effect of Cisplatin (CDDP, a platinum analog; cis-diamminedichloroplatinum [II]) in inhibiting A2780 WT and CDDP-resistant cell growth. Cisplatin is an inorganic metal complex which acts as an alkylating agent [57]. Similar results recorded with doxorubicin (DOX) on MCF-7 DOX-resistant cells when silybin associated with doxorubicin. Doxorubicin ((7S,9S)-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12-dione) is an anthracycline antibiotic isolated from *Streptomyces peucetius* var *caesius* [57]. The effect of silybin-CDDP and silybin-DOX combinations resulted in a synergistic action, as assessed by the Berembaum isobole method [55]. Silymarin demonstrated to have marked inhibition of cell proliferation with almost 50% inhibition in a time dependent manner on the human breast cancer cell line (MDA-MB 468), at 25 μg/mI concentration, after five days of treatment. Its potential anticancer activity was dose dependent and showed a complete inhibition of cancer cells at 50 and 75 μg/mI concentrations at the beginning of Day 2 of exposure [56]. Induction of apoptotic cell death of human prostate cancer (DU145) treated with silibinin is shown to be due to activation of caspase 9 and caspase 3 enzymes [58].

## 4. Evidence from animal studies for anticancer activity of North American medicinal plants

Anticancer and antiproliferative potential of some North American medicinal plants has also been studied in animal studies (Table 3). *In vivo* antitumor activities of *Achyranthes aspera* (L.) (Devil's Horsewhip) on athymic mice, with are subcutaneous xenograft, harboring human pancreatic tumor were demonstrated, using the leaf extract. The leaf extract significantly reduced both tumor weight and volume in mice treated with leaf extract intraperitonealy [14]. Intravenous administration of 40 mg/kg body weight eupatoriopicrin, a sesquiterpene lactone present in *E. cannabinum*, significantly delayed the growth of tumor in Lewis lung tumour-bearing syngeneic C57B1 female mice [22]. A 70% inhibition of tumor growth in PC-3 cells, orthopedically implanted into the dorsal prostatic lobe in athymic nude mice, was observed, upon their receiving 15 mg/kg intraperitoneal *H. perforatum* methanolic extract [24]. Lantadene A is a pentacyclic triterpenoid, isolated from the weed, *Lantana camara* (L) [59]. Feeding of female Swiss albino mice (LACCA) with a dose of 50 mg/kg body weight of Lantadene A twice a week for 20 weeks, showed potential chemopreventive activity. This chemopreventive activity could be linked to the expression of transcriptional factors and a significant decrease in the mRNA expression of AP-1 and c-fos), NF-kB (p-65) and p53 was observed in Lantadene A treated mice skin tumors [59]. Silibinin decreased tumor multiplicity by 71% ($P < 0.01$) in wild type mice, but did not show any such considerable effect in $iNOS^{-/-}$ mice upon oral feeding of 742 mg/kg body weight silibinin for 5 days per week for 18 weeks [60]. Lesser effects of silibinin in $iNOS^{-/-}$ mice suggested that most of its chemopreventive and angiopreventive effects were through its inhibition of iNOS expression in lung tumors [60]. Treatment of a purified diet, containing 0.5% to 1.0% silibinin on a transgenic adenocarcinoma of are mouse prostate (TRAMP) model,

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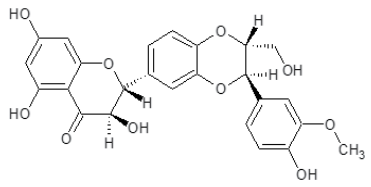
decreased the weight of the tumor in both the prostate and seminal vesicle, when compared with control mice [61].

Treatment of silibinin significantly decreased tumor angiogenesis and proliferation and also there was increased apoptosis in prostate tumor tissue samples in the TRAMP model [61]. The protective effect of silibinin was also demonstrated in mouse skin with tumors caused by acute and chronic UVB-exposure-caused mitogenic and survival signaling and associated biological responses [62]. Mice were treated with silibinin, either topically (9 mg in 200 ml acetone/mouse) or orally (1% of diet), and both administrations strongly inhibited UVB-induced skin tumorigenesis in a long-term study [62]. Thymine dimers are formed in DNA, immediately after UVB irradiation, and are considered as an early and important biomarker for UVB induced DNA damage [62]. A noticeable, 71% reduction ($P < 0.001$) of thymine positive cells was obtained in the mice treated with 1% (w/w) silibinin before the UVB exposure, compared with the UVB alone group [62]. Oral feeding of 200 mg/kg of silibinin for 5 days per week, for 33 days, significantly inhibited human non–small-cell lung cancer cells (NSCLC A549) tumor xenograft growth in nude mice, in a time-dependent manner [63]. This accounted for 58% ($P = 0.003$) reduction in tumor weight per mouse and intraperitoneal administration of 4 mg/kg doxorubicin, once a week for four weeks, showed 61% ($P = 0.005$) reduction in tumor weight. However, interestingly, in silibinin-doxorubicin combination, 76% ($P = 0.002$, versus control) decrease in tumor weight per mouse was observed, that which was significantly different from either treatment alone, showing enhanced efficacy [63].

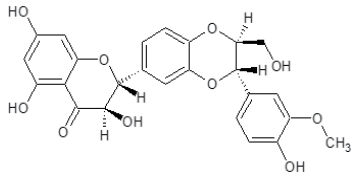
## 5. Mode of action of selected phytochemicals of North American medicinal plants

Apoptosis (programmed cell death) is the principal mechanism through which unwanted or damaged cells are safely eliminated from the body. This programmed cell death is mediated via either an extrinsic apoptotic pathway or an intrinsic apoptotic pathway [65]. These two apoptosis signaling pathways differ in the origin of their apoptosis signal, but converge upon a common pathway [66].

The extrinsic pathway is initiated by the stimulation of the cell surface 'death receptor' due to the binding of death ligand and the intrinsic pathway is also known as the mitochondrial pathway in which an intracellular apoptotic signal initiates the process [68]. Various natural extracts, obtained from medicinal plants grown in North America, have been found to induce apoptosis pathways at different levels (Figure 4 and Table 04). Leaf extract of *Achyranthes aspera* activated caspase-3 and induced caspase-3 mRNA in tumor cells. It also decreased Akt-1 transcription, as well its phosphorylation. Suppression of pAkt-1 and a corresponding activation of caspase 3 by the leaf extract, induced apoptosis of tumor cells [14]. It was also found that maslinic acid, isolated from *O. europaea,* inhibited considerably the expression of Bcl-2 (B-cell lymphoma 2), whilst increasing that of Bax. Maslinic acid stimulated the release of mitochondrial cytochrome-c and activated caspase-9 and caspase-3 [33]. These results showed the activation of the mitochondrial apoptotic pathway, in response to the treatment

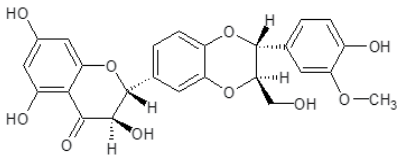


(a) Silybin A

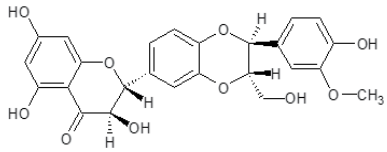


(b) Silybin B

| Metabolite | $IC_{50}$(μg/ml) | | | MOA | Ref |
|---|---|---|---|---|---|
| | MCF7 | OVCA 433 | A2780 | | |
| Silybin | 4.8-24 | 14(L) | 25 | Induce G1 arrest | 56 |
| | | 20(D) | | Inhibits STAT3 activation | 58 |
| | | | | Up-regulates DNA-Protein Kinase-dependent p53 Activation | 67 |

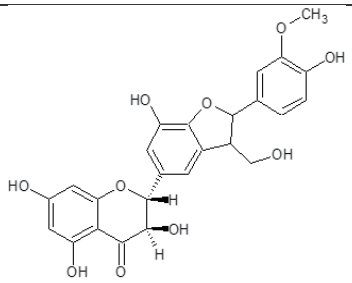


(c) Isosilybin A

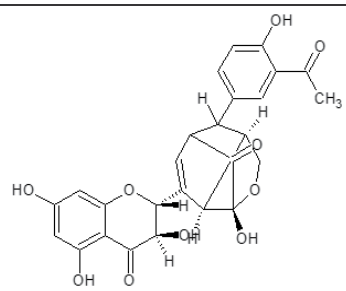


(d) Isosilybin B

| Metabolite | $IC_{50}$(μg/ml)[a] / Apoptosis induction[b] | | | | MOA | Ref |
|---|---|---|---|---|---|---|
| | 22Rv1 | LAPC4 | LNCaP | DU 145 | | |
| Isosilybin A | 90-180[b] | 90-180[b] | 90-180[b] | 32[a] | Activates Akt–NF-kB–AR axis and increases p53 level | 68, 69 |
| Isosilybin B | - | - | - | 20.5[a] | Increases p53 level | 69 |



(e) Silychristin



(f) Silydianin

**$IC_{50}$:** Concentration which inhibited 50% of cell proliferation; **MOA:** Mode of Action; **MCF-7:** Doxorubicin-resistant breast cancer cells; **OVCA 433:** Parental ovarian cancer cells; **A2780:** Drug-resistant ovarian cancer cells; **22Rv1, LAPC4, LNCaP, DU 145:** Human prostate cancer cells

(a) Silybin A – [(2*R*,3*R*)-3,5,7-trihydroxy-2-[(2*R*,3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydrobenzo[*b*] [1,4]dioxin-6-yl]chroman-4-one]: (b) Silybin B – [(2*R*,3*R*)-3,5,7-trihydroxy-2-[(2*S*,3*S*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydrobenzo[*b*] [1,4]dioxin-6-yl]chroman-4-one]: (c) Isosilybin A –[(2*R*,3*R*)-3,5,7-Trihydroxy-2-[(2*R*,3*R*)-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-2,3-dihydrobenzo[1,4]dioxin-6-yl]-4-chromanone]: (d) (c) Isosilybin B –[(2*R*,3*R*)-3,5,7-Trihydroxy-2-[(2*R*,3*R*)-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-2,3-dihydrobenzo[1,4]dioxin-6-yl]-4-chromanone]: (e) Silychristin – [(2R,3R)-3,5,7-trihydroxy-2-[(2R, 3S)-7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-2,3-dihydro-1-benzofuran-5-yl]-2,3-dihydrochromen-4-one]:

**Figure 3.** Major bio-active flavonolignans present in *Silybum marianum*

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of HT29 colon-cancer cells with maslinic acid. The major flavonoid present in *P. lanceolata,* luteolin-7-*O*-β-glucoside, as well as aglycon luteolin, acted as potent poisons for DNA topoisomerase I on cancer cell lines [54]. Silibinin (major bioactive component from *S. marianum*) markedly activated the DNA-PK-p53 pathway for apoptosis, in response to UVB-induced DNA damage [69]. DNA-PK pull-down assay showed that silibinin pre-treatment strongly increased binding of DNA protein kinase with p53 [69].

| Plant | Extraction solvent and concentration | Type of cancer cell line | $IC_{50}$ or growth reduction | Key findings | Ref. |
|---|---|---|---|---|---|
| Annona glabra (Pond apple) | Ethanolic extract of lyophilized plant material in powder form | Human drug-sensitive leukemia (CEM) and its multidrug-resistant-derived (CEM/VLB) cell lines | Leaf-1.00 (CEM/VLB) Pulp-0.65 (CEM/VLB), Seed-0.10 (CEM/VLB) and Leaf-0.30 (CEM), Pulp-0.35 (CEM), Seed-0.07 (CEM) µg/ml | $IC_{50}$ values were significantly lower than Adriamycin (Doxorubicin) (CEM=0.13 µg/ml and CEM/VLB=13.4 µg/ml) indicates its potential for cancer drug discovery programs | 52 |
| Aralia nudicaulis (Wild sarsaparilla) | Methanol extracts of rhizome, stem, leaf and fruit were further partitioned with hexane, ethyl acetate, butanol and water | WiDr (colon), Molt (leukemia), HeLa (cervix) | Hexane rhizome extract 30.1 (WiDr), 7.0 (Molt), 33.33 (HeLa) µg/ml | The concentrations of Rhizome hexane and Fruit hexane required for normal cell death was significantly higher than those required for the cancer cells | 53 |
| Eupatorium cannabinum (Bonesets) | Eupatoriopicrin concentrations of 0.1 - 50 µg/ml in 96% ethanol | FIO 26 (Fibrosarcoma) | 1.5 µg/ml | Possess significant anticancer activity | 22 |
| Foeniculum vulgare (Wild pepper fennel) | Not specified | Breast (MCF-7), liver (HepG2) | - | Remarkable anticancer potential | 23 |
| Hypericum perforatum (St. John's wort) | Methanolic extract | Prostate (PC-3) | 0.42 mg/ml | Extract components synergistically contribute to the | 24 |

| Plant | Extraction solvent and concentration | Type of cancer cell line | $IC_{50}$ or growth reduction | Key findings | Ref. |
|---|---|---|---|---|---|
| | | | | reduction of the PC-3 cells proliferation | |
| Linum Usitatissimum (Common flax) | Ethanol extract | Breast (MCF-7, MDA-MB-231) | Growth reduction of 15.8% in MCF-7 and 11.4% in MDA-MB-231 | Significantly reduced cell growth and induced apoptotic cell death | 31 |
| Olea europrae (Olive) | maslinic acid 0–100 µg/mL | Colon (HT29) | 28.8 µg/ml | Cell proliferation inhibition in a dose-dependent manner and causes apoptotic death | 33 |
| | Aqueous extract and methanol artificial mixture | Breast (MCF-7), Human urinary bladder (T-24), Bovine brain (BBCE) | 72 (MCF-7), 100 (T-24), and 62 (BBCE) for aq. 565 (MCF-7), 135 (T-24), and 42 (BBCE) for methanol µg/ml | Antiproliferative activity of the extracts should mainly be attributed to its identified phytochemicals | 34 |
| Plantago lanceolata (Ribwort plantain) | Methanolic extract | Renal (TK-10), breast (MCF-7), melanoma (UACC-62) | "/>250 (TK-10), 47.2 (MCF-7), 50.6 (UACC-62) µg/ml | Growth of MCF-7 was totally inhibited | 54 |
| | Extracted by maceration with ethanol/water during 72 hr at room temperature | cervix epitheloid (HeLa),breast (MCF-7), colon (HT-29), fetal lung (MRC-5) | 172.3 (HeLa), 142.8 (MCF-7), 405.5 (HT-29), 551.7 (MRC-5) µg/ml | Showed significant antiproliferative activity | 38 |
| Rhodiola rosea (Golden root) | Not specified | Urinary bladder (RT4, UMUC-3, T24, 5637, J82) | 264 (RT4),100 (UMUC-3), 71 (T24), 151 (5637), 165 (J82) µg/ml | Selectively inhibit the growth of cancer cell lines with minimal effect on nonmalignant cells | 41 |
| Saponaria vaccaria (Cowherb) | 70% Methanol extract | colon (WiDr), breast (MDA-MB-231), lung (NCI-417), prostate (PC-3), | 3.8-9.4 (WiDr), 11.4-19.6 (MDA-MB-231), 12.6-18.4 | Dose-dependent growth inhibitory and selective apoptosis-inducing activity. Strong in a breast and a prostate cancer cell lines | 43 |

| Plant | Extraction solvent and concentration | Type of cancer cell line | $IC_{50}$ or growth reduction | Key findings | Ref. |
|---|---|---|---|---|---|
| | | nontumorigenic fibroblast BJ (CRL-2522) | (NCI-417) mg/ml | | |
| Silybum marianum (Milkthistle) | silybin, a flavonoid | Doxorubucin resistant breast (MCF-7), Parental ovarian (OVCA 433), Drug-resistant ovarian (A2780) | 4.8-24 µM (MCF-7), 14 & 20 µM - L & D diastereoisomers respectively (A2780) 25 µg/ml | Dose-dependent growth inhibitory effect on all three cell lines | 55 |
| | Silymarin at a dosages of 10-75 µg/ml in ethanol | Breast (MDA-MB 468) | - - | Inhibits the cell proliferation in a dose- and time dependent manner | 56 |
| | Silibinin in DMSO | Prostate (DU145) | - | Strongly inhibited activation of Stat3 and causes caspase activation and apoptotic death | 58 |
| | Isosilybin A and B | Prostate (LNCaP, 22Rv1) Prostate (DU 145) | Iso A:32 µM (DU 145) Iso B:20 µM (DU 145) | Anti-prostate cancer activity mediated via cell cycle arrest and apoptosis induction | 69 |
| Taraxacum officinale (Dandelions) | Water (lyophilized or reconstituted) | Acute T-cell leukemia (Jurkat clone E6-1), dominant-negative FADD Jurkat cells (clone I 2.1) | - | Effectively and selectively induced apoptosis in human leukemia cell lines in a dose and time dependent manner | 49 |

**Table 2.** Anti-cancer properties of phytochemicals and extracts of medicinal plants revealed from *in vitro* studies using cancer cell lines

| Plant | Preparation | Animal model used | Dosage | Key findings | Ref. |
|---|---|---|---|---|---|
| Achyranthes aspera (Devil's Horsewhip) | 5% suspension in hexane followed by extraction in acetone overnight | Athymic nude mice | 50, 100 and 200 mg/kg extract in 1 ml PBS administered IP | The tumor weight and volume was significantly reduced in the mice treated for 36 days with 50 mg/kg. | 14 |

| Plant | Preparation | Animal model used | Dosage | Key findings | Ref. |
|---|---|---|---|---|---|
| | and residue was dissolved in methanol | | | In one treated mouse tumor completely disappeared | |
| Eupatorium cannabinum (Bonesets) | Eupatoriopicrin, a sesquiterpene lactone | Syngeneic C57B1 female mice | i.v. injection of 20 or 40 mg/kg | Significantly stronger growth delay of both lung tumours and fibrosarcoma | 22 |
| Hypericum perforatum (Orange root) | Methanolic extract | Human prostatic carcinoma cell line orthotopically implanted athymic male nude mice | ip with a dose of 15 mg/kg dissolved in 1% DMSO | Inhibited tumor growth by 70% with no observed side effects | 24 |
| Lantana camara (Wild sage) | Lantadene A, pentacyclic triterpenoid | Female Swiss albino mice (LACCA) | 50 mg/kg body weight twice a week for 20 weeks | Activity could be linked to the expression of transcriptional factors | 59 |
| Silybum marianum (Milkthistle) | Silibinin | Lung - Male B6/129-Nos2tm1Lau ($iNOS^{-/-}$) and B6/129PF2 WT mice | 742 mg/kg body weight for 5 d/wk for 18 weeks | Significantly decreases urethane-induced tumor number and size in WT mice. Decreased tumor multiplicity in WT mice, but not in $iNOS^{-/-}$ mice | 60 |
| | Silibinin | Prostate - A transgenic adenocarcinoma of mouse prostate (TRAMP) model | Purified diet containing 0% and 1% (w/w) silibinin until | Decreased the weight of tumor + prostate + seminal vesicle. Significantly decreased tumor angiogenesis and proliferation and increased apoptosis also. | 61 |
| | Topically applied silibinin in acetone or oral feeding of silibinin | Skin – mouse | 9 mg in 200 ml acetone/mouse or 1% in diet | silibinin (both topical and oral) strongly inhibited UVB-induced skin tumorigenesis in long-term study | 62 |
| | Silibinin | Skin - SKH-1 hairless mouse | 1% (w/w) silibinin in diet for 2 weeks | Strong suppression of UVB-induced damage by dietary feeding of silibinin | 63 |
| | Silibinin | Athymic (BALB/c,nu/nu) male nude mice | 200 mg/kg body weight, 5 d/wk for 33 days | Significantly inhibits human NSCLC A549 tumor xenograft growth in a time dependent manner | 64 |

**Table 3.** Anti-cancer properties of medicinal plants revealed from *in vivo* studies using experimental animals

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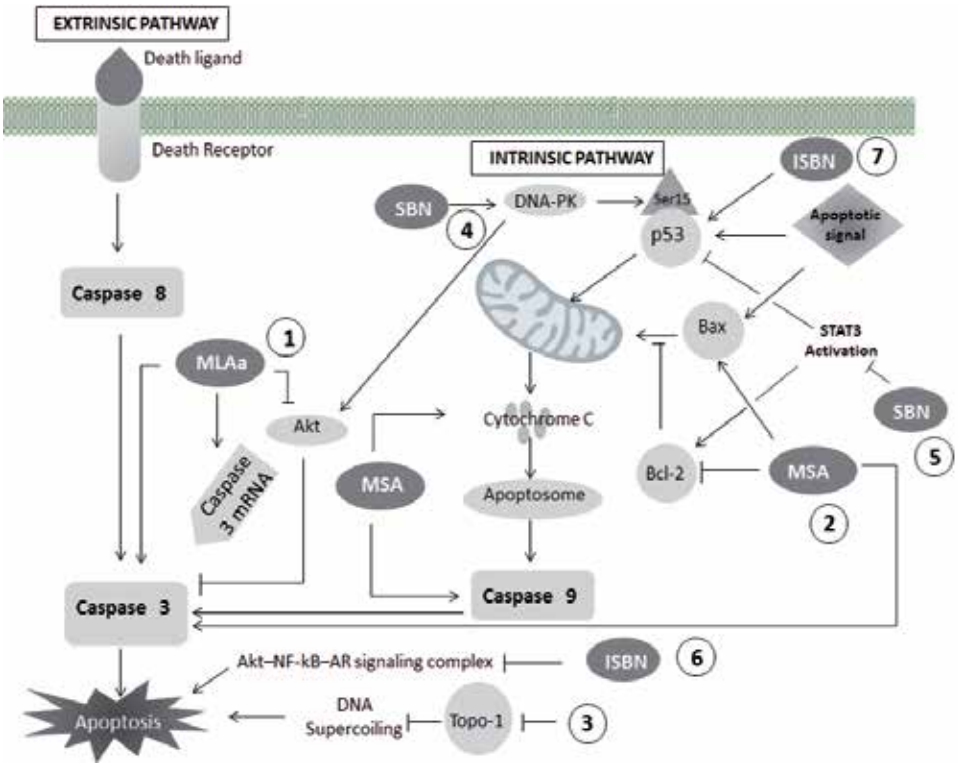


**Figure 4.** Schematic representation of current knowledge of mode of action of some selected anticancer phytochemicals in North America (in a hypothetical cancer cell).

Akt (Protein kinase B); Bcl-2 (Protein kinase B); Bax (Bcl-2–associated X protein); Topo-1 (Topoisomerase 1); p53 (tumor protein 53); Ser15 (Serine 15); NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells); AR (Androgen Receptor)

1. Methanolic leaf extract of *Achyranthes aspera* (MLAa) induces caspase -3 mRNA and suppress expression of the kinase Akt-1. Apoptosis is induced by activation of caspase-3 and inhibiting Akt-phosphorylation.
2. The mechanism of Maslinic acid (MSA) (isolated from *Olea europaea*) is regulated via Bcl-2 inhibition and Bax induction, producing mitochondrial disruption, cytochrome-c release, leading finally to the activation of caspases 9 and caspase 3.
3. Luteolin-7-*O*-β-glucoside and its aglycon, luteolin (major bio-active constituents of *Plantago lanceolata*) showed DNA topoisomerase I poison activities and Topoisomerase mediated DNA damage might be the possible mechanism which induce apoptosis.
4. Silibinin (SBN) (extracted from *Silybum marianum*) pretreatment enhance DNA-PK (DNA Protein kinase) associated kinase activity as well as the physical interaction of p53 with DNA-PK and it preferentially activates the DNA-PK-p53 pathway for apoptosis.
5. SBN inhibits active Stat3 phosphorylation, and causes caspase activation and apoptosis.

6. Isosilybin A (ISBN) (extracted from *Silybum marianum*) activates apoptotic machinery in human prostate cancer cells via targeting Akt–NF-kB–AR axis.

7. ISBN increases p53 protein levels.

| Plant | Mode of action | References |
|---|---|---|
| Achyranthes aspera (Devil's Horsewhip) | Significantly induced caspase-3 mRNA and suppressed expression of the pro survival kinase Akt-1. Apoptosis was induced by activation of caspase-3 and inhibiting Akt phosphorylation. | 14 |
| Olea europaea (Olive) | Activation of the mitochondrial apoptotic pathway | 33 |
| | Significant block of $G_1$ to S phase transition manifested by the increase of cell number in $G_0/G_1$ phase | 37 |
| Plantago lanceolata (Ribwort plantain) | The topoisomerase-mediated DNA damage seems to be a candidate mechanism, by which some flavonoids may exert their cytotoxic potential | 54 |
| Silybum marianum (Milkthistle) | Induces $G_1$ arrest in cell cycle progression | 56 |
| | Up-regulates DNA-protein kinase-dependent p53 activation to enhance UVB-induced apoptosis | 67 |
| | | 68 |
| | Activates apoptotic machinery in human prostate cancer cells via targeting Akt–NF-kB–AR axis | 58 |
| | | 69 |
| | Inhibits active Stat3 phosphorylation, and causes caspase activation | |
| | Increases total p53 levels | |
| Podophyllum peltatum (Mayapple) | Inhibition of microtubule assembly | 70 |

**Table 4.** Mode of action of anticancer activity of phytochemicals present in selected North American medicinal plants

## 6. Conclusion

Currently, natural products, especially plant secondary metabolites such as isoprenoids, phenolics and alkaloids, have been demonstrated to be the leading providers of novel anticancer agents. Thiese important groups of phytochemicals represent a vast majority of chemical groups, including alkaloids, flavonoids, flavonols, flavanols, terpenes and terpenoids, phenols, flavonolignans and steroids. Potential anticancer properties of these phytochemicals have been shown by both cell culture (*in vitro* methods) and animal (*in vivo* methods) studies. However, *in vitro* and *in vivo* findings should be strengthened by valid human clinical trial data before introducing to the medicine cabinet as natural therapeutics or drugs.

## Abbreviations

**CEM**, Human drug-sensitive leukemia cells; **CEM/VLB**, Human multidrug-resistant-derived leukemia cells; **Jurkat clone E6-1**, Acute T-cell leukemia cells; **WiDr** and **HT29**, Human colon

cancer cells; **Molt**, Human leukemia cancer cell; **FIO 26**, Human fibrosarcoma cells; **MCF-7** and **MDA-MB-231**, Human breast cancer cells; **HepG2**, Human hepatocarcinoma cells; **LNCaP**, **22Rv1**, **PC-3** and **DU145**, Human prostate cancer cells; **RT4**, **UMUC-3**, **T24**, **5637** and **J82**, Human urinary bladder cancer cells; **BBCE**, Bovine brain capillary endothelial cells, **TK-10**, Human renal cancer cells; **UACC-62**, Human melanoma cells; **HeLa**, Human cervical epithelioid cells; **MRC-5**, Fetal lung cancer cells; **NCI-417**, Human lung cancer cells; **CRL-2522**, Human nontumorigenic fibroblast cells; **OVCA 433**, Parental ovarian cancer cells; **A2780**, Drug-resistant ovarian cancer cells.

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Chapter 7

# A New Perspective on the Development of Cholesterol-Lowering Products

Sandhya V.G. Nair and Yanwen Wang

Additional information is available at the end of the chapter

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## 1. Introduction

### 1.1. Health impact of cardiovascular disease (CVD)

Cardiovascular disease (CVD) is the principal cause of death worldwide, representing nearly 30% of the annual global mortality and 10% of global health burden [1]. The current status of CVD is now on international scale; which can be considered as the commonest chronic illness in both developed and developing countries, causing the most deaths and the greatest impact on morbidity [2]. In 2006, CVD was the leading cause of death for Canadians, representing 30% of all deaths [3]. A total number of 1.3 million Canadians are diagnosed having heart disease accounting for 5% among those above 12 years and 23% at 75 years and older. The increased rate of obesity and diabetes combined with further aging of the population will likely lead to an increase in the number of people with CVD in the future. This will compromise the health of Canadians, put a strain on the health care system, and have a significant economic impact on Canada [4]. Similarly, over the past five decades the prevalence of CVD has steadily increased in economically developing countries [5]. These countries will account for 76% of an estimated 25 million death due to CVD in 2020 [6]. On an international basis, by 2020 CVD will reach nearly epidemic proportions and become the cause of more deaths, disability and economic loss than any others group of diseases. The number of fatalities by CVD projected to increase to over 20 million a year by 2020 and over 24 million a year by 2030 [7]. Apparently, understanding the aetiology of CVD and accordingly develop preventive and therapeutic approaches to address this health threat continues to be critically important in the next decades although significant achievements have been made in the past decades.

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## 2. Risk factors of CVD

The aetiology of CVD is multifactorial, complex and still not completely understood. However, there is now a general agreement that elevated total cholesterol, LDL-cholesterol and triacylglycerol levels, low HDL-cholesterol concentrations, smoking, high blood pressure, hyperglycemia and diabetes are all risk factors of CVD. Physical inactivity, obesity, diet and low socio-economic status are thought to be predisposing risk factors which work, at least in part, as promoter on other risk factors. These factors predispose to develop syndrome X or metabolic syndrome, which is characterised by obesity, hypertension, dyslipoproteinaemia, and disturbed glucose tolerance [8]. Some other factors such as elevated prothrombotic factors, markers of inflammation, elevated homocysteine, elevated lipoprotein (a) and some psychological factors show associations with CVD [6]. Nevertheless, the aetiology of CVD is far from clear [9], and most factors are unmodifiable while others can be modified through the change of diet habits and lifestyles.

### 2.1. Unmodifiable risk factors

#### *2.1.1. Ageing*

Cardiovascular diseases, such as atherosclerosis, coronary heart disease and resultant heart failure reach epidemic proportions among older persons. Aging leads to arterial stiffening that results in aortic dilation and wall thickening along with increased collagen level. Potential age-associated changes in the tissue levels or responses to growth factors, catecholamines, angiotensin II, endothelin tumor growth factors β ( TGF β) or fibroblast growth factors influences myocardial or vascular cells. Deficit in myocardial beta adregenic receptor signalling, decline in omega-3 polyunsaturated fatty acids and increased reactive oxygen species generation occur with aging, which enhances $Ca^{2+}$ influx [10]. The clinical manifestations and prognosis of CVD and resultant heart failure worsen with ageing. Over 83 percent of people who die of coronary heart disease are 65 or older. Thus, age, per se, is the major risk factor for CVD [10].

#### *2.1.2. Gender*

There is a marked difference in CVD risk between sexes [11]. Incidence of CVD was approximately 3-fold and mortality about 5-fold greater in men thanin women [12]. Among middle-aged people,incidence of coronary heart disease is 2 to 5 times more in men than in women, and this sexratio varies between populations. The role of major risk factors such as lipid abnormalities, high blood pressure, smoking, obesity and diabetes in the development of CVD is well established amongmen (Jousilahti, Vartiainen et al. 1999). In women, significnat shift in their physiological function and heath profiles occurs during and the postmenopausal. When entering into middle ages, women tend to have lower LDL cholesterol and higher HDL cholesterol values than men of similar age. Following menopause, total cholesterol, LDL cholesterol and triglycerides levels increases while HDL levels remain unchanged or decrease

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slightly. These alterations in lipid values are thought to be related in part to loss of protective effects of estrogen [13].

*2.1.3. Heredity (including race)*

The prevalence of CVD considerably varies by race/ ethnicity. Genetic predispositions are a result of gene mutations, which alter the biological function expressed by the original genes (polymorphisms) and increases an individual risk for the disease. Several polymorphism and linkage markers have been identified as being correlated to the onset of CVD. For example, M235T polymorphism of the angiotensinogen gene is linked to hypertension and later to the development of CVD [14]. In many developed countries, racial and ethnic minorities bear disproportionate burden of heart disease. Significant differences in socioeconomic status and conventional heart disease risk factors exist among racial/ethnic groups in the United States, Canada and United Kingdom (Ludwig, Ebbeling et al. 2002). The rate of heart disease among the racial and ethnic group in the United States vary widely, with African Americans having rates a half to three fold greater than Asians, depending on the gender [15]. It is also well documented that the prevalence of CVD is higher in several minority populations (Hispanics and African Americans) in comparison to whites. [16]. However, these differences do not fully account for the observed disparities in disease prevalence, suggesting the presence of other biological factors [17].

**2.2. Modifiable risk factors**

*2.2.1. Obesity*

Obesity is an independent risk factor for CVD. It is a chronic metabolic disorder associated with increased morbidity and mortality. Obesity may affect atherosclerosis through many risk factors such as dyslipidemia, hypertension, glucose intolerance, and increased chronic inflammatory and prothrombotic state. Obesity causes a variety of adaptations/ alterations in cardiac structure and function due to excessive adipose tissue accumulation, even in the absence of comorbidities [18]. Thus, it increases cardiac workload that leads to heart failure, coronary heart disease, sudden cardiac death, and arterial fibrillation [19]. In many cases, these events result in mortality and morbidity. By favourably modifying blood lipids, in particular LDL cholesterol, lowering blood pressure, controlling blood sugar, decreasing proinflammatory cytokines and adhesion molecules, weight loss may prevent the progression of atherosclerosis or the occurrence of acute coronary heart events in the obese high-risk populations [18].

*2.2.2. High blood cholesterol*

A high concentration of serum cholesterol is a major risk factor for coronary heart disease [20]. The relationship between abnormal plasma cholesterol fractions and increased CVD risk was described 60 years ago [21]. The excessive cholesterol, especially cholesterol transported/ carried by low density lipoproteins that contain protein apolipoprotein (apo) B100 contributes to the formation of atherosclerotic plaques in arteries. Accordingly, cholesterol that is carried

by LDL particles is called “bad cholesterol”. by contrast, HDL cholesterol which transports esterified cholesterol from the periphery to the liver is considered more cardioprotective and sometimes referred to as “good cholesterol” [22]. The ratio of LDL to HDL cholesterol is more important than LDL or HDL cholesterol concentration and has been widely used to evaluate susceptibility to the development of heart disease. For a healthy person, it is recommended to maintain the LDL/HDL ratio below 3.5.

*2.2.3. High blood pressure*

Hypertension is a highly prevalent major contributor to atherosclerotic cardiovascular disease. It accelerates atherogenesis, imparting a 2- to 3-fold coronary heart disease (CHD) and lethal sequel [23]. In most cases, hypertension results from excessive vasoconstriction of the small arterioles throughout the body, raising the diastolic pressure. Because of high peripheral resistance, the heart needs to generate more force to overcome the resistance created by the constricted arterioles and supply adequate blood to the tissues, which leads to a compensatory rise in systolic blood pressure. This excess of systolic and diastolic blood pressures causes excessive vasoconstriction. Thus, increased levels of systolic and diastolic blood pressure are associated with an increased risk of CVD events [24]. When high blood pressures co-exist with obesity, smoking, hypercholesterolemia, and/or diabetes, the risk of heart attack and stroke increases several times.

*2.2.4. Physical inactivity*

Sedentary lifestyle is associated with almost twice the risk of developing coronary heart disease compared with their active counterparts. Regular physical activity plays a crucial role in the prevention of CVD. High levels of physical activity are associatedwith substantial reductions in CVD risk, and total mortalitydecreases by 20% to 30% for the increase of every 1000 kcal/wk of energy expenditure resulting from physical activity [25]. Regular exercise has a favourable effect on many of the establishedrisk factors of CVD. Exercisepromotes weight reduction, reduce blood pressure, "bad” cholesterol (LDL level), and total cholesterol,and can raise the "good" cholesterol (HDL) [26].

*2.2.5. Diabetes mellitus*

Diabetes has long been recognized to be an independent riskfactor for CVD. Type-1 diabetes ortype-2 diabetes is at high risk for several cardiovasculardisorders: coronary heart disease, stroke, peripheral arterialdisease, cardiomyopathy, and congestive heart failure. Closely linked to type-2 diabetes are several metabolic risk factors such as hypertension, atherogenic dyslipidemia which is associated with insulin resistance that relatedto coronary heart disease. Cardiovascularcomplications are now the leading causes of illness and death in the diabetic patient. The incidence of diabetes rises with advancing age, obese and overweight persons and in the populations (race/ethnicity) who areparticularly susceptible to diabetes [27].

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*2.2.6. Tobacco smoke*

Smokingnearly doubles the risk of heart disease. Smoking acts synergisticallywith other risk factors, substantially increasing the risk ofCVD [28]. The exact toxic componentsof cigarette smoke and the mechanisms involved in smoking-relatedcardiovascular dysfunction are notclearly elucidated, but smoking increasesinflammation, thrombosis, and oxidation of LDLcholesterol. Smokers have significantly higherserum total cholesterol, LDL cholesterol and triacylglycerol levels, while having lower blood concentration of HDL cholesterol than non-smokers [29]. Cigarette smoke exposure increases oxidativestress as a potential mechanism for initiating cardiovasculardysfunction. Cigarette smoke exposure decreases the plasma activity of paraoxonase, an enzymethat protects against LDL oxidation. Smoking is also found to be an independent predictor of newcoronary lesion formation and thrombosis [30].

In spite of identification of many unmodifiable and modifiable risk factors, there are still several paradoxes in the pathogenesis of CVD that cannot be sufficiently explained; mortality from CVD is relatively low despite a high intake of saturated fatty acids [31] or a high incidence of CVD without having the expected risk indicators [32]. CVD mortality rate in urban populations is higher compared with rural populations despite a very low fat intake [33]. All these paradoxes support the assumption that some important factors in the aetiology of CVD are currently unknown. However, it has been well established that elevated blood total cholesterol, especially LDL cholesterol levels is one of the primary risk factors that contribute to the development of atherosclerosis and ultimately CVD. Therefore, in the following sections, we focus mainly on the atherosclerosis, cholesterol metabolism and homeostasis, benefits versus side effects of current cholesterol-lowering products, and our perspectives on the development of future cholesterol-lowering products.

## 3. Atherosclerosis and CVD

Atherosclerosis is a disease of the arterial wall that is characterized by cholesterol accumulation and culminates in potentially life-threatening conditions such as heart attack, stroke and angina. The build-up of cholesterol in the walls of arteries is a hallmark of atherosclerosis. However, the process starts is poorly understood. The atherogenic process starts at an early age with the deposition in blood vessel walls of lipids such as cholesterol, derived from lipoproteins circulating in the bloodstream, which leads to the formation of the characteristic 'fatty streaks'. Inflammatory white blood cells congregate at these damaged areas through their interaction with adhesion molecules expressed by cells in the endothelial layer, which lines the inside of blood vessels. This event then sets off a cascade of inflammatory process and further lipid deposition, leading eventually to full blown atherosclerosis with plaque formation in the artery wall. Atherosclerosis is thus viewed as a chronic inflammatory disease of the blood vessel wall [34], [35]. Oxidized LDL contributes to atherogenesis and is an early event of atherosclerosis. WhenLDL particles become trapped in an artery, they can undergoprogressive oxidation and be internalized by macrophages bymeans of the scavenger receptors on the surfaces of these cells. The internalization leads to the formation of lipid peroxidesand

facilitates the accumulation of cholesterol esters, resultingin the formation of foam cells. As the fatty streak progresses, smooth muscle cells (not normally present in the subendothelial space) migrate from the media to the subendothelial space where they proliferate and produce connective tissue to form a fibrous cap, which represents the second phase of atherosclerosis. Finally, complicated lesions occur, which can manifest calcification, hemorrhage, ulceration and thrombosis [35, 36]. All these changes and events lead to the hardening and thickening of artery wall, reducing or blocking blood flow. Atherosclerosis is a silent and asymptomatic disease until complications arise with thrombosis and occurrence of clinical symptoms [37]. The clinical manifestation of atherosclerotic plaque formation is acute vascular occlusion due to the formation of a thrombus or clot which can lead to ischemia of vital organs, such as heart causing myocardial infarction, brain resulting in strokes and lower extremites causing peripheral artery disease. Oxidized LDL contributes to atherothrombosis by inducing endothelial cell apoptosis, and thus plaque erosion, by impairing the anticoagulant balance in endothelium, stimulating tissue factor production by smooth muscle cells, and inducing apoptosis in macrophages [38]. It is reasonably thinking that maintaining a healthy cholesterol or lipid profile is critically important to the health cardiovascular system.

## 4. Cholesterol homeostasis

Cholesterol, the characteristic steroid alcohol of animal tissues, performs a number of essential functions in the body. For example, cholesterol is a structural component of cell membranes and modulates cell membrane fluidity; cholesterol is a precursor of bile acids, steroid hormones and vitamin D. It is therefore of critical importance that the cells of the body be assured a continuous supply of cholesterol. To meet this need, a complex series of transport, biosynthetic and regulatory mechanism has evolved. The liver plays a central role in the regulation of the body's cholesterol homeostasis. For example, cholesterol enters the liver's cholesterol pool from a number of sources including dietary cholesterol, as well as cholesterol synthesized de novo by extra hepatic tissues as well as by the liver itself. Cholesterol is eliminated from the liver as unmodified cholesterol in the bile or it can be converted to bile acids that are secreted in bile into the intestinal lumen.

Cholesterol, similar with other lipids (triacylglycerols and phospholipids) do not circulate as independent molecules but are carried by specific apolipoproteins to form macromolecular complexes, called lipoproteins. Plasma lipoproteins keep their component lipids soluble during circulation and provide an efficient mechanism for transporting their lipid contents to (and from) the tissues. Four major groups of lipoproteins have been identified and they are important physiologically and in clinical diagnosis. They are chylomicrons, very low density lipoproteins (VLDL or pre β lipoprotein), LDL (β lipoprotein) and HDL ($\alpha$ lipoprotein). They differ in their relative composition of cholesterol, triacylglyceroles, phospholipids and apoproteins.

Chylomicrons, the largest and least dense lipoproteins, predominately transport triacylglycerols to adipose tissue and muscle, but also deliver the absorbed dietary and billiary

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cholesterol to the liver. Once most of the triacylglycerols have been delivered to the adipose tissue and muscle, the remnants of the lipoprotein, including cholesterol, apo-E, and apo-B48 are then delivered to and taken up by the liver through interaction with the chylomicron remnant receptor.

VLDL is smaller and more dense than chylomicrons. VLDL contains triacylglycerols, some cholesterol and cholesteryl esters and the apoproteins; apo-B100, apo-CI, apo-CII, apo-CIII, and apoE. VLDL delivers triacylglycerols and cholesteryl esters from the liver and distribute them throughout the body. When VLDL moves into the circulating blood, it is converted first to intermediate density lipoproteins (IDL) and then into low density lipoproteins (LDL). Lipoprotein lipase serves to remove the majority of fatty acids from both the VLDL and IDL, thus increasing the density of the lipoproteins while maintaining cholesterol and cholesteryl ester concentrations. The removal of fatty acids and the loss of all apolipoproteins except apoB-100 and apo(a) results in the formation of LDL.

LDL is the primary blood carrier of cholesterol and delivers cholesterol to all tissues. LDL can be absorbed by the liver and other tissues via receptor mediated endocytosis. The LDL receptor recognizes the main apolipoprotein of LDL, apo-B100, resulting in the intake of LDL and subsequently enzymatic hydrolysis of cholesteryl ester. A positive correlation exists between the incidence of coronary atherosclerosis and the plasma concentration of LDL cholesterol.

High density lipoprotein is the smallest but most dense lipoproteins in the body. Nascent HDL or HDL contains several types of apolipoproteins including apo-AI, II & IV, apo-CI, II & III, apoD and apoE. HDL contains protein, phospholipids, cholesteryl esters, and cholesterol. HDL is produced as a protein-rich particle in the liver and intestine, and serves as a circulating source of Apo-CI & II and ApoE proteins. The HDL protein particle accumulates cholesteryl esters through the esterification of cholesterol by lecithin:cholesterol acyl-transferase (LCAT). LCAT is activated by apo-AI on HDL. HDL returns the liver where cholesterol is removed by reverse cholesterol transport, thus, serving as a scavenger of free cholesterol. Cholesteryl ester transfer protein (CETP) facilitates the transfer of cholesteryl esters from HDL to VLDL, IDL, and LDL in exchange of triacylglycerol, relieving the inhibition of LCAT activity in HDL.

## 5. Hyperlipidemia and causes

Hyperlipidemia, most common form of dyslipidemia, refers to elevation of lipoproteins and/or lipids. HDL, LDL, and VLDL vary in their atherogenicities. High levels of cholesterol particularly LDL cholesterol together with low levels ofHDL cholesterol increase the risks for developing atherosclerosis [39]. Hyperlipidemia itself usually causes no symptoms but can lead to the development of symptomatic vascular disease, including coronary artery disease and peripheral arterial disease. There are two different types of hyperlipidemia, primary and secondary hyperlipidemia:

1. Primary hyperlipidemia is generally due to genetic causes, such as a mutation in a receptor or binding protein. This type of hyperlipidemia is often linked to family history. For

instance, defects in the essential components of lipid transportation and metabolism inherited from family. Examples include familial defect in LDL receptor or apo B-100 (diminished LDL clearance and hypercholesterolemia), familial lipoprotein lipase deficiency (hypertriglyceridemia), and combination of multiple unknown defect and known familial defects (combined hyperlipidemia).

2. Secondary hyperlipidemia arises due to other underlying causes, such as sedentary lifestyle coupled with the excessive dietary intakes of saturated fat, cholesterol and trans-fats, in addition to many other disease conditions and drug uses. These factors include obesity, diabetes mellitus, hyperhomocystinemia, smoking, alcohol intake, chronic kidney disease, hypothyroidism, primary biliary cirrhosis and other cholestatic liver diseases, and drugs, such as thiazides, β-blockers, retinoids, estrogen and progesterons, and glucocorticoids.

## 6. Metabolic pathways involved in cholesterol homeostasis

### 6.1. Biosynthesis

Cholesterol levels in the body derived from *de novo* biosynthesis and diet. The majority of cholesterol utilized by healthy adults is synthesized in the liver, which accounts for about 70% of the daily cholesterol. Virtually all cells containing nucleus are capable of cholesterol synthesis, which occurs in endoplasmic reticulum and the cytosol. Biosynthesis of cholesterol generally takes place in the endoplasmic reticulum of hepatic cells and begins with acetyl-CoA, which is mainly derived from fatty acid oxidation reaction in the mitochondria. The conversion of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate by HMG-CoA reductase is the rate-limiting step of cholesterol biosynthesis and is under strict regulatory control. Thus, HMG-CoA reductase is one of important targets of cholesterol lowering drugs. The development of statin drugs is a very successful story of discovering and applying HMG-CoA reductase inhibitor to lower hypercholesterolemia.

### 6.2. Absorption

Dietary cholesterol is absorbed within the lumen of the small intestine. Bile salts produced from cholesterol in the liver interact with phospholipids to produce a biliary micelle that is transported via bile into the lumen. Dietary cholesterol in the lumen is easily incorporated into the micelles and together with the biliary cholesterol can be absorbed into the enterocytes. In the enterocytes, absorbed cholesterol is esterified by acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2), which is found in both the intestine and liver. Reducing the absorption of cholesterol of dietary ad billiary sources has become another key area in cholesterol research and product development. A typical example is plant sterols/stanols that have long found as effective inhibitors of cholesterol absorption. These molecules inhibit cholesterol absorption by competitively inhibiting with cholesterol for incorporation into micelles. Recently, inhibitors, such as ezetimibe, that block the absorption of cholesterol into the enterocytes through

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suppressing the activity of cholesterol transporters have also been used to reduce absorption of dietary cholesterol.

### 6.3. Transport

Chylomicrons deliver absorbed dietary and biliary cholesterol from the enterocytes to the liver. During the process, triacylglycerols are released with assistance of lipoprotein lipase and taken up by adipose tissues and muscle, the remnants of the lipoprotein then delivered to, and taken up by, the liver through interaction with the chylomicron remnant receptor. In the liver, absorbed cholesterol, together with synthesized cholesterol and cholesterol transported back from peripheral tissues and LDL receptor-mediated uptake go through several metabolic pathways and secreted out from different outputs. One of them is the secretion in VLDL back into the bloodstream. VLDL removes triacylglycerols and cholesteryl esters from the liver and distributes them throughout the body. Endothelial Lipoprotein lipase remove the majority of fatty acids from both the VLDL and IDL, thus increasing the cholesterol and cholesteryl ester concentrationsand apoB-100 results in LDL. LDL is the primary plasma carrier of cholesterol, which can be taken up by the liver and other tissues via receptor-mediated endocytosis. The cytoplasmic domain of LDL receptor facilitates the formation of coated pits which is the receptor-rich regions of the membrane. The ligand binding domain of the receptor recognizes apo-B100 on LDL, resulting in the formation of a clathrin-coated vesicle that buds from the inner surface of the cell membrane. ATP-dependent proton pumps lower the pH inside the vesicle resulting in dissociation of LDL from its receptor. After loss of the clathrin coat, the vesicles fuse with lysozomes, resulting in peptide and cholesteryl ester enzymatic hydrolysis. On the other hand, HDL is the small and rich in lipoproteins. The HDL protein particle accumulates cholesteryl esters by the esterification of cholesterol with lecithin:cholesterol acyl-transferase (LCAT). In the plasma, these particles undergo aseries of remodeling steps involving two HDL-associated proteins:phospholipid transfer protein (PLTP) and CETP. The primary role of PLTP is in thetransfer of surface remnants, which contain apolipoproteinsand phospholipids originating from triglyceride-rich lipoproteins,to pre-ß–HDL. PLTP has also been implicatedin mediating fusion of HDL particles to generate pre-ß–HDLand CE-rich HDL. CETP promotesboth transfer and exchange of hydrophobic lipids, CE, and triacylglycerols between lipoproteins. HDL can acquire cholesterol from cell membranes and transfer cholesteryl esters to VLDL and LDL via the transferase activity of apoD. More importantly, HDL can return to the liver where cholesterol is removed by reverse cholesterol transport, thus serving as a scavenger of free cholesterol. Scavenging activity of HDL initiates by accepting cholesterol from tissues in smaller $HDL_3$ via the ATP-binding Cassette transporter -1 (ABC-1). The cholesterol in $HDL_3$ is then esterified by LCAT, increasing the size of the particles to form the less dense $HDL_2$. The cycle is completed by the reformation of $HDL_3$ either after selective delivery of cholesteryl esters to the liver via the scavenger receptor-B1 or by the hydrolysis of $HDL_2$ phospholipid and triacylglycerol by hepatic lipase. $HDL_2$ concentration is inversely related to the incidence of coronary atherosclerosis. The enzyme cholesterol esterase controls

the hydrolysis of these stored cholesterol esters, yielding bioavailable cholesterol and fatty acids.

### 6.4. Excretion of cholesterol

About 1 g of cholesterol is eliminated from the body per day, approximately equivalent to the amount that absorbed cholesterol and synthesize cholesterol. Approximately, half is excreted in the feces after conversion to bile acids in liver, and the remainder is excreted as cholesterol. Bile acids serve to remove unwanted cholesterol from the body and to aid in lipid digestion in the intestine. 7$\alpha$-hydroxylase, the rate limiting enzyme of bile acid biosynthesis converts cholesterol into 7-hydroxycholesterol. 7-hydroxycholesterol is converted to one of the two primary bile acids, cholic acid and chenodeoxycholic acid. Bile acids are then delivered to the intestines where they aid in the absorption of lipids. Some of bile acids are modified to form secondary bile acids (lithocholic acid and deoxycholic acid) in the intestine by intestinal bacteria. However, the majority of bile acids delivered to intestine are recycled by re-absorption in the ileum and returned to the liver by enterohepatic circulation. In liver, glyco- and tauroconjugate bile acids are formed and stored in gall bladder, from where they are released into the intestinal lumen for aid fat/lipids digestion and absorption.

## 7. Regulation of specific pathways and its influence on cholesterol homeostasis

Blood cholesterol concentration is a result of balance between cholesterol input and cholesterol output. When the input is surpass the output, blood cholesterol increases and by contrast, when cholesterol input is less than the output blood cholesterol levels decrease. Cholesterol input is attributed from the intestinal absorption of dietary and biliary cholesterol and cholesterol biosynthesis. On the other hand, the cholesterol output is mainly from LDL-receptor mediated LDL-cholesterol clearance, reverse transport by HDL, cholesterol catabolism by converting into bile acids, cholesterol and bile acids secretion in bile into the intestine lumen, and fecal excretion.

Metabolic nuclear receptors serve a central role in maintainingcellular and whole-body cholesterol homeostasis [40]. Two important transcriptional mechanisms to regulate cholesterol metabolism are the pathways mediated by sterol responsive element– binding protein (SREBP) and liver X receptor (LXR), which tightly regulate intracellular sterol concentrations. The SREBP pathway ensures that there is sufficientcholesterol to meet cellular requirements by directly activating expression of genesinvolved in the synthesis and uptake of cholesterol, and lipogenesis [41]. In the setting ofexcess free or unesterified cholesterol, SREBP-dependentgene expression is suppressed. LXR and farnesoid X receptor (FXR),together with other members of the nuclear receptor superfamilypromote sterol storage, transport, and catabolism to prevent cholesterol accumulation [42]. LXRs respond to elevated cholesterol levels via transactivationof genes involved in sterol transport (ABCA1, ABCG1, ABCG5,and ABCG8), cholesterol efflux and high-density lipoprotein(HDL) metabolism (ABCA1, APOE, CETP, and

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PLTP), and sterol catabolism(CYP7A1) [42]. Other members of metabolicnuclear receptor family include receptors for bile acids (CAR and PXR), and fatty acids (peroxisome proliferaotr-activated receptors). Throughthe coordinated regulation of gene transcription, these nuclearreceptors regulate the key aspects of cellular and whole-body sterolhomeostasis, including cholesterol absorption and synthesis, lipoprotein synthesisand remodeling, lipoprotein uptake by peripheral tissues, reversecholesterol transport, and bile acid synthesis and absorption.

The amount of cholesterol that is synthesized in the liver is tightly regulated by dietary cholesterol levels. When dietary intake of cholesterol is high, synthesis is decreased and when dietary intake is low, synthesis is increased. However, cholesterol produced in other tissues is under no such feedback control. Cholesterol and similar oxysterols (the oxygenated derivatives of cholesterol, such as 22(R)-hydroxycholesterol, 2 4(S)-hydroxycholesterol, 27-hydroxycholesterol, and cholestenoic acid) act as regulatory molecules to maintain healthy levels of cholesterol. In tissues, many factor influence cholesterol balance through every cholesterol metabolic pathways.

### 7.1. LDL receptor-mediated cholesterol clearance

Cellular cholesterol increase is due to the uptake of cholesterol containing lipoproteins by receptors. LDL receptor regulates the cellular transport of LDL particles. One mechanism for regulating LDL receptor expression and controlling the expression of all the enzymes in the cholesterol biosynthetic pathway involves sterol-sensitive response elements (SREs). SREs are found in the promoters of the genes coding for the enzymes of cholesterol biosynthesis pathway and LDL receptors. Transcription factors of SRE activation are SREBPs. Three major SREBP isoforms, SREBP-1a, -1c, and -2, have been identified and differ in relative abundance in the liver and other various tissues. SREBP-1a is a potent activator of all SREBP-responsive genes and functions to maintain basal levels of cholesterol and fatty acid synthesis. SREBP-1c selectively activates genes involved in fatty acid synthesis, while SREBP-2 preferentially regulates genes important for cholesterol homeostasis by activating the transcription of HMG-CoA synthase, HMG-CoA reductase, LDL receptor [41].

Due to their ability to bind SREs, SREBP-2 plays an instrumental role in cholesterol homeostasis. These transcription-regulatory proteins are bound by another protein called SREBP cleavage activating proteins (SCAPs). SCAP, in turn, can bind reversibly with another endoplasmic reticulum-resident membrane protein, INSIG. SCAPs bind to SREBP-2 in the endoplasmic reticulum where a regulatory domain within SCAP responds to the level of oxysterols present in the cell. When the intracellular cholesterol and oxysterols concentrations decrease, the SREBP/SCAP complex moves to Golgi apparatus, leaving INSIG. Two proteases localized in Golgi, site-1 and -2 proteases (S1P and S2P) cleave SREBP-2 to release the transcription activation domain of SREBP-2. SREBP-2 preferentially activates transcription of target genes of LDL receptor [41]. When oxysterol levels are high, the SCAP/SREBP complex remains in the endoplasmic reticulum, preventing cleaved SREBP-2 from promoting gene expression. In addition to the up-regulation of LDLR transcription, nuclear SREBP-2 increases the transcription of PCSK9, a sterol-responsive protein that accelerates LDLR turnover in the

liver, thereby limiting lipoprotein uptake. As high concentrations of cellular cholesterol suppress SREBP-2 cleavage and release from endoplasmic reticulum, PCSK9 transcription is reduced, which subsequently increases LDLR levels, helping to maintain cholesterol homeostasis [43].

### 7.2. Regulation of cholesterol biosynthesis

SREBPs directly activate the expression of more than 30 genes dedicated to the synthesis and uptake of cholesterol, fatty acids, triacylglycerols, and phospholipids, as well as the reduced nicotinamide adenine dinucleotide phosphate (NADPH) cofactor required to synthesize these molecules. SREBP-2–responsive genes in the cholesterol biosynthetic pathway include those for the enzymes HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase, and squalene synthase. SREBP-1c and SREBP-2 activate three genes required to generate NADPH, which is consumed at multiple stages in these lipid biosynthetic pathways [41]. High cholesterol/oxysterol levels acting on SCAP ultimately stop the maturation of SREBPs, resulting in the down regulation of key enzymes such as HMG-CoA reductase, thus, reducing the amount of cholesterol produced by the liver. To compensate the decreased cholesterol synthesis a homeostatic response in which cells increase the density of LDL receptors on their surfaces. This increases the clearance rate of LDL particles from the plasma and reduces plasma LDL cholesterol and its related health risks. The decrease in cholesterol synthesis also promotes an increase of HDL, thus, clearing even more cholesterol from the plasma.

Elevated levels of cellular cholesterol are accompanied by the increased production of oxysterols, which are specificligands of LXRs, allowing LXRs to function as cholesterol sensors [44]. LXRs respond to elevated cholesterol levels via transactivationof genes involved in sterol transport (ABCA1, ABCG1, ABCG5,and ABCG8), cholesterol efflux and HDL metabolism (ABCA1, APOE, CETP, and PLTP), and sterol catabolism(CYP7A1). Additionally, LXRs also play a central role in regulatingcellular lipid content through activation of SREBP-1c, whichis the master regulator of de novo lipogenesis [40]. In response to activation, LXRs act in a coordinated fashion to maintain cholesterol homeostasis by directing the tissue-specific expression of genes involved in sterol transport and metabolism [45]. A principal function of LXR in macrophages is to promote cholesterol removal from the cell through the induction of ABCA1, ABCG1, and apolipoprotein E. LXR also induces genes involvedin lipoprotein metabolism, including LPL, CETP, and PLTP [45].

### 7.3. Regulation of cholesterol absorption and secretion

At the intestine, cholesterol is absorbed into enterocytes by a mechanism involving Niemann Pick C1-like protein 1 (NPC1L1) [46]. The NPC1L1 protein is abundant on intestinal brush border membranes. It functions as a sterol transporter to mediate intestinal cholesterol absorption and counterbalances hepatobiliary cholesterol excretion [46]. NPC1L1, is not under control of a nuclear receptor LXR [47]. In the enterocyte, cholesterol is readily esterified by the action of acyl-CoA:cholesterol acyltransferase 2 (ACAT2) and released into lymph in association with chylomicrons. The ATP-binding cassette (ABC) transporter protein ABCA1 andthe ABC half-transporters, ABCG5 and ABCG8, are LXR target genesin the intestine and partici-

pate in cholesterol absorption. ABCA1 and ABCG5/8 counteract cholesterol absorption via effluxof cholesterol from the enterocyte into the gut lumen. LXR agonists exerttheir effect on cholesterol absorption through upregulationof ABCG5 and ABCG8, which is necessary for the majority of sterols secreted into bile [40, 48].

### 7.4. Regulation of cholesterol transport

The hepatic nuclear receptor, PPAR$\alpha$, exert control over many aspects of reversecholesterol transport. Hepatic synthesisof apoA-I and apoA-II, the two major apolipoproteins in discoidalHDL ( $HDL_2$), is regulated via PPAR$\alpha$ activation and transcriptional regulation ofROR$\alpha$ (NR1F1), a widely expressed nuclear receptor that is activatedby cholesterol or cholesterol sulfate ligands [49]. ABCA1, which helps in reverse transport from peripheral tissues, is PPAR$\gamma$/LXR-regulated cholesterol/phospholipid transporter [50]. PLTP is activatedby both LXR and FXR whereas CETP is transactivated by LXR [40,51].

### 7.5. Excretion of cholesterol

Bile acids are synthesized in hepatocytes and this production is tightly controlled by the nuclear receptor transcription factors, LXR-$\alpha$ and LXR-$\beta$. Activation of LXRs by specific oxysterol derivatives leads to the regulation of bile acid synthesis by stimulating cholesterol 7$\alpha$-hydroxylase (*CYP7A1)* transcription to convertcholesterol to bile acids. FXR, a bile acid receptor, plays a central role of lipid metabolism in liver cells. FXR may play the major roles in bileacid metabolism, reverse cholesterol transport, and protecthepatocytes against cholestasis by feedback inhibition ofbile acid synthesis by CYP7A1; stimulation of bile acid effluxfrom hepatocytes by bile salt export pump; inhibition of bile acid uptakeinto hepatocytes by $Na^+$-taurocholate co-transporting polypeptide; and regulation of reverse cholesteroltransport by inducing ApoCII and PLTP [40, 52].

## 8. Cholesterol-lowering drugs

Mainly cholesterol lowering drugs have been developed and used clinically, which includes 1) HMG-CoA reductase inhibitors, e.g., atorvastatin, fluvastatin, lovastatin, pravastatin, rosuvastatin and simvastatin, 2) bile acid sequestrants — colesevelam, cholestyramine and colestipol — and nicotinic acid (niacin), and 3) cholesterol absorption inhibitor - ezetimibe. Other available drugs are gemfibrozil, fenofibrate and clofibrate which are fibric acid derivatives primarily used for lowering high triglyceride levels

### 8.1. Statins – Benefits versus side effects

Statins or inhibitors of 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase are widely used to reduce the risk of cardiovascular events and death. Statins includes lovastatin, which is a fungal metabolite, and many synthetic derivatives, pravastatin, atorvastatin and simvastatin etc. Statins target predominantly hepatocytes and inhibit HMG-CoA reductase,

the enzyme that converts HMG-CoA into mevalonic acid, a cholesterol precursor. Statins alter the conformation of the enzyme when they bind to its active site. This prevents HMG-CoA reductase from attaining a functional structure. The change in conformation at the active site makes these drugs very effective and specific [53]. Binding of statins to HMG-CoA reductase is reversible, and their affinity for the enzyme is in the nanomolar range, as compared to the natural substrate, which has micromolar affinity [54]. The inhibition of HMG-CoA reductase determines the reduction of intracellular cholesterol, inducing the activation of a protease which slices SREBPs from the endoplasmic reticulum. SREBPs are translocated into nucleus, where they increase the gene expression for LDL receptor. The reduction of cholesterol in hepatocytes leads to the increase of hepatic LDL receptors that leads to the reduction of circulating LDL and of its precursors (intermediate density - IDL and very low density- VLDL lipoproteins). All statins reduce LDL cholesterol non-linearly, dose-dependent, and after administration of a single daily dose [53]. Statins inhibit hepatic synthesis of apolipoprotein B-100, determining a reduction of the synthesis, secretion of triacylglycerols-rich lipoproteins, and an increase of receptors for apolipoproteins B/E. Statins have a modest effect on HDL increase, and no influence on lipoprotein(s) concentration. Statins can also prevent LDL oxidation by preserving the activity of the endogenous antioxidant system, like superoxide dismutase [53].

In general, the currently used statins are well tolerated and have a good safety profile [55]. Statins acting as inhibitor of HMG-CoA reductase, inhibits all the pathway in which mevalonate is a precursor such as synthesis of nonsteroid isoprenoids such as farnesylpyrophosphate (FPP), geranylgeranylpyrophosphate (GGPP), dolichol and side chain of coenzyme Q which play an essential role in cellular physiology. Coenzyme Q is a component of mitochondrial respiratory chain and a lipid-soluble antioxidant. Farnesyl- and geranylgeranyl groups are needed for protein isoprenylation and formation of small GTP-binding proteins including Ras, Rho and Rab, which are involved in signal transduction pathways. Inhibition of isoprenoid synthesis by statins decreases the activity of these proteins and modifies the respective signalling pathways; the mechanism responsible for cholesterol-independent pleiotropic effects of statins. The best recognized and most commonly reported adverse effect of statins are muscle adverse effect with muscle pain, fatigue and weakness as well as rhabdomyolysis. Rhabdomyolysis is the most severe form of statin induced myopathy, characterized by marked increase in CK activity, myoglobinemia, myoglobinuria, and myoglobin induced acute renal failure. These symptoms arising on statins are shown to be reversed with discontinuation. Coenzyme Q10 deficiency produces mitochondrial encephalomyopathy, resulting in fatigue, muscle symptoms, and cognitive problems. Gastrointestinal and neurological symptoms, psychiatric symptoms, sleep problems, glucose elevations, and a range of other symptoms are also reported on statins [56].

### 8.2. Ezetimibe – Benefits and side effects

Ezetimibe is the first of a new class of highly selective cholesterol absorption inhibitors. It does not inhibit cholesterol synthesis in the liver or increase bile acid excretion. It belongs to a class of lipid-lowering compounds that selectively inhibits the intestinal absorption of cholesterol

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and sterols. Ezetimibe's pharmacological effect is complementary to that of the statins [57]. Mechanism of action of ezetimibe involves inhibiting the absorption of cholesterol in the small intestine. Through a mechanism that is not yet fully elucidated, ezetimibe appears to block a protein transporter called Niemann-Pick C1-like 1 protein (NPC1L1) that is located at the apical membrane of the small intestine enterocytes [58]. Unlike other cholesterol-lowering agents, ezetimibe localizes and appears to act at the brush border of the small intestine and inhibits the absorption of dietary and biliary cholesterol in the small intestine, leading to a decrease in the delivery of intestinal cholesterol to the liver. This leads to a reduction of hepatic cholesterol stores and an increase in clearance of cholesterol from the blood. Ezetimibe has been demonstrated to have no significant effect on the plasma concentrations of the fat-soluble vitamins A, D, and E [57]. No published reports could be identified that assessed the potential impact of ezetimibe therapy on other traditional CVD risk factors, including blood pressure and obesity. Recent reports have raised concerns about an association between ezetimibe and an increased incidence of cancer [58]. Ezetimibe may rarely cause hepatotoxicity, severe cholestatic hepatitis, or acute autoimmune hepatitis [59].

Ezetimibe is proved to be more effective in combination with statin drugs in lowering LDL-c than monotherapy. Ezetimibe has an additive and at times synergistic effect on the reduction of LDL-C and total cholesterol (TC) concentrations when combined with statin therapy. Ezetimibe has not been associated with increased rates of myopathy or rhabdomyolysis, whether used alone or in combination with statins, although there have been some case reports of myopathy attributed to this agent. Moreover, ezetimibe has been associated with mild elevations of liver transaminases, mainly in combination with a statin. Other side effects are extremely rare. It should be noted, however, there are no long-term safety data or outcome studies for ezetimibe yet [60].

### 8.3. Niacin (Nicotinic acid)

Niacin is the most potent HDL increasing drug currently available and it also can efficaciously lowers triglycerides and LDL cholesterol. It is the only lipid-lowering drug that considerably lowers lipoprotein(a). Beneficial effect of niacin to reduce triglycerides and apolipoprotein-B containing lipoproteins (e.g., VLDL and LDL) are mainly through: a) decreasing fatty acid mobilization from adipose tissue triglyceride stores, and b) inhibiting hepatocyte diacylglycerol acyltransferase and triglyceride synthesis leading to increased intracellular apo B degradation and subsequent decreased secretion of VLDL and LDL particles. Niacin raises HDL by decreasing the fractional catabolic rate of HDL-apoAI without affecting the synthetic rates. Additionally, niacin selectively increases the plasma levels of lipoprotein-AI (HDL subfraction without apoAII), a cardioprotective subfraction of HDL in patients with low HDL. Recent studies indicate that niacin selectively inhibits the uptake/removal of HDL-apoAI (but not HDL-cholesterol ester) by hepatocytes, thereby increasing the capacity of retained HDL-apoAI to augment cholesterol efflux through reverse cholesterol transport pathway [61]. Niacin treatment is associated with a number of side effects, including headache, itching and gastrointestinal disturbances, but these are generally mild. The most severe of the side effects is flushing, and this is sufficiently severe to negatively affect compliance [62].

### 8.4. Bile acid sequestrant

Bile acid sequestrant or bile binding anion (Chloride) exchange resins that is effective in reducing total cholesterol and LDL cholesterol levels. The primary and direct action of the bile acid sequestrants is to bind to bile acids in the gut and thus interrupt the enterohepatic recirculation of bile acids [63]. Three key enzyme are affected by bile acid sequestrant, which are phosphatidic acid phosphatase, cholesterol 7-alpha-hydroxylase and HMG-CoA reductase. Activation of phosphatidic acid phosphatase promotes hepatic triglyceride synthesis, induces secretion of triglyceride-rich VLDL particles and consequently increases plasma triglyceride levels. The activation of hepatic cholesterol 7-alpha-hydroxylase promotes the conversion of intracellular cholesterol to bile acids. The decrease in intracellular cholesterol stores, in turn, increases LDL receptor expression on hepatocyte membranes and consequently, increases receptor-mediated fractional catabolism of LDL or LDL uptake by liver cells. Reduction of intracellular cholesterol may also increase the synthesis of cholesterol through activation of HMG-CoA reductase. The potential loss of the bile acid sequestrant's cholesterol-lowering efficacy can be overcome by adding HMG-CoA reductase inhibitor (statins). Finally, bile acid sequestrants promote apoprotein AI synthesis and tend to raise high-HDL cholesterol levels, primarily by increasing plasma HDL-2 concentrations. Three drugs in this class are synthetic cholestyramine, colestipol, colesevelam. The side effect profile of the bile acid sequestrants is tolerable, with most complaints related to effects on the gastrointestinal tract and the bulkiness of resins [64].

### 8.5. Fibrates

Fibrates are primarily effective for the treatment of hypertriglyceridemia or mixed hyperlipidemia by stimulating the peroxisomal β-oxidation pathway. Their main action is to lower plasma triglyceride levels, but they also reduce total and LDL cholesterol concentrations and induce a moderate increase in HDL cholesterol. Fibrates act by stimulating the activity of peroxisome proliferator-activated receptor (PPAR)-$\alpha$, a member of the PPAR subfamily of nuclear receptors [65]. It controls the transcription of regulatory genes of fatty acids and cholesterol metabolism. It inhibits the synthesis and secretion of triglycerides by the liver and a stimulation of the degradation of triglyceride-rich lipoproteins. This increased clearance of triglycerides results from a stimulation of the expression of lipoprotein lipase and a decreased expression and concentration of apolipoprotein-CIII, an inhibitor of lipoprotein lipase activity. PPAR-$\alpha$ activation modifies the expression of several key genes controlling HDL cholesterol metabolism and reverse transport of cholesterol [65]. Several fibrate drugs such as ciprofibrate, bezafibrate, fenofibrate, and gemfibrozil has revolutionized lipid-lowering but research has shown the prolonged use of some of these drugs like clofibrate and ciprofibrate causes peroxisome proliferation leading to hepatomegaly and tumor formation in the liver of rodents [66].

A recent report demonstrated that between the periods 1988-1994 and 1999-2002, mean total cholesterol and mean LDL cholesterol declined in American adults. Coincidently, during this time there also was an increase in the percentage of adults receiving lipid-lowering medications. However, among adults not receiving lipid-lowering medications, trends in lipids were

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similar to those reported for adults overall. Among obese adults, mean total cholesterol, non–HDL cholesterol, LDL cholesterol, and geometric mean triglycerides declined between 1988 and 2010[67]. These data suggest that in addition to the increased use of lipid-lowering medications, something else must have been involved in the overall reduction of blood total cholesterol and LDL cholesterol. Although those factors, the application of natural lipid-lowering products plays a critical role and is thus described below.

## 9. Cholesterol-lowering natural products

### 9.1. Plant sterols/ stanols

Plant sterols/ stanols, naturally occurring in foods of plant origin, perform similar biological functions to cholesterol, and contain a similar chemical structure. They differ from cholesterol only in the presence of either an extra methyl or ethyl group. Absorption efficiency for plant sterols in humans is less (2-5%) than that of cholesterol (60%). The most common forms are unsaturated plant sterols β-sitosterol, campesterol, and stigmasterol and the saturated sitostanol and campestanol [68]. Plant sterols reduce the absorption of both dietary and biliary cholesterol from the intestinal tract by 30%–50% [69]. The exact mechanism is yet fully elucidated while it is generally assumed that the presence of increased quantities of plant sterols in the gut lowers the micellar solubility of cholesterol, therefore lowering the amount of cholesterol available for absorption [69]. Intake of phytosterol and stanols at an average of 2 g/day has been shown to lower low density lipoprotein cholesterol (LDL-C) by 10-15%. The effect appeared to be peaked at intakes of 2 g/d, with little additional benefit being achieved at intakes higher than 2.5 g/d. The recommended daily intake of phytosterols is 2 g/d in humans [70]. Plant sterols and their derivatives reduce plasma cholesterol levels independently from the mRNA expression of ABCG5 and ABCG8 transporters [71]. Food products such as margarine, milk, yoghurt, and cereal products enriched with plant sterols/stanols are promoted as functional foods to help lower serum cholesterol levels. Human and animal studies have shown that plant sterol and stanol esters are non-toxic [72]. There have been concerns raised over the reduced absorption of some fat soluble vitamins from the use of plant sterols. For example, plant sterols and stanols have been shown to reduce β-carotene, $\alpha$-carotene, and vitamin E levels by around 25%, 10%, and 8%, respectively [72]. Used alone in the diet, or as an adjuvant to drug therapy, or in combination with other functional food components, plant sterols/stanols-enriched products are generally effective at reducing serum total and LDL-C [72], and thus the most popular natural ingredient in cholesterol-lowering product market.

### 9.2. Soy products

Soy foods have been consumed for centuries in Asian countries. Consumption of soy foods contribute to lower incidences of coronary heart diseases, atherosclerosis, type 2 diabetes, and decreased risk of certain types of carcinogenesis such as breast and prostate cancers [73]. Animal and human studies have also shown that consumption of soy protein or associated isoflavones has beneficial impacts such as lowering liver or blood triglyceride, total and LDL

cholesterol levels, increasing HDL cholesterol and the ratio of HDL/LDL cholesterol [73, 74]. Soy protein regulates SREBP-1 expression by modulating serum insulin concentration, thus preventing the development of fatty liver [75]. Isoflavones are major soy phytoestrogens present in soy foods and Genistin, daidzin, and glycitein are the main soy isoflavones (Xiao 2008). Soy and soy bioactive components are well-tolerated and the adverse effects reported are gastrointestinal symptoms (e.g., diarrhea), followed by menstrual complaints (e.g., prolonged periods, amenorrhea) headache, dizziness, and musculoskeletal complaints [76].

### 9.3. Dietary fibre

Dietary fibre is one of the most studied dietary components associated with cardiovascular benefits. It is a complex of non-digestible carbohydrates and lignin that are intrinsic and intact in plants and are resistant to digestion and absorption in the small intestine. Dietary fibre can modulate body weight and promotes beneficial physiological effects such as laxation, reduction in blood cholesterol and postprandial blood glucose [77]. Traditionally, dietary fibre has been classified on the basis of its solubility in water (soluble or insoluble). Foods rich in fibre need to be chewed longer, leading to an increase in the time needed to eat and the feeling of satiety. Fibres which make up viscous solutions also delay the passage of food from the stomach to duodenum and contribute to an increase in satiety and a decrease in energy consumption [78]. In the intestine, the incorporation of fibre in food may complicate the interaction between digestive enzymes and their substrates, thus slowing down the absorption of nutrients[77]. The hypocholesterolemic action of fibre is partly mediated by a lower absorption of intestinal bile acid because the interruption of the enterohepatic bile acid circulation, thus increasing faecal bile acid loss, and its de novo synthesis in liver. The physicochemical properties of soluble fibre result in important modifications in volume, bulk and viscosity in the intestinal lumen, which will alter metabolic pathways of hepatic cholesterol and lipoprotein metabolism, also resulting in lowering of plasma LDL cholesterol [79]. Dietary fibre increases the enzymatic activity of cholesterol-7-$\alpha$-hydroxylase, contributing to a higher depletion of hepatic cholesterol but increased endogenous cholesterol synthesis. However, there is an increase in the number of LDL receptors and in the recruitment of the esterified cholesterol from the circulating LDL particles. Several types of soluble dietary fibre such as pectin, glucomannan, psyllium can decrease plasma total cholesterol and LDL cholesterol. Epidemiological evidences showed a stronger association of cardiovascular protection with soluble fibre than insoluble fibre. Insoluble fibre such as that from wheat or cellulose has not been reported to have any significant effect on blood cholesterol [78]. Consumption of too much high fibre foods that have not been cooked can cause side effects of abdominal bloating and gas.

### 9.4. Flaxseed lignans

Flax seed is the richest source of natural lignans, with secoisolariciresinol diglucoside (SDG) being the principal lignan compound. Flaxseed or flaxseed meal have cardioprotective properties and can suppress atherosclerosis by virtue of its antioxidant properties due to the presence of flaxseed lignans. Lignan reduces serum triglycerides and LDL and raises HDL

cholesterol. In addition, flaxseed oil possesses anti-inflammatory properties and reduces platelet aggregation as well [80]. Flaxseed lignans along with soy isoflavones are phytoestrogens commonly consumed in the human diet.

### 9.5. Polyunsaturated fatty acids and omega-3 fatty acids

An old assumption regarding fatty acids and their effects on atherosclerosis was that monounsaturated fatty acids were neutral, saturated fatty acids were bad, and polyunsaturated fatty acids were good. However, a study conducted by Scott Grundy and Fred Mattson in 1985 turned the "world of monounsaturated fatty acids" around[81]. They demonstrated that diets rich in saturated fatty acids caused a high LDL cholesterol/HDL cholesterol ratio, and that substitution of monounsaturated fatty acid for saturated fatty acids reduced LDL cholesterol but did not reduce HDL cholesterol. Consequently the LDL cholesterol/HDL cholesterol ratio was the lowest with monounsaturated fatty acids, given that polyunsaturated fatty acids reduced HDL cholesterol as well as LDL cholesterol. A similar phenomenon was observed in a 5-year study in male African green monkeys[82]. In the monkey studies, average HDL cholesterol was 50 mg/dl in the polyunsaturated fatty acids group versus 86 and 81 mg/dl in the saturated fatty acids and monounsaturated fatty acids groups, respectively. Average plasma LDL cholesterol concentrations in the polyunsaturated fatty acids and monounsaturated fatty acids-fed monkeys were 157 and 167 mg/dl, respectively (no significant difference between them) versus 257 mg/dl in the saturated fatty acids-fed animals.

The influences of dietary fatty acids on blood cholesterol profiles are also related to diet composition[83]. It is well accepted that the consumption of saturated fatty acids increases LDL cholesterol, whereas carbohydrates, monounsaturated fatty acids and polyunsaturated fatty acids do not. The effect of fatty acids on blood lipid profiles also depends on heath conditions. Among individuals who are insulin resistant, a low-fat, high-carbohydrate diet typically has an adverse effect on lipid profiles. In addition to lowering HDL cholesterol, it also increases triacylglycerols and LDL cholesterol. Consequently, a moderate fat diet in which unsaturated fatty acids replace saturated fatty acids and carbohydrates are not augmented is advised to lower LDL cholesterol[83].

Fish oil (marine n-3 fatty acids, eicosapentaenoic acid, and docosahexaenoic acid), whether from dietary sources or fish oil supplements, exhibit cardioprotective effects and reduce mortality due to cardiovascular diseases. Fish oil provides cell membrane stabilization, anti-inflammatory, antiatherogenic effects and suppression of cardiac arrhythmias[84]. Omega-3 fatty acids lower moderately the blood pressure through primarily the improvement of vascular endothelial cell function whilst a multitude of mechanisms may be involved85. Polyunsaturated fatty acids lower triacylglycerols, which has also been important in cardioprotection and the management of insulin resistance and diabetes. The effect of omega-3 fatty acids on blood cholesterol is inconsistent effect. In general, omega-3 fatty acids do not offer a benefit of directly lowering blood cholesterol. Instead, in some studies fish oil has been found to cause a small rise in LDL-cholesterol; however a change in the LDL particle size from the smaller more atherogenic form to the larger less damaging particle size have been noted85.

Health Canada has recently reconsidered the classification of food products with disease risk reduction claims or therapeutic claims in light of clarified principles for the classification of foods at the Food-Natural Health Product interface (http://www.hc-sc.gc.ca/fn-an/label-etiquet/claims-reclam/assess-evalu/sat-mono-poly-fat-gras-eng.php). Health Canada has concluded that the results of the updated literature review are consistent with the 2002 report provided by the Institute of Medicine (IOM), which forms the basis of the US and Canada dietary guidance, on the replacement of saturated fat with unsaturated fat for blood cholesterol lowering. In other words, scientific evidence exists in support of the therapeutic claim linking the replacement of saturated fat with unsaturated fat to a reduction of blood cholesterol. It is stated that the claim is relevant and generally applicable to the Canadian population as a high proportion of the Canadian population is hyperlipidemic. It is allowed now by the Health Canada's to put therapeutic claim statements such as "Replacing saturated fats with polyunsaturated and monounsaturated fats (from vegetable oils) helps lower/reduce cholesterol" in vegetable oils and foods containing vegetable oils when specific conditions for the food carrying the claim are met.

### 9.6. Olive oil

Olive oil can reduce LDL and raise high-density lipoprotein cholesterol and decrease lipid damage due to oxidative stress. In addition, olive oil reduces inflammatory and thrombogenic status, endothelial dysfunction, and blood pressure[86].

### 9.7. Green tea products

Tea catechin-especially (-)-epigallocatechin-3-gallate-inhibits the expression of soluble adhesion molecules including vascular adhesion molecule-1 and intercellular adhesion molecule-1, endothelial cell inflammatory markers, decreased oxidized LDL, and prevents the development of atherosclerosis and[87].

Certainly, there are more natural products available, for instance a number of antioxidants and phenolic compounds, to lower blood cholesterol levels. However, the big challenges that the natural products have been facing in the past years lie in their relatively lower efficacies as compared with the cholesterol-lowering drugs. The apparent advantages of natural products are their better safety profiles. In order to promote the market share of cholesterol-lowering natural products in competing with the drugs, it is critical to develop new products that can offer better efficacies than the current natural products, without losing safety or introducing increased toxic or severe side effects. Considering that the majority of the current natural products fall into the same category of cholesterol absorption inhibitor, the future direction of research and development of cholesterol-lowering products, novel distinct pathways or targets should be focused. Herewith, we will provide some brief thoughts on new approaches that we believe worth to tackle into, with a hope that the new mind in the research and development direction and focus would help to discover and develop novel natural products with significantly improved cholesterol-lowering efficacy working through distinct mechanisms than the currently available natural products, without apparent side effects.

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## 10. New perspectives on the development of natural cholesterol-lowering products

Cardiovascular friendly natural products are those nutritional supplements, which can provide potential health benefits in cardiovascular diseases (CVD). Experimental, epidemiological and clinical data indicates that dietary nutrients and supplements have profound cardioprotective effects in the primary as well as secondary prevention of coronary heart disease [88]. Except for the aforementioned several cholesterol-lowering natural products, the plant alkaloid berberine has recently been introduced to the filed of lipid-lowering products. Berberine has shown a moderate or comparable cholesterol- and triacylglycerol-lowering effect in humans [89]. More importantly, recent studies have demonstrated that berberine combined with plant stanols improves cholesterol-lowering efficacy through a synergistic action on cholesterol absorption and reducing plasma triacylglycerols in animals [90], [91], with the efficacies are comparable or even better cholesterol-lowering drugs, statins. The product has been patent-protected and at the preclinical stage. Clinical trials are warranted before moving this potentially highly effective natural product to the lipid-lowering market.

### 10.1. Current status in the development of cholesterol-lowering products

An initial approach to control the modifiable risk factors of hyperlipidemia relies on the changes of diet, lifestyle, and other factors such as smoking. When all these fail, pharmaceutical intervention is necessary. Many of the current available drugs are those either inhibit cholesterol synthesis, or increase synthesis of bile acids and excretion. The majority of the current research focuses on lowering the "bad" LDL cholesterol, with less attention on increasing "good" HDL cholesterol. A treatment with a combination that can lower LDL cholesterol and increase HDL cholesterol would certainly provide more benefits than single approach for the treatment of hyperlipidemia, atherosclerosis and cardiovascular disease.

#### *10.1.1. Lowering LDL cholesterol*

Statins drugs, which are currently widely prescribed, lower LDL cholesterol primarily via inhibiting cholesterol biosynthesis and a secondary effect of increase LDL receptor and LDL clearance. However this family of drugs has shown several undesirable side-effects. Niacin, can also lower LDL cholesterol but not efficiently as statin but it is only one drug proved to increase HDL cholesterol with very less side effects. Therefore, a combination of niacin and statins might be superior to these drugs alone for lowering lipid levels. The scientific evidence of long-term safety and tolerability of these combination therapies are key determinants for good compliance and cardiovascular benefits.

Proprotein convertase subtilisin kexin-9 (PCSK9) is a sterol-responsive protein that accelerates LDLR turnover in the liver. When PCSK9 transcription is reduced, there will be subsequently increases LDLR levels helping to maintain cholesterol homeostasis. The transcription of PCSK9 is increased by SREBP-2, which also regulate the transcription of many other genes. Therefore,

a pharmacologically specific inhibitor of PCSK9 will have atherogenic effect by increasing LDLR levels without affecting other genes regulated by SREBP-2.

*10.1.2. Increasing HDL levels*

Low HDL-cholesterol (HDL-C) is a strong and independent cardiovascular risk marker. Niacin is the only drug proved to increase HDLC but associated with flushing as a side-effect. Niacin-induced flushing involves both $PGD_2$ from mast cells and serotonin from platelets. The possibility of administering or formulating niacin together with flavonoid luteolin was recently shown to inhibit niacin flush in rats. Further investigation on inhibitors on side-effect can warrant an effective medicine for CVD[92]. Aleglitazar, a dual PPAR$\alpha/\gamma$ agonist, proven has beneficial effects on both lipid and glucose parameters [93]. Activation of PPAR$\alpha$ may increase ApoA1, apolipoprotein of HDL and increased the synthesis of LPL which hydrolyzes VLDL, IDL and LDL. PPAR$\alpha$ agonist, with good safety profile, may have a therapeutic role in modifying cardiovascular risk factors.

*10.1.3. CETP inhibition*

Besides established strategies to increase HDL-C, e.g. with nicotinic acid, CETP (Cholesteryl ester transfer protein)-inhibition is a promising new therapeutic option. The failure of torcetrapib, the first CETP-inhibitor, seems to be attributed to "off-target" effects. Treatment with the newer CETP-inhibitors dalcetrapib and anacetrapib has been shown to be efficacious and safe - but their usefulness in clinical practice remains to be determined in ongoing clinical trials [94].

*10.1.4. Inhibition of cannabinoid receptors*

G protein-coupled cannabinoid receptor, CB-1 of the endocannabinoid system, plays a crucial role in regulating feeding pattern, lipid metabolism, and energy homeostasis. CB-1 receptors are located in the central nervous system and peripheral tissues including adipocytes, pancreas, gut, liver, and muscle. Rimonabant, a selective CB-1 antagonist drug to be developed for weight loss via increasing energy expenditure actually also increases HDL-cholesterol and triglycerides.However, the clinical efficacy and safety of these new antiobesity compounds are yet to be determined. Additional cannabinoid receptor blockers have been developed and are in testing [95].

*10.1.5. Nuclear receptor modifiers*

Identification of nuclear receptor LXR and FXR as regulatorof genes in bile acid and cholesterol metabolism has providedpotential new targets for screening cholesterol-lowering drugsby manipulating bile acid synthesis, transport, and absorption. Therapies targeted to LXR and FXR would be ideal for drug developmentbecause nuclear receptors are activated by natural and syntheticligands, which could be identified by high-throughput screening [96].

*10.1.6. Acyl-coenzyme A: Cholesterol acyltransferas (ACAT) inhibitors*

ACAT 1 and ACAT2 play an important role in cellular cholesterol esterification and thus modulate intestinal cholesterol absorption and hepatic lipoprotein secretion. ACAT2 is proposed to play a central role in cholesterol absorption from the intestine. ACAT1, on the other hand is widely distributed in tissues, and plays a pivotal role in cholesterol metabolism in macrophages and steroidogenic tissues. It is anticipated that inhibitors of ACAT2 activity could be developed that would decrease cholesterol absorption, whereas specific ACAT1 inhibitors could be used to reduce foam cell formation and prevent atherosclerosis. However, the results of studies have indicated that ACAT1 inhibition is not a good strategy and, in fact, could have detrimental consequence [97]. In contrast, mice lacking ACAT2 exhibited attractive metabolic findings. These include a restricted capacity to absorb cholesterol and protection against diet-induced hypercholesterolemia and gallstone formation [98, 99]. Further, ACAT2 inhibitor reduces cholesterol esters in apolipoprotein B-containing lipoproteins and protects from atherosclerosis in murine models of the disease [100]. A fundamental question remains: would ACAT2 specific inhibition in humans, either pharmacologically or with anti-sense oligonucleotides, prevent or reduce atherosclerosis? "The sole test of the validity of an idea is experiment." Until a potent and specific inhibitor of ACAT2 is tested in humans, the hypothesis remains uncertain [101].

## 10.2. Future perspectives of cholesterol-lowering products and their potential impact on CVD

*10.2.1. Identification of new molecular targets*

Target identification is an essential first step in drug development. Although cholesterol metabolism has been studied for decades and relatively well understood, the specific molecular targets that can be used for drug discovery and development are far from well-known. It is believe that with the development and use of modern molecular technology and techniques in nutritional and physiological research, more targets will be indentified and used for future drug development.

*10.2.2. Application of gene silencing technology*

Apart from the identification of new targets, several new approaches have been introduced or shown great potential for the development of new cholesterol-lowering products. Of them is gene-silencing technology. One technology that has generated a lot of excitement is RNA interference (RNAi) [102]. For example, clinical trials have been launched using RNA interference approaches to reduce PCSK9 expression or specific antibodies targeting and inhibiting PCSK9 interaction with the LDL receptor. They constitute very promising approaches to reducing cholesterol levels and coronary heart disease. Understanding of PCSK9 and its potential as a therapeutic target through which to reduce LDL cholesterol for prevention and treatment of coronary heart disease has been of great interest recently [103]. The administration of chemically modified small interference RNAs (siRNAs) results in silencing of a target mRNA, such as apolipoprotein B (apoB) mRNA in liver and jejunum and decreasing plasma

levels of apoB protein. ApoB is a molecule involved in the metabolism of cholesterol; the concentrations of this protein in human blood samples correlate with those of cholesterol. Higher levels of both compounds are associated with an increased risk of coronary heart disease. Intravenous injections of the siRNA-cholesterol conjugates in mice resulted in a lowering of the levels of blood cholesterol comparable to that in mice in which the apoB gene had been deleted. These results demonstrate that siRNA can be delivered systemically to target the liver and suggest that RNAi has the potential to become a new therapeutic for the treatment of metabolic diseases [102].

*10.2.3. Development of target-specific inhibitors or enhancers*

For those molecular targets that have several different isoforms, the biggest challenge in the drug development is the specificity. A good example is ACAT inhibitors. To date, it has been demonstrated that there are two sioforms, ACAT1 and ACAT2. ACAT1 is expressed universally while ACAT2 is expressed mainly in the liver and small intestine. Both isoforms can esterify cholesterol and other sterols. However, ACAT1 primarily works on the esterification of plant sterols and other sterol products, while ACAT2 mainly converts cholesterol to its esters. Due to their different distribution and activities in the body, a universal inhibitor of both enzymes has been shown to be detrimental and recent humans trials are disappointing [104, 105]. The study of Bell et al. in 2006 breathes life back into the idea of ACAT2-specific inhibition [106]. In atherosclerosis-prone mice, ACAT2 was specifically ihibhied in the liver with antisense oligonucleotides. Biweekly intraperitoneal injections, which reduced ACAT2 expression by a remarkable 80%, decreased diet-induced hypercholesterolemia and sharply reduced cholesterol ester deposition in the aorta. The specific inhibition can be achieved from the antisense gene technology or small molecules that inhibit only ACAT2. There is no mature product in this category and lots more work needs to be done.

It has been demonstrated by numerous studies that inhibition of cholesterol absorption is associated with much less side-effects than other approaches. It remains challenge but highly promising that a potent inhibitor of cholesterol absorption would sufficiently lower blood cholesterol or keep blood cholesterol levels within the recommended healthy range. To achieve this goal, other approaches have to be take in addition to the specific inhibitor of cholesterol absorption, such as a combination of two or more products, in particular the combinations that work synergistically through one or multiple pathways of cholesterol homeostasis. Recent studies on the combination of the plant alkaloid berberine and plant sterols/stanols have shown an excellent example of this approach. The researchers have demonstrated consistently that plant sterols or stanols and berberine have a moderate effect to lower blood cholesterol levels in different animal models. However, when plant sterols/stanols were combined with berberine, a significant synergism was produced. They remarkably improved cholesterol-lowering efficacy by synergistically inhibiting cholesterol absorption [90,91].

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## 11. Conclusions

Cardiovascular disease has long been the lead cause of mortality and morbidity in the developed countries and similar situation has arisen in recent years in the developing countries. The increased prevalence of CVD is due to the significantly improved food supply and processing and rapid shift of lifestyle from active to sedentary nature, resulting increased incidence of obesity and overweight. Although the development and clinical use of statin drugs and other cholesterol-lowering drugs has been stabilize the mortality resulted from CVD since last decade, the incidence has been stably rising. According, the control of risk factors for atherosclerosis and CVD remains critical. The primary risk factor is hypercholesterolemia. There still exists a strong demand to develop novel products, especially from distinct cholesterol metabolic pathways or molecular targets and from different new approaches as mentioned above. It is optimistic that in the near future, new cholesterol-lowering products, either drugs or natural products with higher efficacies, better specificity and better safe profiles will be developed.

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Chapter 8

# A Glance at the Complexity of Nutrition and the Prostate: Considering Molecular Targets to Unravel the Most Recent Controversy Between Omega-3 Fatty Acids and Their Impact on Prostate Cancer Risk

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Additional information is available at the end of the chapter

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## 1. Introduction

### 1.1. Pathophysiology of prostate cancer

Considered part of both the male reproductive and urinary systems, the prostate gland (or prostate) is oval shaped, and is variable in size (ranging from 20 – 30 grams in adult males)[1]. The prostate gland consists of different types of cells, including gland (epithelial) cells, muscle cells and fibrous cells [2]. The overall structure of the prostate is divided in two ways: by zone, a classification more often used in pathology [2, 3] or by lobe, more often used in anatomy [2]. Prostate associated diseases usually present with urinary and sexual dysfunction and the initial symptoms related to such often necessitate diagnostic testing to indicate the presence of a certain disease state [4, 5]. Although the accuracy and use of Digital Rectal Examination (DRE) and Prostate Specific Antigen (PSA) remains controversial, these tests can be used alone, or in combination, to detect benign conditions (including benign prostatic hyperplasia (BPH) and prostatitis) or predict most forms of PCa [6, 7]. Subsequent prognosis of PCa is confirmed by prostate biopsy and involves pathological staging (Tumor/ Nodes/ Metastasis (TNM) System) and grading (Gleason Score) [8-10]. Broadly defined as the malignant growth of cells of the prostate gland, PCa occurs in many different zones of the prostate [1, 11, 12]. PCa (mostly 75%) arise in the peripheral zone, and to a lesser extent in the central and transitional zones [13]. Similar to the pattern of most epithelial cancers, cells within the prostate undergo an accumulation of genetic changes whereby the functions of cellular control are lost as the cell and tissue undergo phenotype changes from normal to prostatic intraepithelial neoplasia (PIN) [14]. This

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progressively leads from to an acute high-grade PIN (HGPIN) to superficial cancers, and then to invasive disease [11, 12] (See Figure 1). The most common form of PCa (around 95%) originates in the glandular/epithelial tissue, and is coined prostatic adenocarcinoma; thus, this term has emerged synonymously with PCa [13]. The residual forms of PCa are termed nonadenocarcinoma, and present as less commonly occurring cancers, but are often more aggressive. Such can be categorized as epithelial (such as squamous cell carcinoma) and nonepithelial (such as osteo- and angio-sarcoma), and also include others which rarely develop in the prostate and are derived from primary tumors of the bladder and urethra (such as transitional cell carcinoma) [13]. The diagram below gives a general overview of prostate location, the division of zones and the general progression of PCa as the cell and tissue phenotype changes from normal to A. prostatic intraepithelial neoplasia (PIN), to B. increasing and severe high-grade PIN (HGPIN), then to C. superficial cancers, and finally to D. invasive disease [11, 12, 15].

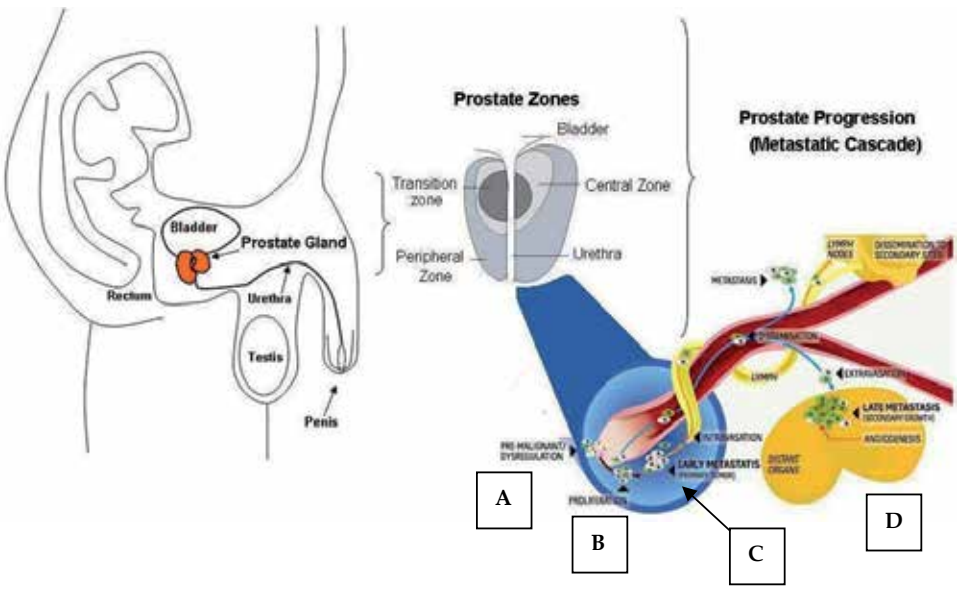


**Figure 1.** GA Tobin, 2011. An Overview of the Pathogenesis of PCa.

While the specific causes of PCa remain unknown, the only established risk factors for PCa are age, race and family history [16]. However, it is well accepted that the stimulation of androgens over a prolonged period contributes to the development of PCa [17]; thus, the androgen receptor (AR), along with its various cofactors play an important role in PCa. Nonetheless, progression of PCa to androgen independence (or the hormone refractory state) is one of the primary reasons for PCa-related deaths today [18]. PCa is the sixth most commonly diagnosed cancer worldwide [19]. For Canadian men, PCa is the most prevalent (based on 15 years) and frequently diagnosed non-dermatological cancer [20]. Like most other solid malignancies, PCa

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can extend to distant organs such as the liver, lungs and brain, with an abnormally high tendency for metastasizing to the bone [21, 22]. When distant metastasis occurs, the 5 year survival rate drops to 31% [20]. Although Prostate Specific Antigen (PSA) values, TNM staging, Gleason Score, and tumor biomarkers are considered significant in their ability to predict patient outcome, alone or when incorporated into various nomogram models, there is much controversy about their accuracy. Nonetheless, treatment regimens are largely based on these combined indicators.

Standard treatments for PCa can range from watchful waiting to surgery, radiation therapy, hormone therapy, chemotherapy, and biological therapy, alone or in combination [23-26]. Additionally, in North America, a minimum of 30% of men with diagnosed prostate disease choose to use complementary and/or alternative medical (CAM) therapy [27, 28] including primarily herbal biological agents, vitamins, supplements and dietary intervention [29]. This trend is likely due to progressive research on differences in global distribution, indicating that prolonged diet and micronutrient intake intervals may possibly control whether PCa remains latent or develops into a clinically significant disease [30-34]. In particular, the role of polyunsaturated fatty acids (PUFA) has gained considerable importance in PCa in the last several years. However, the molecular basis for these observations has not been fully explained. Genetic (and epigenetic) alterations and specific signaling pathways [35] are considered important in the multi-step process of cancer [36]. Thus, dietary interventions which target signaling pathways and downstream genes known to be impacted in PCa may be a worthwhile and effective approach in developing novel therapies.

## 2. Endocrine and molecular biology and controls in prostate cancer

### 2.1. Hormones and prostate cancer

Charles Huggins introduced the phenomenon of androgen removal as a plausible means for prostate cancer treatment in men after his observation that castration in canines caused shrinkage of prostate tumors [37]. Similar to normal prostate cells, prostate cancer cells are androgen dependent at the outset with a normally functioning androgen receptor (the receptor only responds to androgen) [37]. These initial studies on the dependence of the prostate gland on androgens formed the principle behind androgen deprivation therapy. There are several avenues that can be utilized to remove androgens, and the decision to do so is largely dependent on the stage or severity of cancer. Early cancers commonly target 5alpha($\alpha$)-reductase inhibition in an effort to decrease levels of the active hormone dihydrotestosterone (DHT). In fact, in the Prostate Cancer Prevention Trial (PCPT), finasteride (a 5$\alpha$-reductase inhibitor) has been reported to lower the incidence of prostate cancer in men by 25% [38]. The initial concern about finasteride causing more severe cancers was refuted. However, despite this, the FDA has published advice concerning the use of finasteride and an increased risk of high-grade PCa [39]. Interestingly, the PCPT study is the one which Brasky *et al.* used to extrapolate data for their recent findings regarding n-3 PUFA and prostate cancer [40]. AR antagonists can also be used to block both testicular and adrenally produced androgens from binding to the androgen

receptor and initiating gene transcription [41]. Additionally, testicular-derived androgen production can be blocked by luteinizing hormone (LH) inhibition [42]. For more aggressive cancers, these previously mentioned methods can be used in combination blocking both androgen production and their actions [41].

Despite the fact that most prostate cancers respond well to androgen deprivation, prostate cancer cells develop survival mechanisms involving the androgen receptor that can render these treatments ineffective; this represents the progression from androgen sensitive to androgen insensitive disease. Central to this role, the AR develops abnormal signaling functions that are not seen in normal AR signaling in androgen sensitive prostate cancer progression. There are several mechanisms through which the androgen receptor can participate in androgen insensitive prostate cancer. Amplification of the AR creates a hypersensitivity to even low amounts of circulating androgens [43]. The AR can also become promiscuous; mutations of the ligand binding domain can allow for aberrant AR activation by factors other than androgens (i.e: estrogen or other steroid hormones) and increased AR activity can also occur when such mutations are found in coregulators [44]. The AR can become an outlaw receptor - receptive to transactivation, phosphorylation and activation by other signaling pathways and peptide growth factors (i.e: insulin growth factor -1 (IGF-1) in the absence of androgen [45]. Other modified functions of the AR include activation of other pro-proliferative and anti-apoptotic factors that can bypass the AR pathway, eliminating any dependence on androgens for these physiological effects. For example: bcl-2, an anti-apoptotic protein, is not expressed in normal prostate epithelium, however in the absence of AR function, bcl-2 is produced [46]. Thus, effective treatment is dependent in the ability of the AR to develop these modified survival mechanisms.

The topic of testosterone production is worthy outside the realm of AR function in androgen insensitive prostate cancer. It is well known that the testes are the main site of testosterone production. Therapies that reduce testosterone production by targeting the testes do not affect adrenal androgen synthesis which is increased in androgen insensitive prostate cancer, thus compensating for the reduced testicular androgen production. In such cases, a broad spectrum cytochrome P450 inhibitor may be recommended, although their use may introduce side effects so extensive that their utility is limited [47].

### 2.2. Molecular biology of prostate cancer

Hanahan and Weinberg, in 2011, summarize the unique biological capabilities of cancer [48]. The hallmarks of cancer outlined within include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Interestingly, in this updated review, genomic stability and inflammation are noted as underlying these characteristics as they expedite acquisition and foster multiple functions, respectively. Additionally, two emerging hallmarks - reprogramming of energy metabolism and evading immune destruction are discussed, as well as the dimensions of tumor complexity including the ability of these acquired traits to create the tumor microenvironment [48]. We agree that the recognition of all of these concepts, alone or in combination, provide guidance in the development of new cancer treatments. The

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paragraphs to follow are meant to provide a basic overview of some of the aforementioned concepts and their relevance in prostate cancer. While we have not specifically discussed tumor microenvironment or two of the above-mentioned emerging hallmarks and their relevance to prostate cancer in this chapter, such topics of importance subsequently emerge in broader areas of discussion.

#### *2.2.1. Sustaining proliferative signaling*

Although the host may provide a source of growth factors, such are not essential for the survival of cancer cells. Cancer cells can evade the requirement for exogenous growth factors in several ways, including the production of their own growth factors. Cancer cell mutations can result in constitutively active growth factors and growth factor receptor pathways. Thus the initiation of the signaling cascade occurs without the required binding of the growth factor ligand to the receptor; cell growth occurs in the absence of ligand. In this regard, mutations in both the ras family and epidermal growth factor (EGF) receptors are seen in prostate cancer [49]. Another example of such includes autocrine and/or paracrine production of IGF-1 by prostate cancer cells [50], as stromal prostate cancer cells and the adjacent epithelium produces IGF-1 and expresses IGF-1 receptor, respectively.

#### *2.2.2. Evading growth suppressors*

Pathway hypersensitivity can result when cancer cells over-express growth factor receptors in the presence of normal physiological levels of a growth factor. PCa cells frequently increase their expression of Human Epidermal Growth Factor Receptor (HER)2 and the androgen receptor [51]. In this way, small amounts of androgen that can direct large increases in the expression of pro-carcinogenic genes or AR regulated gene expression can even occur in the absence of androgens.

#### *2.2.3. Resisting cell death*

The body eliminates damaged or dysregulated cells through intrinsic mechanisms including tumor suppressor pathways responsible for the mediation of DNA repair, cell cycle arrest, apoptosis and senescence [52]. However, some of these damaged cells harbor mutations and/or have the ability to produce factors that allow them to survive. Proliferation of these cells, if not kept in check, results in tumors. The expression of Bcl-2, an anti-apoptotic protein indicates that a cell may not respond to cell death cues. Production of Bcl-2 is high in prostate cancer cells [46], rendering them resistant to apoptosis.

#### *2.2.4. Enabling replicative immortality*

The balance between growth and anti-growth signaling results in normal cell growth. Differentiation of cells occurs only when cells halt the growth program. Thus, rapidly proliferating (undifferentiated) cells form a mass of cells commonly referred to as a tumor. Such unimpeded

growth is usually less differentiated than normal cells. Under normal conditions, transforming growth factor beta (TGFβ) is an anti-growth factor stimulating epithelial cells to a terminally differentiated state. However, this property is dysregulated in prostate cancer development [53]. As TGF-β signals through retinoblastoma (Rb) tumor suppressor gene [54], it is assumed to cause an increase in cell cycle inhibitors, such as p21. Thus, the loss of Rb in prostate cancer renders the pro-differentiation, anti-proliferation effects of TGF-β null [55].

The ability of cancer cells to replicate many more times over their natural lifespan distinguishes them from normal cells. Inherent defects in prostate cancer cells, such as losses of cell cycle inhibitors, including p21 or p27 enable this phenomenon. As with other cancer cell types, dysregulated telomere maintenance provides another explanation. The small DNA fragments at the ends of chromosomes are known as telomeres [56]. Telomere shortening to a desired length, following cell reproduction, signals a cell to stop replicating. However, cancer cells express elevated levels of telomerase, thus allowing cancer cells to replicate beyond their normal programmed number of replications by maintaining telomere length [38].

### *2.2.5. Inducing angiogenesis, activating invasion and metastasis*

In order to expand in size, cancer cells require the support of a vasculature system. Judah Folkman's pioneering work in cancer revealed this unconditional requirement for sustained angiogenesis in cancer [57], accompanied by increased requirements for oxygen and nutrient supply, as well as waste removal; the latter is due to the ability of cancer cells to rapidly proliferate. In prostate cancer, and other cancers, vascular endothelial growth factor (VEGF) production is elevated [58]. This paracrine factor stimulates nearby endothelial cells and epithelial cells to form irregular vasculature associated with tumorigenesis. This confirms the notion that cancer cells produce factors that recruit endothelial progenitor cells and epithelial cells are capable of forming primitive vascular channels.

To further survive along the cancer cascade, and even in the presence of increased angiogenesis, cells must metastasize as the tissue of origin is capacity limiting with respect to tumor burden [57]. Metastasis of cancer cells involves degradation of the basement membrane at the site of origin and penetration to the bloodstream and/or lymph. Although few cells survive this journey through the lymph and blood stream, those that penetrate into the tissue of destination complete the metastatic cascade by invading and colonizing to secondary sites.

Cadherins, a class of membrane receptors, can undergo isoform switching (known as "cadherin switching") during normal development which allows cell types to segregate from one another. Within this family, particular cadherin members promote cell motility and invasion control via growth factor receptor signaling and pathway signaling components [59]. In tumor cells, this activity ceases resulting in aggressive tumor cells with an ability to escape the origin of the tumor and metastasize.

Therefore, the functional significance of these specific cadherins and cadherin switching provides insight into the molecular mechanism underlying tumor progression and offers an opportunity for the development of novel molecular targets for anti-cancer therapy. Integrity

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of the basement membrane and maintenance of epithelial integrity involves E-cadherin (epithelial calcium-dependent adhesion), a Type 1 transmembrane protein. The extracellular component of E-cadherin is responsible for homophilic interactions, while the cytoplasmic component of E-cadherin binds to beta (β-) and gamma (γ-) catenin [60]. While the loss of E-cadherin signifies the ability of a cancer cell to leave the prostate [61], it would be remiss to think that this loss alone is responsible for tumor cell invasion considering the dimensions of tumor microenvironment [48]. Aside from changes in cell-to-cell and cell-to-matrix adhesion, tumor invasion involves additional cellular events including cell migration and proteolytic degradation of the extracellular matrix (ECM), and other events which alone or in combination can affect cell signaling pathways. The initial suggestion that E-cadherin downregulation may result in the activation of specific signaling pathways triggering tumor cell invasion has been explored. Because of its dual role as a cytoplasmic cellular adhesion complex component and its fundamental role in Wnt-mediated signal transduction, β-catenin has emerged as a prime contender for activating such signaling pathways. Additionally, levels of matrix metalloproteases (MMPs) also increase in prostate cancer and degrade stromal and basement membrane components, enabling cancer cells to escape the tissue of origin [62]. Overall, such factors that allow the progression of the metastatic cascade, rather than the original tumor, are responsible for cancer death.

### *2.2.6. Inflammation*

Although inflammation has been long recognized in the development of cancer [63, 64], *chronic* inflammation has emerged as one of the enabling characteristics of human cancer [48]. Chronic inflammation can be caused by numerous factors including infectious and non-infectious agents and / or other environmental aspects including hormonal changes and dietary interventions [65, 66]. Fostering multiple functions related to cancer, inflammation cuts across broad areas of study including genetics, epidemiology, molecular pathology, histopathology, immunology and animal modelling to name a few. In other words, it would be difficult to capture an association between cancer and inflammation by way of any one over-arching theory. A diverse amalgamation of evidence exist which points to the role of the inflammatory response in physiological maintenance including tissue homeostasis and the healing process involved succeeding injury or damage. Rakoff-Nahoum (2006) has provided an excellent overview of this dual role of inflammation in tumor development [52]. The inter-relationship of cancer and inflammation with respect to inflammatory cells (and their mediators) and signaling pathways involved that have been published in the past suggests that the inflammatory system can affect tumor development and inhibit the development of cancer [67-72]. The topic of inflammation in prostate carcinogenesis has been recently updated in a thorough review authored by Sfanoes and DeMarzo (2012) encompassing recent advances in prostate risk and development with respect to prostatic inflammation stimuli, immunobiology, inflammatory pathways and cytokines, proliferative inflammatory atrophy as a risk factor lesion to prostate cancer development, and the role of nutritional or other anti-inflammatory compounds [66]. While this exceptional review articulates the vast effects of inflammation in prostate cancer, we will aim to highlight evidence to date as related to some promising anti-inflammatory natural products while focusing on PUFA.

## 3. Promising anti-inflammatory natural products in PCa

The role of inflammation in prostate cancer etiology stems from studies accessing the relationship between intake of anti-inflammatory dietary compounds and prostate cancer risk. Epidemiological evidence associates or significantly correlates consumption of tomato [73], soy and green tea [74, 75] with decreased prostate cancer risk. Furthermore, animal studies confirm that the anti-inflammatory properties of both soy and green tea cause a decrease in prostate cancer [74, 75]. One study involving Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice fed a diet enriched with processed whole tomatoes reported benefits including increased survival, delayed progression from PIN to PCa cancer and a decreased incidence of poorly differentiated PCa cancer [76]. Prostate cancer cell lines treated with phytoestrogens, specifically genistein and daidzein, indicate a decrease in prostate cancer risk, however this effect seems to be linked to epigenetic modifications of DNA [77].

### 3.1. PUFA and prostate cancer

Interest in dietary fats and disease etiologies has emerged because of their known anti-inflammatory properties. Examples of dietary fat which are essential in a variety of mammalian biological processes and impact PCa include omega-3 (n-3) and omega-6 (n-6) PUFA [78, 79]. In published cell line and xenograft studies, n-6 PUFA (linoleic acid and arachidonic acid) typically exert a growth-promoting effect, while n-3 PUFA (EPA and DHA) have growth-inhibitory effects [80-85]. *In vitro* and animal studies suggest a trend of opposite effects for cancer development with respect to n-3 and n-6 PUFA. The n-3 PUFA, such as EPA and DHA, indicate a suppression of tumor carcinogenesis, however n-6 PUFA seems to promote tumor development. From an epidemiological perspective, evidence of an association between particular PUFA and PCa is inconsistent, with many studies reporting no association between dietary intake of n-3 or n-6 PUFA and the risk of PCa [86-92], however both pre-clinical *in vivo* and *in vitro* studies clearly indicate that there are biological mechanisms by which omega-3 PUFA can arrest growth in both PCa cells and tumors. Supplementation of n-3 PUFA in animal studies failed to produce an effect on prostate tumor growth or other markers of PCa progression and did not significantly reduce tumor growth in a PCa mouse model [93-96]. Treatment of human PCa cells with n-3 PUFA has been shown to consistently inhibit proliferation and /or increase programmed cell death, affect gene expression and deter properties of invasive human PCa in cells supporting the notion that EPA and DHA supplementation could preclude or limit the growth of prostate tumors [80, 85, 94, 97-102]. In general, and as found in a recent case-control study, while proinflammatory n-6 PUFA may present an increase in PCa risk, the anti-inflammatory properties of n-3 PUFA have been noted in their association with decreased risk [103]. Interestingly, this same study reported that mutations in the inflammation and mitogenesis related gene (COX-2), combined with low nutritional consumption of n-3 PUFA, had a higher risk of PCa; this risk was lowered with an increase in the dietary intake of n-3 PUFA [103]. Inflammatory gene expression is usually negatively associated with cancer stage and prognosis [104]. Despite these trends, a recent epidemiology study by Brasky (2011) contradicts the protective role of n-3 PUFA in PCa by reporting a positive association with

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DHA for high-grade disease[40]. Specifically the findings indicate that higher blood concentrations of n-3 PUFA are associated with increased risk of high-grade prostate cancer (Gleason score 8–10), and higher concentrations of n-6 PUFA were associated with decreased risk [40]. Dr. Brasky has indicated that these findings *"turn what we know—or rather what we think we know—about diet, inflammation and the development of prostate cancer on its head and shine a light on the complexity of studying the association between nutrition and the risk of various chronic diseases* [105]." Could it be that what is deemed good and promoted for heart and brain health may be essentially harmful to the prostate? Consider the following: Data analyzed to come to this vastly different conclusion was based on a subset of more than 3200 (half of which developed PCa throughout the duration of the study) from the overall study population of 19,000 [40]. This nationwide randomized clinical trial was initiated to test the efficacy of the drug finasteride to prevent prostate cancer and considered unique as biopsy was used to confirm the absence or presence of prostate cancer in all study subjects [105]. The original intent of the study was to prove the hypothesis that n-3 PUFA would reduce and n-6 PUFA and trans-fatty acids would increase prostate cancer risk, pre-empted by two important known factors; that n-3 fatty acids originating mainly in fish and their oil supplements have anti-inflammatory properties and chronic inflammation is known to increase the risk of several cancers. All this combined with an inconsistent history of studies and the fact that the mechanisms behind the influence of n- 3 PUFA on the risk of developing high-grade PCa have not been identified, warrants further research. Nonetheless, we are presented with evidence of substantive biological activity, and thus we must explore other mechanisms, aside from inflammation, that may play a greater role in the development of certain prostate cancers. To embark upon this currently debatable journey, there are key factors concerning prostate cancer and dietary interventions that should be considered, particularly n-3 PUFA. The sections to follow are meant to generate, by example and based on the current literature, some thoughts on how to proceed in this area of research to determine the actual role of n-3 PUFA in prostate cancer.

## 4. Looking forward: Considerations for further research in n-3 PUFA and prostate cancer

### 4.1. Prostate cancer signaling pathways and n-3 PUFA

Signaling pathways and their associated molecules often have a dual role in events such as homeostasis, tissue repair, and tumorigenesis. For example, the Wnt/β-catenin pathway is critical in maintaining steady-state proliferation and tumorigenesis of tissues [106]. Numerous studies to date indicate that n-3 PUFA treatment can affect cell signaling partially through AKT (protein kinase B), mTOR (mammalian target of rapamycin) and NF-κB (nuclear factor kappa B) associated pathways [94, 95, 99, 107]. Treatment with n-3 PUFA has been indicated in the inhibition of human hepatocellular carcinoma and cholangiocarcinoma cell growth by blocking the Wnt/β-catenin pathway [108, 109], and DHA treatment has been shown to inhibit the production of β-catenin in colon cancer cells [110-112]. However, the effects of n-3 PUFA on Wnt/β-catenin signaling in PCa remain largely unknown. A single study in PCa indicates

that fat-1 gene (cloned from *Caenorhabditis elegans*) that encodes for a n-3 PUFA desaturase (which converts n-6 PUFA to n-3 PUFA) expression was shown to reduce phosphorylation of glycogen synthase kinase-3β (GSK-3β), a major element in the Wnt/β-catenin pathway, resulting in subsequent down-regulation of both β-catenin and cyclin D1 thereby inhibiting PCa cell proliferation [113].

*4.1.1. An introduction to Wnt/β-catenin signaling*

The Wnt family constitutes 19 highly conserved glycoprotein members in mammals [114]. The most significant molecule implicated in the canonical Wnt cascade is β-catenin, a cytoplasmic protein regulated by a multi-protein destruction complex made up of Axin, adenomatous polyposis coli (APC), GSK-3β and casein kinase 1 (CK1) [115]. In the absence of Wnt signaling, the destruction complex stimulates the phosphorylation of β-catenin by GSK-3β, leading to subsequent ubiquitination and proteasomal degradation [116, 117]. Conversely, in the presence of Wnt signaling, Wnt ligand-frizzled (FZD) binding causes disheveled (DVL) protein dissociation of the β-catenin destruction complex, blocking the phosphorylation of β- catenin, and leading to β-catenin accumulation in the nucleus [116, 117]. Nuclear β-catenin functions as a transcription co-factor of the Tcf/Lef family and leads to the activation of Wnt target genes implicated in cell proliferation, differentiation and apoptosis, including c-myc, cyclin D1, Akt, MMP-7, and AR [114, 116-122].

*4.1.2. Using Wnt/β-catenin signaling as a model to determine the pathogenic role of n-3 PUFA in prostate cancer*

Modular in nature, the activity of the Wnt/β-catenin pathway can be modified through several points of intervention. The fundamental event in Wnt/β-catenin signaling occurs in the nucleus and is the result of stabilized β-catenin recruiting Tcf/Lef transcription factors that modulate the expression of oncogenes, such as c-myc and cyclin D1[123-128]. Abnormal expression of β-catenin has been observed in up to 71% of prostate tumor specimens [129-131], is elevated in more than 20% of advanced prostate tumors [132] and is associated with advanced stage PCa [130, 133, 134]. APC alterations alone are considered prognostic with respect to an unfavorable outcome [135] even though it varies with respect to inactivation in PCa reporting somatic loss ranging from 2– 43% [132, 136-138] and promoter hypermethylation in up to 90% of PCa [135, 139, 140]. Mutations in Axin-1 have been identified in 14% of advanced PCa, and several Axin-1 mutations and polymorphisms have been noted in PCa cell lines [138]. DVL-1, is a pathway regulator involved in Axin recruitment and inactivation, is significantly overexpressed in PCa and has also shown a positive correlation with PCa grade [141]. Pathway activators, including WNT-1, -2, -5A, and -6 have been highly overexpressed in primary PCa compared to normal prostate [142-145], and WNT-1 and -2 have been indicated as having a role in invasive PCa [143, 146]. Conversely, but also resulting in stabilized β-catenin, pathway inhibitors are commonly downregulated in PCa. Dickkopf-related protein (DKK)-1 expression is lower in PCa tissue in comparison to normal prostate tissue samples and furthermore is significantly reduced during progression to metastasis [143, 147]. Secreted frizzled-related protein (SFRP)- 1 and DKK-3 show decreased expression in prostate tumor cells [148-151]

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Downregulation of SFRP-1 was also reported in PCa cell lines [152]. It is interesting to note that SFRP-4 overexpression is associated with decreased proliferation, decreased anchorage-independent growth and decreased invasiveness in a PCa cell line, and additionally predicts a good prognosis in PCa [153, 154]. WIF-1, another Wnt antagonist is downregulated at the mRNA (23%) and protein level (64%) of PCa [155]. Recently, FZD-3 inhibition by soy protein was shown to suppress the growth of PCa cell lines [156]. Similarly, FZD-5 was highly upregulated in the prostate tissue of advanced PCa cancer patients and was downregulated by zoledronic acid in PC3 cells, but not DU145 cells [157]. Appropriate steps to determine the role of n-3 PUFA in prostate cancer may include investigating whether n-3 PUFA treatment will affect subcellular gene expression and localization of β-catenin. Beyond confirmation of the molecular mechanism(s) by which n-3 PUFA inhibits Wnt/β-catenin, subsequent experiments could target degradation of cytoplasmic β-catenin and activation of the Wnt receptor complex. Table 1 summarizes dysregulation of numerous pathway components which may be responsible for PCa tumor development/progression and as such represents potential targets for further investigation to determine n-3 PUFA/Wnt/β-catenin interactions in PCa.

#### *4.1.3. Clinical significance of targeting the Wnt/β-catenin pathway in PCa with n-3 PUFA*

Despite the noted progress of hormone-based drugs as a therapy, PCa remains as one of the primary causes of cancer deaths worldwide [20, 101]. Thus, new and improved therapeutic strategies to prevent PCa and inhibit its progression are needed. In this regard, n-3 PUFA presents tremendous opportunity as a therapeutic intervention in PCa. There is enough convincing data available to show a positive correlation between Wnt/β-catenin activation and PCa progression. It is also well established now that n-3 FAs supplementation can impact PCa development and progression *in vitro.* However, whether these effects of n-3 PUFA on PCa can occur at least in part through inhibition of Wnt/β−catenin signaling remain largely unknown. Thus, the incorporation of variations as suggested above could start by targeting nuclear interaction and progressing back through the pathway to the point of surface expression interactions of the Wnt-FRZ-LRP Receptor Complex. Furthermore, AR expression is positively correlated with an increase in cytoplasmic/nuclear β-catenin levels in epithelial cells of the prostate [175]. β-catenin interacts with AR and acts as a co-activator of AR to increase its transcriptional activity in response to androgen [17]. This leads to activation of AR target genes implicated in PCa progression. Therefore it is possible that n-3 PUFA can inhibit aberrant expression and activation of AR. As β-catenin is considered to be a ligand-dependent coactivator of AR transcription [17], the effects of n-3 PUFA on AR expression and β-catenin/ AR crosstalk is an area requiring exploration.

A series of experiments to specifically target different components of the Wnt/β-catenin pathway as noted above would be a rational approach to determining the effects of n-3 PUFA in PCa. Goss's recent textbook reviews some innovative strategies that have been utilized to antagonize signaling at various levels of the Wnt/β-catenin signaling [176]. These range from blocking β-catenin-mediated transcription to modulating the catenin destruction complex to attenuating extracellular signaling through the Wnt receptors [176]. Figure 2 illustrates the Wnt/β-catenin pathway and could serve as a model to incorporate multiple targets of interest

| Pathway Component | Alteration(s) | References |
|---|---|---|
| **A. Wnts** | | |
| Wnt-1 | Increased Expression | [142] |
| Wnt-2 | Increased Expression | [143, 146, 158] |
| Wnt-5A | Increased Expression | [131, 143, 158] |
| Wnt-6 | Increased Expression | [144] |
| **B. Secreted Wnt Antagonists** | | |
| SFRP-1 | Decreased Expression | [148, 149] |
| SFRP-4 | Decreased Expression | [153, 154] |
| WIF-1 | Decreased Expression | [155] |
| DKK-3 | Decreased Expression | [150-152] |
| DKK-1 | Decreased Expression | [143, 147] |
| **C. Wnt Receptors/coreceptors** | | |
| FZD-3 | Increased Expression | [156] |
| FZD-5 | Increased Expression | [159] |
| **D. Pathway Regulators** | | |
| DVL-1 | Increased Expression | [141] |
| **E. Destruction Complex Components** | | |
| β-catenin | Nucleus/cytoplasm accumulation | [129-132, 134, 142, 160-163] |
| APC | Decreased or Loss of Expression | [132, 134-137, 139, 140, 164, 165] |
| GSK-3β | Increased activity | [165-170] |
| Axin-1 | Increased Expression | [138] |
| **F. Tcf/Lef Transcription Factor** | | |
| Tcf-4 | Increased Expression | [142, 171] |
| **F. Genes** | | |
| CyclinD1 | Upregulation | [123, 124] |
| c-myc | Upregulation | [125-128, 172-174] |

**Table 1.** Components of the Wnt/β–catenin Pathway Implicated in PCa: Alterations Of Primary PCa Tissues and PCa Cell Lines Compared to Normal Prostate.

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to be explored in n-3 PUFA and PCa. Therefore, research focusing on targeting components of the Wnt/β-catenin pathway or other cellular pathways as a means of inhibiting PCa development and progression with a promising prospect, n-3 PUFA seems reasonable.

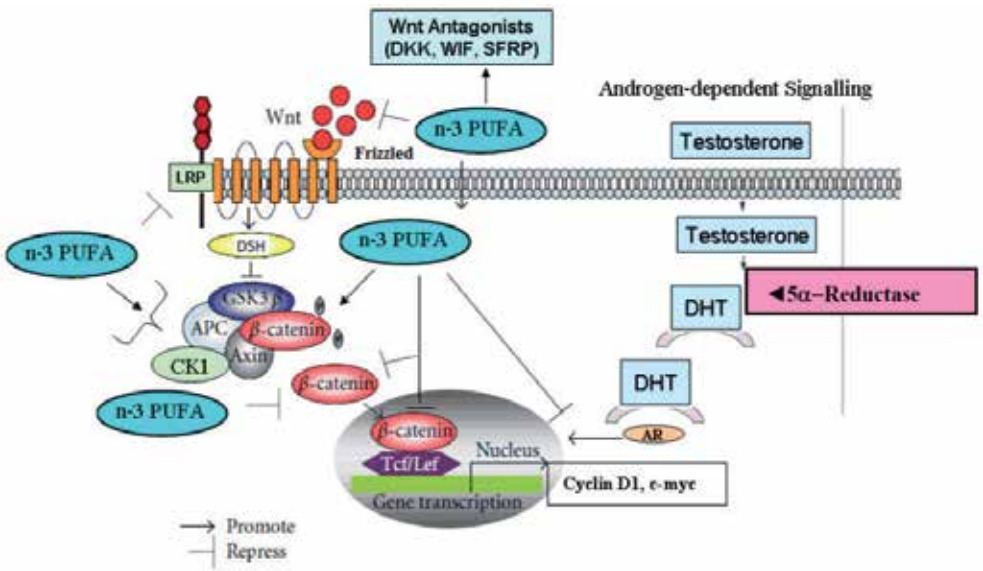


**Figure 2.** Tobin, GA. 2012. Adapted from articles under the Creative Commons Attribution License Copyright © 2011 Chi-Tai Yeh *et al*. [177] and from Lattouf *et al*. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Urology [178, 179]. Copyright © 1969). A hypothetical diagram of n-3 PUFA and n-3 PUFA/ androgen receptor induced inhibition of Wnt/β-catenin signaling pathway in PCa (directly and indirectly). When the Wnt signal is absent, a multi-complex destruction unit consisting of CK1/2, GSK3β, APC, and Axin forms the target for ubiquitination and degradation, namely a hyper-phosphorylated β-catenin. In the presence of Wnt, Fzd receptors recruit DVL to the plasma membrane to inactivate Axin. Overall, binding of Wnt ligand to a Frizzled/LRP receptor complex leads to stabilization of β-catenin. Subsequent interaction with nuclear Tcf/Lef proteins, increases the expression of genes such as cyclin D1 and c-myc. Additionally, as noted above the effects of n-3 PUFA on AR expression and β-catenin/AR crosstalk should be considered.

## 5. Choosing a model: *in vitro* and *in vivo* prostate cancer models and their relevance to human disease

Unpredictable pre-clinical models have been credited for the absence of effective treatments in Phase II and III trials [180, 181]. In fact, up to 70% attrition has been reported in Phase II trials; lack of efficacy accounts for about 30% of failures [180]. In the past, drug efficiency was normally assessed during the preclinical phase by xenografts of human prostate cancer cells. The limited number of cells lines that have been utilized in the past mostly include Prostate Cancer (PC)3, Lymph Node Carcinoma of the Prostate (LNCaP) and DU145 (DU-145). *In vivo* (ectopic or orthotopic xenograft models) and *in vitro* studies employing such remain at a disadvantage as these cells lack features found in chemically-induced cancers. The relevance

of such models to human prostate cancer is debatable. Nonetheless, these models are valuable in initial research and preclinical assessments. While disease heterogeneity of prostate cancer continues to challenge the development of clinically relevant models, prostate cancer treatment approaches are reliant upon the development and incorporation of relevant preclinical models to confirm appropriate therapeutic targets and biomarkers. Perhaps the most significant milestone and best solution to date in providing researchers with a clinically relevant model, is the evolution of mouse models designed to intentionally inhibit or express a specific gene function by introducing foreign DNA. Over the past 15 years, these Genetically Engineered Mice (GEM) have become valuable tools in studying any combination of oncogenes expressed in specific tissues or conditionally through the tissue-specific removal of tumor suppressors [182-186]. The goal of GEM modeling is to render the best clinical and molecular characteristics of human cancer.

### 5.1. Prostate cancer cell lines

An exhaustive list of prostate cancer cell lines is freely available via the British Columbia Cancer Research Centre's Prostate Cancer Cell Line Database and Health Canada (website: http://capcellines.ca). This site was created to provide prostate cancer researchers with valuable information regarding cell lines to be utilized in their research. Information is available for 114 cell lines in total with information inclusive of origin, tumor forming ability in mice, doubling time (and other relevant growth factors), karyotype, as well as the status of ribonucleic acid (RNA) and protein for androgen receptor (AR), Prostate Specific Antigen (PSA), prostate-specific human kallikrein (hK2) and creatine kinase (CK). Prostate cancer xenograft models are also included as well as other relevant markers, too numerous to mention. This site conveniently includes cell lines deemed controversial in nature as well as those that have been reported as contaminated. It would seem that currently, and based on a recent search of PubMed, that the most widely used prostate cancer cell lines include PC-3, LNCaP and DU145.

Cell culture experiments provide a practical and financially viable means of investigating PCa research questions by providing preliminary and mechanistic data that justifies further research. Cell culture approaches do have limitations. In general, consider that the conditions represented in cell culture are artificial. The ideal concentrations of factors replicated to characterize the ideal environment are by no means representative of physiologic conditions. Combine this with the fact that cell culture experiments often utilize a single, clonally identical cell line of similar origin in identically constant conditions (i.e: flask culture). This is especially problematic in prostate cancer research due to the complexity of the interconnected paracrine and endocrine communications system between epithelium, stroma and endocrine organs (i.e: the pituitary) [52]. This use of a single cell type removes the ability to detect effects that a distant organ may have on the agent being tested. A co-culture *in vitro* model may prove to be a more reasonable approach. Additionally, the epithelial layer of the prostate is surrounded by a stromal layer containing fibrobasts and blood vessels, among other cell types. In cell culture models, communication of surrounding and distant tissues is not represented. In this manner, the effect of a therapeutic to surrounding and distant normal tissue is speculative. Further-

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more, cell culture represents only one end of the carcinogenic spectrum as it is often conducted on transformed cells. Aside from the ability of such cells to develop various escape / survival mechanisms, this concept negates long-term exposure. Throughout a person's lifespan, exposure to agents may have varying effects prior to, during or after the transformation process to neoplastic tissue.

Choosing an appropriate cell line is largely dependent on the type of study being conducted. For example, based on the model of study represented in this chapter, the LNCaP line would be an appropriate choice to study n-3 PUFA and β-catenin as these cells are androgen sensitive, prostate-specific antigen (PSA) positive, and maintain malignant properties, even though they have low metastatic potential [187]. Furthermore, this cell line endogenously expresses β-catenin [188] and thus represents an ideal tool to elucidate the interactions of n-3PUFA/ β-catenin in PCa.

### 5.2. Animal models of prostate cancer

Although animal experiments are both costly and time-consuming, they are vital in the transition to Phase 1 clinical investigations. As PCa occurs naturally both in dogs and in certain strains of rats, these species represent options for animal modeling [189]. Although the dog most closely resembles human PCa characteristics [190], their use is limited and unrealistic for a variety of reasons including androgen independent tumor growth, long latency period, prohibitive costs, long gestation period, and difficulty of genetic manipulation [189]. Some strains of rats, although well characterized and capable of developing a wide range of PCa phenotypes [191], present different issues including scarcity of tumors, variability in phenotypes, long latency periods, and inability to represent metastatic disease [189]. Utilization of genetically engineered rat models for PCa will likely increase as the development of knockout rats advance [192]. In the meantime, despite anatomical differences between the murine and human prostate gland [189] and other weaknesses, mouse models have emerged as the choice for investigating the stepwise progression of PCa. Compared to earlier *in vivo* prostate cancer experiments, where rodent models were exposed to carcinogens or hormones, recent technological advances (i.e.: tissue specific promoters and conditional gene deletions) have provided multiple models for researchers to investigate genetic defects [189]. Each model has its strengths and weaknesses. For example, the transgenic mouse models of PCa that express the Simian Virus 40 (SV40) early region have a highly prognostic gene signature for cancer, however mouse prostate expressing the large probasin promoter directed by SV40-large T-antigen (LPB–Tag) is not consistent in its ability to mimic the full spectrum of PCa as it only progresses to mPIN and rarely results in progression to adenocarcinoma formation [193]. Likely the most popular mouse model, TRAMP is based on such technology and develops cancer as a result of SV40 T antigen expression resulting in many genetic defects including p53 and Rb (tumor suppressor genes) loss [54]. Because cancer is not the result of a single genetic mutation, this multi-hit effect is desirable. However, consider that certain aspects of this model may not be relevant to human disease, including viral transformation. Additionally this model does not follow the normal human prostate cancer course as it is very aggressive and progresses to advanced cancer very rapidly. Consider that it may take up to 20 years for PIN to

develop, 10 or more years to advance from PIN to HGPIN and then to an early form of latent cancer, while a clinical diagnosis of PCa may occur anywhere from three to 15 years [11].

It would be reiterative to include a thorough review of mouse models utilized in prostate cancer research following the publication of such excellent work by both Valkenburg *et al.* [189] and Hensley *et al* [194]. In this section we will summarize commonly utilized transgenic mouse models; later we will discuss the reliability of mouse models to study disease effects, specifically n-3 PUFA. Table 2 summarizes the characteristics of several commonly used transgeneic mouse models.

| Model | Genetic Manipulation | Characteristics | Reference |
|---|---|---|---|
| Apt121/Rbf | Fragment of SV40 Transgene to dominantly inactivate Rb | PIN at age 4 weeks and micro-invasive carcinoma at age 8 months. | [195] |
| PTEN (Phosphatase and tensin homologue deleted on chromosome 10) | Germline Knockout | Invasive carcinoma age 30 weeks; metastasis to lymph node and lung. | [196] |
| | Conditional Knockout | High grade PIN with advancement to invasive metastasis. | [194] |
| Hi-Myc (high Myc transgene expression) | Myc transgene Expression | PIN at age 2 weeks and invasive carcinoma at age 6-12 months with lymphovascular invasion. | [197] |
| LADY (LPB promoter driving the large-T antigen) | SV40 Trangene Expression | Neoplasis by age 10 weeks. Undifferentiated adenocarcinoma and invasive neuroendocrine tumors from age 5 - 9 months and metastasis to lymph node, liver and lung. | [194, 198] |
| Nkx3.1/Pten | Compound Deletion (Pten(+/-) Nkx3.1 (-/-)) | High-grade PIN at age 6 months; invasion by age 2 months and metastasis to lymph node. | [194, 199] |
| TRAMP (TRansgenic Adenocarcinoma of the Mouse Prostate) | SV40 Transgene Expression | Progressive PIN at age 6 weeks. Invasive adenocarcinoma and lymph node, liver, lung and skeletal metastasis by age 6 months. Large, poorly-differentiated neuroendocrine tumors by age 16 weeks. | [182, 194] |

**Table 2.** Common Transgenic Murine Models of Prostate Cancer

In choosing a mouse model, one must consider whether the study is aimed at treatment or prevention and the ability of a model to produce prostate phenotypes or other key features

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relevant to human disease. In dietary intervention studies, such as n-3 PUFA, it is important to consider whether or not models are resistant to the tumor suppressive effects of such treatments. For example, Smolinski (2011) indicates that the TRAMP model may not be ideal for studying the effects of bioactive lipids on prostate carcinogenesis. In fact, in the context of a high fat diet, including fish oil and corn oil as the primary source of lipids, the TRAMP model is reported to be resistant to the tumor suppressive effects of n-3 PUFA [200]. Thus, choosing an inappropriate mouse model may negate significant findings and lead to the conclusion that certain dietary interventions have no effect on a disease when in fact they may be responsible for disease risk or progression. When engaging in novel or controversial research such as discussed in this chapter, a rational tactic before confirming a mouse model may include the completion of a pilot study to ensure that mice are not resistant to the protective effects of n-3 PUFA and to confirm an appropriate cohort size. The goal should be to identify at least a statistically significant 15% difference in mean tumor size within an 80% confidence interval. Based on the proposed research model discussed in this chapter, the transgenic 12T-7s/Catnb $^{lox(ex3)}$/ PB*Cre4* (designated as LPB–Tag/DA β-catenin) mouse would likely be ideal and was developed recently as a model to study the expression of stabilized β-catenin in PCa and represents the full spectrum of PCa progression to invasive adenocarcinoma [169]. Additionally, this model represents AR activity and offers a reasonable study period, with an end-point of 20–22 weeks [169].

## 6. n-3 PUFA specific factors that make a difference: Source, purity and EPA: DHA ratio

If the general question on n-3 PUFA and PCa surrounds whether or not there is an increase or decrease in PCa cancer risk, then consideration of confounding factors for n-3 PUFA intake, including source, purity and ratio, should be addressed primarily. The foremost sources of essential fatty acids for the majority of studies are fish derived. Consider that both animal protein [201] and accumulated environmental pollutants [202] both have been linked to prostate cancer. In this regard, it is fitting that the development of n-3 PUFA as a pharmaceutical is already underway for other indications as the approval process normally removes the risk of contamination by methyl mercury, arsenic or other pollutants that are often seen in ocean or land based food sources protein [201]. Currently, there are three commercially significant formulations of pharmaceutical grade n-3 PUFAs and numerous over the counter supplements available for potential use in PCa. The noted differences between all of these formulas are purity, source and/or the ratio of EPA to DHA. Lovasa has received United States Food and Drug Administration (USFDA) approval to lower very high triglyceride levels (hypertriglyceridemia) [203]. In other major markets outside the United States, including Europe, Lovasa is known as Omacor, and can be prescribed to patients as a montherapy for hypertriglyceridemia, in combination therapy with a statin for mixed dyslipidemia, or as a secondary preventative therapeutic following myocardial infarction [204]. The expiration of GlaxoSmithKline's patent for Lovasa in September [2012] will likely increase availability of generic replacements. Such equally effective, but lower priced versions could supply markets

for already proven indications as well as for subsequently approved disease indications, including for example, PCa. AMR-101, also marketed as Vascepa, received USFDA approval in July, 2012 [205]. Similar to Lovasa, this approval was based on effective treatment of hypertriglyceridemia but with the added ability to impact people with high triglycerides; the latter indication has been filed and is under review pending outcomes in clinical trials [206]. Epanova, another promising n-PUFA formula, is undergoing clinical trials for similar indications as previously mentioned for Lovasa, and USFDA approval is forthcoming [207]. Other dietary supplements, containing various amounts and ratios of n-3 PUFA which are sold over the counter in the United States and Canada would likely be based on values issued by the Institute of Medicine and National Academies as described below. Such unregulated formulary preparations do not undergo clinical trials. Studies relating to n-3 PUFA pharmaceutical preparations have focused on treating cardiovascular disease with no clinical consideration for PCa. Albeit, men diagnosed with PCa have accepted the use of such nutraceuticals and thus there is a role in ensuring that this approach constitutes safe and effective therapies. Although studies around the prescription formulas have demonstrated a very good safety and tolerability profile, the focus has been in treating cardiovascular disease and thus there has been no concern over potential adverse effects for other diseases.

Another proposed explanation for inconsistent findings in the role of n-3 PUFA in prostate cancer relates to absolute intake versus ratio of n-3 to n-6 PUFA. The latter may be more relevant for prostate cancer risk considering that the recommended dietary ratio of n-6/n-3 PUFA for health benefits are variable and range from 1:1-4:1, yet dietary intake in such studies, relative to the Western diet could contain 10 or more times the amount of n-6 compared to n-3 PUFA [208]. Also consider that in gathering and thoroughly reviewing all information toward this chapter, we were unable to find an *official* recommended daily allowance for n-3 PUFA for adults or children. In Canada and the United States, the general public and health professionals (including healthcare policy makers and public health officials) use a set of five nutrient based reference values issued by the Institute of Medicine and National Academies for a wide variety of food and dietary applications [209]. These dietary reference intakes (DRI), are inclusive of components including: estimated average requirements (EAR), recommended daily allowance (RDA), adequate intake (AI), tolerable upper intake level (UL) and an acceptable macronutrient distribution range (AMDR). Considered to be the official standard for federal agencies, these values are used to issue dietary guidance or policy directives for the health and well-being of individuals. In the absence of a DRI specific to EPA and DHA, the National Academies have recommended an AMDR of 10% for another n-3 PUFA, namely alpha-linolenic acid (ALA) to be consumed as EPA and/or DHA equating to a much lower value (about 100 mg/day) than currently recommended by many groups worldwide. For example, a number of nutritionist and physicians recommend as much as 450 milligrams of n-3 PUFA in either DHA or EPA form per day to promote a healthy diet [79, 210], a fact alone which warrants further studies to determine DRI, specifically UL. More importantly, if the effects of n-3 PUFA represent different disease specifications, then it is crucial to establish DRIs for DHA and EPA individually, and in combination. Considering the contradictory evidence that excessive DHA may be a possible cause for some types of aggressive prostate cancer, the expansion of this field of research may want to revisit the incorporation of such basic factors as the ratio of EPA to DHA

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as a beginning to rationalize some existing data. Altogether, in proceeding with such studies, it would be important to consider all forms of n-3 PUFA, as well as a variety of ratios and then proper incorporation of such into cell-culture media or animal models; all while considering relevant study models to reflect relevance to human disease. However, while supplementation in cell based and animal studies may produce positive results, such may not be relevant to actual human therapies.

The existing results for n-3 PUFA and prostate are contradictory, especially in the absence of consistent epidemiological data. Therefore, incorporation of n-3 PUFA related factors such as source, purity and EPA/ DHA ratios are crucial while employing *in vitro* and *in vivo* models representative of the entire PCa cascade.

## 7. Other considerations

### 7.1. Markers of tumor growth

Epidemiologic studies have shown that lower serum insulin-like growth factor (IGF)-1 levels and increased IGF binding protein-3 (IGFBP-3) levels are associated with decreased PCa risk [211]. Unlike humans, rodents do not have a PSA counterpart for monitoring prostate cancer initiation and progression, however prostatic secretory protein (PSP)-94 has been established as a serum tumor marker [212]. However, measurement of mouse serum for IGF-I, IGFBP-3 and PSP-94 can be assayed using enzyme-linked immunosorbent assay (ELISA) using mouse-specific antibodies and recombinant mouse standards (where applicable) and by methods previously described [213]. Therefore, such biomarkers could be used as indicators in both human and mouse studies in conjunction with other parameters and may be correlated with fatty acid analysis.

### 7.2. Fatty acid analysis: Serum blood levels of EPA and DHA

Selection of fatty acid concentration and methods of introducing fatty acids to cell cultures in such experiments are documented [214, 215]. Like most *in vitro* studies, data obtained may provide indicators of how to progress to the next levels of research. More relevant to human disease outcome, the fatty acid composition of serum (or plasma) phospholipid is an established valid biochemical marker for assessing the physiological status of various fatty acids including predictive correlations with the dietary intakes omega-3 PUFA, including EPA and DHA. [216, 217]. Serum fatty acid analysis by gas chromatography seems to be the standard for such experiments [218, 219] for both humans and animals.

## 8. Conclusions

As such, the features of prostate cancer, including long latency, high prevalence, significant mortality and morbidity, combined with the already accepted use of CAM, in particular n-3

PUFA presents an excellent opportunity for nutritional intervention. However, until we unravel this controversial enigma of how n-3 PUFA affects prostate cancer, much remains to be explored and examined. More research is required to support or refute the value of n-3 PUFA in prostate cancer. And thus the search continues as to how one should proceed with fatty acid intake, including supplementation, as a prophylactic or therapeutic. Currently there is not enough evidence to discount the noted beneficial effects of n-3 PUFA intake to prevent heart disease as they seem to outweigh any harm related to prostate cancer risk [40]. However, further research is required to clarify the precise mechanisms of n-3 PUFA and its role in human disease, in particular cancer. Without such, we cannot develop novel strategies for n-3 PUFA to target molecular pathways (inflammatory or other involved processes) in the prevention, development or treatment of prostate cancer or other cancers. Expansion of research in this area is warranted.

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Section 4

# Bioactive Compounds in Health and Disease

Chapter 9

# Mechanisms and Treatment of Photoaging and Photodamage

Marianna Kulka

Additional information is available at the end of the chapter

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## 1. Introduction

Damage caused by excessive, long-term sun exposure contributes to signs of ageing and is most readily recognizable on our external surface, the skin. The damaging effects of the sun are mainly caused by ultraviolet radiation (UVR) which, on earth, consists of UVA (320 to 400 nm) and UVB (280 to 320 nm). The third type of UV radiation, UVC, also called short-wave or ionizing radiation, is absorbed by gasses in our atmosphere, does not reach the earth's surface and does not normally contribute to photodamage of skin. Ozone is one of the gases that absorbs UVC – fortunately, since UVC's ionizing effects can significantly alter cellular structures and molecules. Although UVB and UVA radiation does not have enough energy to ionize atoms, it can alter chemical bonds in molecules, thus altering their structure and functions. Skin cells are particularly susceptible to alterations mediated principally by the creation of free radicals and oxidative stress and changes in DNA structure can result in constitutive expression of protocongenes and ultimately the development of cancerous skin lesions.

Through its changes of molecular structures, UV radiation causes a series of biochemical and structural changes in skin tissues. Some of these effects, such as the induction of vitamin D production, are actually very beneficial to skin health. Niels Ryberg Finsen, a Danish physician and scientist, was one of the first to show that light can be both beneficial and detrimental to human skin and his works *Om Lysets Indvirkninger paa Huden* ("On the effects of light on the skin"), published in 1893 and *Om Anvendelse i Medicinen af koncentrerede kemiske Lysstraaler* ("The use of concentrated chemical light rays in medicine"), published in 1896 created the paradigm on which photoaging and photodamage research was based for the next fifty years. Finsen won the Nobel Prize in Physiology in 1903 for his work on phototherapy in which he showed that certain wavelengths of light (UV in particular) could treat *lupus*

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*vulgaris*, cutaneous tuberculosis. However, UV radiation can also initiate some deleterious effects that fundamentally change the structure and function of the skin and its constituent components. In fact, it has been shown that repeated, excessive sun exposure can initiate and perpetuate an inflammatory response that, given time, causes breakdown of the skin's immunological functions.

This review will focus on the effects of sun exposure on the skin microenvironment – particularly the structural proteins and immune cells that are key players in the skin's protective functions. There is sufficient evidence to suggest that photodamage is associated with a low level of chronic inflammation that ultimately breaks down the skin's structure and results in some of the manifestations of skin aging. Consequently, skin treatments that target these processes are primarily focused on rebuilding the skin's architecture and promoting its biochemical regeneration. While some treatments have been found to have some beneficial effects to the underlying cellular immune systems regulating skin inflammation and regeneration, others may, in fact, be deleterious to these processes. In this review, I will discuss some naturally-derived bioactive compounds and formulations that have been shown or are suggested to be effective at modulating the pathophysiological changes associated with photodamage and photoaging. Bioactive compounds that can address these immunological changes are uniquely poised to not only reduce the damage associated with photodamage but may ultimately improve the resiliency and protective nature of the skin surface.

## 2. Skin is a protective organ

### 2.1. The three layers of defense

Healthy human skin is an important physical barrier to the environment and protects the body from a variety of insults. It is the largest human organ and comprises approximately 15 percent of a person's body weight and covers about 1.5 to 2.0 $m^2$ of our surface area. Skin is mainly composed of water (70%), protein (25%) and lipids (2%) forming an effective barrier to dehydration, pathogens and mechanical insults such as abrasion. In a mechanical sense, the main function of the skin is to serve as a protective barrier to keep good things in (water, nutrients and heat) and keep bad things out (pathogens, UV radiation, toxins).

In order to understand the essential functions of the skin, it is important to review the complexity of this large organ. The skin is composed of three main layers, the uppermost epidermis, the lower dermis and the hypodermis (Fig. 1). Each of these layers is composed of a specific set of cells that perform an essential function in that layer. The epidermis contains keratinocytes, melanocytes and Langerhans cells which are critical for the structural and functional integrity of the epidermis. Keratinocytes are the major population of cells and originate in the bottom-most stem-cell pool in the stratum spinosum (Fig. 1). The epidermis itself is composed of five layers: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. The uppermost layer of the skin, the keratinous stratum corneum, renews itself every 3-5 weeks and is principally composed of dead keratinocytes (see section below on keratinocytes for more detail). Keratinocytes have the capacity to

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renew themselves every 4 weeks but this process can be increased during injury or inflammation. The underlying dermis is a vascularized, connective tissue containing nerve endings, glands and lymphatic vessels and provides structural and nutritional support to the upper epidermal cells. The dermis is, in fact, the a mucopolysaccharide matrix composed of collagen and elastin fibers. In this layer, there is a great variety of cells including mast cells, fibroblasts, macrophages and Langerhans cells all of which contribute to the immune response observed in some skin pathologies such as atopic dermatitis. These cells also maintain and regulate the essential healthy functions of skin such as repairing injury, preventing infection, regulating circulation, preventing dehydration and providing nutrition. Beneath the dermis is the superficial fascia, or hypodermis, which is comprised primarily of fat tissue.

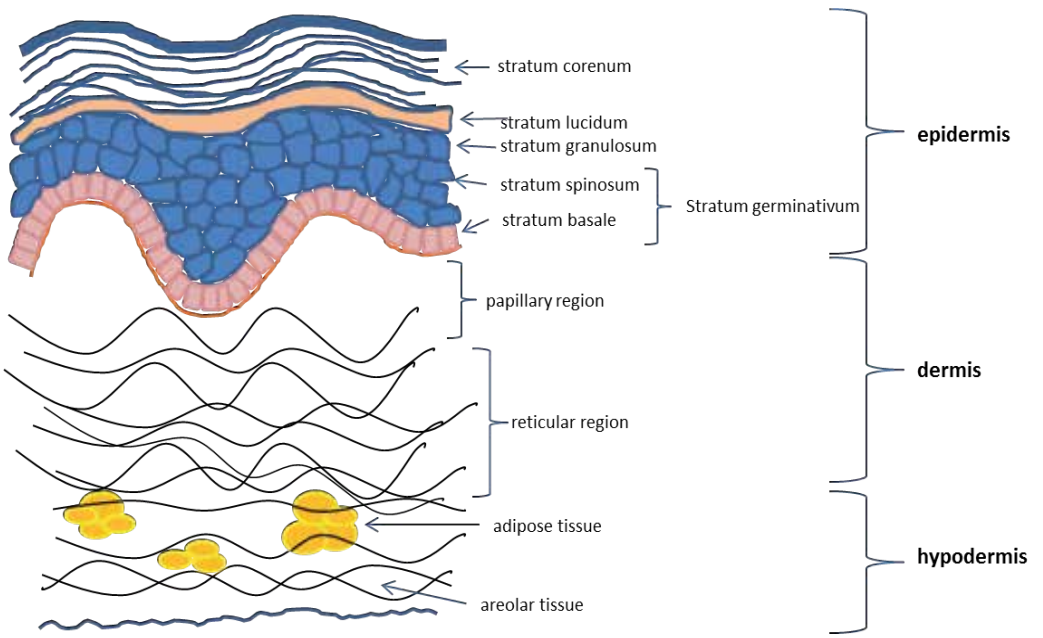


**Figure 1.** Structure of skin. Skin is comprised of three main layers as shown. The epidermis contains keratinocytes, melanocytes and Langerhans cells which are critical for the structural and functional integrity of the epidermis. The epidermis itself is composed of five layers: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. The strata granulosum, spinosum and basale all contain keratinocytes whereas the strata corneum and lucidum have only dead keratinized cells. The underlying dermis is a vascularized, connective tissue containing nerve endings, glands and lymphatic vessels. Beneath the dermis is the superficial fascia, or hypodermis, which is comprised primarily of fat tissue.

### 2.2. Melanin and melanogenesis — A protective response

As a result of its optical properties, the stratum corneum scatters photons and reflects UV radiation, providing some degree of protection. However, a portion of UV radiation penetrates this uppermost layer and can potentially damage the delicate structures beneath. Mel-

anin plays an important role in the skin's function as a protective barrier by absorbing UV radiation and thus protecting the underlying cells from DNA damage. The word melanin comes from the Greek word *melan*, meaning black. Melanin is a polymeric pigment derived from tyrosine and, in humans, it is synthesized in two main forms: the brown/black eumelanin and the cysteine-rich red/brown pheomelanin. The chemical structures of these two types of melanin are different resulting in different properties and functions (Fig. 2). Whereas eumelanin is composed of dihydroxyindole carboxylic acids, pheomelanin is a polymer of benxothiazine units. The carboxylic structure may account for pheomelanin's weak shielding capacity against ultraviolet radiation relative to eumelanin. In fact, pheomelanin has been shown to amplify UVA-induced reactive oxygen species (ROS)[1]. For this reason, pheomelanin is sometimes referred to as a UV-sensitizer and in some instances has been considered to have a weak carcinogenic effect in terms of melanoma formation.

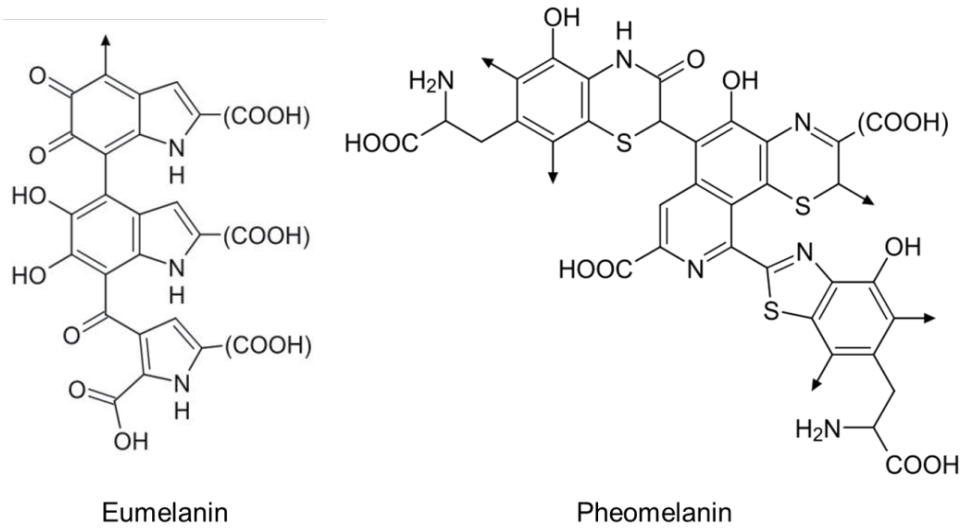


**Figure 2.** Chemical structure of eumelanin and pheomelanin.

Melanogenesis refers to the biochemical process by which melanin is formed. Melanocytes are the principal cell type responsible for the production and distribution of melanin in the skin. Since melanocyte differentiation and proliferation is linked very closely with their ability to produce melanin, melanogenesis is sometimes used to describe the process of melanocyte viability and reproduction. Indeed, many cosmetic products on the market that profess to alter or manipulate melanogenesis are actually cytotoxic to melanocyte and by killing healthy melanocytes they indirectly decrease the amount of melanin in the human epidermis. For example, hydroquinone (HQ), which is an U.S. Food and Drug Administration (FDA) -approved product, is used in 2 – 4 percent concentration as a skin-lightening agent but is considered to be cytotoxic to melanocytes[2]. Interestingly, some antibiotics used to

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treat ear infections, such as amikacin, are cytotoxic to melanocytes as well and one of its principal side-effects is ototoxicity[3].

One of the most obvious and controversial side-effects of exposing our skin to UV radiation is an increase in skin pigmentation. Certainly the issue of tanning and tanning beds has recently gained a great deal of attention because it has been shown that exposure to prolonged UV radiation (either from tanning beds or outside exposure) can cause melanoma and an effort is underway to limit the amount of time that people, especially children, spend tanning in any setting. The process of tanning is a protective mechanism and the skin's way of protecting its internal biochemical reactions from UV damage. Increased pigmentation is caused by increased production of melanin which is able to absorb some UV radiation and thus protect more sensitive biological pathways in the cell[4]. Epidemiological studies have shown that people who have high levels of constitutive pigment in their skin and are able to "tan well" are less likely to develop skin cancer[5], [6].

We still do not fully understand the precise mechanisms that initiate the malignancies associated with skin cancer and we certainly have an incomplete understanding of how photodamage and repair functions in people of different skin colors. However, mouse models and human studies have shown that albinos have a lower incidence of melanoma than their fair skinned counterparts[7] suggesting that melanin cannot just function as a protective UV-energy absorber. Recently, several groups have shown that melanin, especially pheomelanin (a yellow/red form of melanin), acts as a potent UVB photosensitizer to induce DNA damage and cause apoptosis in mouse skin. These groups believe that pheomelanin contributes to UV-induced DNA damage that is incompletely repaired. They further believe that this DNA damage maps to specific sequences of BRAF and N-RAS genes, both of which are frequently mutated in human melanoma[8], [9].

Some organic and inorganic compounds mimic the effects of melanin by absorbing or scattering UVR due to their particulate nature – often in the nanoscale. These chemicals are processed and made available as lotions, sprays, gels or other topical applications commonly known as sunscreens. There are 17 different chemicals that have been approved by the U.S. FDA as active ingredients in sunscreens. Oxybenzone (benzopehone-3) is the most widely used sunscreen chemical worldwide but other chemicals include octinoxate, avobenzone, and octyl salicylate. Some of the physical sunscreens are micro or nano-ionized to create particulate formulations capable of forming a protective UV barrier on the skin's surface. Titanium dioxide ($TiO_2$) are often used as a inorganic protective sunscreens in cosmetic products and to adjust their protective factor (SPF), they are often coupled with organic chemicals such as p-amino benzoic acid (PABA) which absorbs UVB but not UVA radiation. Recently, some have suggested that these nanoparticles (NP; around 200 nm) may penetrate the skin and modify the skin's immune-environment. However, several groups have shown that although nanoparticles are unable to penetrate healthy, intact human epidermis[10] they can significantly disturb cell functions through direct contact when the skin barrier is compromised[11], [12]. In fact, NP can reach the epidermal layer via hair follicles in certain circumstances such as laser-treatment[13] or inflammation[14], [15] but most compounds, although targeting the follicular region, do not permeate past the pilosebaceous region[16].

## 3. The production of melanin

### 3.1. Tyrosinase

Tyrosinase is responsible for the hydroxylation of monophenol and conversion of an *o*-diphenol to the corresponding *o*-quinone which then undergoes several modifications to become melanin (Fig. 3). Tyrosinases are ubiquitously expressed by both plants and animals, and although they may vary in structure, they all contain copper as an important cofactor. Human tyrosinase is a transmembrane protein and is sorted into specialized organelles in melanocytes called melanosomes such that melanin is ultimately synthesized and stored within these melanosomes[17]. Tyrosinase is an excellent example of convergent evolution in that although both animals and plants express tyrosinase, and although tyrosinase essentially performs the same function in all of these organisms, the structure and enzymatic requirements of these proteins are diverse. Regardless, tyrosinase regulates both the type and amount of melanin synthesized in the specialized cells that contain it. In humans, tyrosinase is encoded by the *tyr* gene which when mutated causes albinism in humans[18]. In Caucasians, tyr mutations can be identified in 56% of patients with autosomal recessive ocular albinism, characterized by reduced pigmentation of the eye[19].

### 3.2. Microphthalmia-Associated Transcription Factor (MITF)

Transcription of *tyr* is controlled by microphthalmia-associated transcription factor (MITF). MITF acts both as a transcription activator to promote expression of genes involved in melanogenesis within the cell cycle and as a transcriptional repressor of genes involved in invasion, making elevated MITF levels a possible biomarker for melanoma[20]. In humans, MITF regulates the expression of tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2 (TRP-2). A complex regulatory network precisely modulates MITF expression and activation and therefore controls tyrosinase and melanogenesis. Therefore, targeting only tyrosinase or only MITF is destined to be either an ineffectual or a temporary intervention. It is likely that combination therapy that targets MITF, tyrosinase and the other regulatory pathways is more likely to produce a measurable and prolonged effect on skin pigmentation.

One of the key pathways in the stimulation of melanocytes in response to UV radiation is the adenylate cyclase pathway. UV radiation mediates the synthesis and release of alpha-melanocyte-stimulating hormone ($\alpha$-MSH) which then promotes pigmentation by binding to the melanocortin 1 receptor (MC1R) on the surface of melanocytes. In people with the red-hair/fair-skin phenotype, the MC1R gene is non-signaling which leads to the increased risk for the development of melanoma. The production of cyclic adenosine monophosphate (cAMP) downstream of MC1R activates the receptors of MITF. MITF then participates in the conversion of tyrosine into melanin pigments by stimulating the transcription of melanocyte-specific genes. Therapeutic agents that are able to inhibit MITF through the down regulation of MC1R activity are likely to be particularly effective in reducing melanocyte activity.

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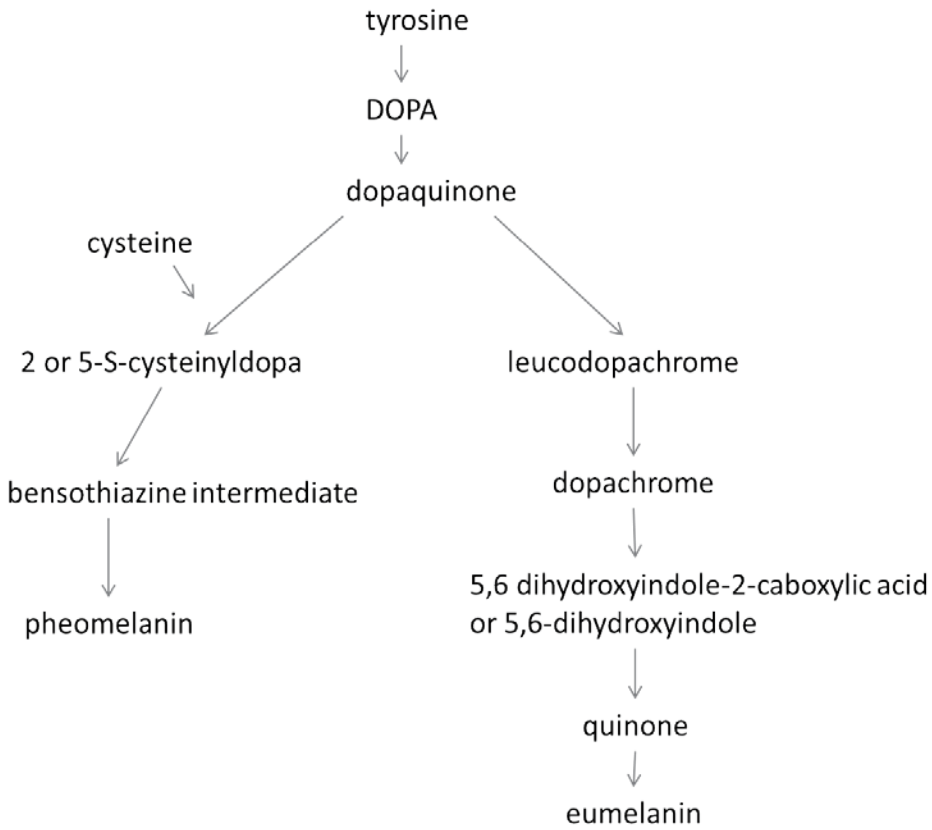


**Figure 3.** Melanin biosynthesis.

### 3.3. Keratinocytes

Keratinocytes are an important structural cell of the epidermis and comprise the majority of the cells in this layer of the skin. Throughout their life cycle, keratinocytes can take on many forms. Keratinocytes are produced by mitosis in the most basal layer of the epidermis when they begin to produce a great deal of keratin. Eventually, older keratinocytes are pushed up to the upper layers by the rapidly dividing keratinocytes beneath them until they reach the stratum granulosum where they begin to drastically change their morphological and biochemical functions. In the stratum granulosum, keratinocytes lose their organelles and acquire a flattened appearance. Due to their production of keratin, these cells become strong and their tightly bound structure makes the outermost keratinocyte layer strong and relatively impermeable.

An important role of keratinocytes is the transport of melanin through the layers of the skin. Once melanin is produced by melanocytes, the pigments are packaged into melanosomes that are subsequently translocated along the dendrites and then captured and aggregated by

keratinocytes. The mechanism of transfer has long been a mystery, however a recent study summarizes the transfer mechanism in three steps: (i) the melanocyte's dendrite attaches to the surface of the keratinocyte, (ii) a portion of the melanocyte's dendrite sheds in order to deposit the melanosomes on the surface of the keratinocyte, (ii) and finally, the keratinocyte engulfs the melanosome package[21].

## 4. Natural ingredients that reduce pigmentation

Since a number of naturally derived bioactive molecules have been found to reduce skin pigmentation, the cosmetic industry has focused on the development of these compounds for use in their products. These compounds inhibit the production of melanin at different stages of melanogenesis. There are several compounds that specifically inhibit tyrosinase. For example, terrein, a bioactive fungal metabolite agent isolated from the *Penicilllium* species, has been shown to strongly decrease the production of tyrosinase by down-regulating MITF through dual pathways[22]. One pathway of these pathways may involve the activation of extracellular signal-regulated kinase (ERK) which has been reported to reduce melanin synthesis[23]. A second pathway recently identified may involve the ubiquitination and subsequent breakdown of tyrosinase itself[24].

Flavonoids, in particular, have received a great deal attention for their potential pigment-reducing activity because they are seen as "natural" and are associated with less side-effects than conventional medications and synthetically-designed compounds. Flavonoids are polyphenolic compounds typically found in plants. These compounds are ideal candidates for cosmetic purposes because of their potent bio-activities and low toxicity. Aloesin, a compound isolated from aloe extracts, has been shown to successfully reduce tyrosinase *in vitro* by inhibiting the hydroxylase activities[25], [26]. Resveratrol, a compound found in red wine, appears to have an affinity for tyrosinase and, by an unknown mechanism, reduces tyrosinase activity and MITF expression[27] although some studies have questioned its ability to reduce tyrosinase expression when used alone[28]. Flavonoids isolated from licorice are able to inhibit mushroom tyrosinase[29] and a skin lightening agent that uses licorice extract in its formulation (comprised of many bioactive ingredients) has been shown to reduce pigmentation in patients[30]. There are many reports of flavonoids from various sources interfering with or reducing the activity of tyrosinase, and although they all show some efficacy at reducing the biochemical pathways that lead to pigmentation, it is still unclear whether targeting these pathways in human skin would necessarily lead to reduced melanin formation *in vivo*.

Melanin formation is a complex process and there are compounds that may inhibit melanogenesis without necessarily inhibiting tyrosinase function. Anti-oxidants such as fermented rice bran and vanillic acid can reduce expression of MITF, MC1R, and associated biochemical pathways, without affecting tyrosinase activity,[31], [32] possibly due to their ability to impair peroxidase activity. Anti-oxidants, in general, may potentiate the MC1R pathway which itself is able to reduce downstream levels of hydrogen peroxide and promote nucleo-

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tide excision repair mechanisms in response to UV radiation[33]. The process of melanogenesis involves cell-to-cell communication between keratinocytes and melanosomes and compound such as niacinamide, a bioactive form of niacin (vitamin B3), has been shown to reduce pigmentation both *in vitro* and *in vivo* by downregulating the amount of melanosomes transferred to keratinocytes[34].

## 5. Photoaging and skin cells

### 5.1. Photoaging

As skin ages, a number of changes occur in the structure of the connective tissues. Healthy human dermis consists primarily of an extracellular matrix and a fibrous component formed by collagen and elastic fibers. The complex network of collagen accounts for approximately 80 percent of the dry weight of the skin and it is the component that provides the resistance to deformation. Elastic fibers comprise 2 to 4 percent of the dry weight of the skin and provide elasticity. Glycosaminoglycans (complex sugars, including hyaluronic acid) and water provide the viscous component of the deformation itself. As skin undergoes normal aging, a general reduction in the production of collagen fibers and proteins that make up the extracellular matrix contributes the formation of wrinkles and flaccid skin. Similarly an increased rate of degeneration of elastic fibers leads to the loss of elasticity.

Many of the changes that occur in skin structure as a result of aging are expressed prematurely in photodamaged skin, a phenomenon known as photoaging. Photoaging refers to the various cellular processes associated with senescence that result from photodamage. These processes are characterized by two events referred to as intrinsic (chronoaging) and extrinsic (photoaging) ageing. Leveque et al. first described the effects of chronic sun exposure on the mechanical properties of the skin and showed that long term sun exposure caused a decrease in skin extensibility and elastic recovery[35]. The skin also appeared thicker, more rigid and less susceptible to deformation. Berardesca and Maibach confirmed these findings using controlled ultraviolet (UVA) treatment and showed that even short term, low UVA and UVB exposure can impair skin elasticity and that inflammation and edema are characteristic of this process[36]. Other clinical features of photoaging include wrinkles, dyspigmentation, yellow hue, laxity, vascular ectasia, and malignancies. Adult populations in European and North American countries experience a prevalence of photoaging as high as 80 to 90 percent.

## 6. Photodamage and impact on skin cells

### 6.1. Photodamage

Photodamage refers to the damage of human skin by ultraviolet light, specifically UVA and UVB, and although the detailed mechanisms of photodamage are not fully understood,

some general features have been described and are used as a standard by which topical treatments are judged. Physiological consequences of UVA radiation include thickening of the stratum corneum, epidermal hyperplasia, depletion of Langerhans cells, and dermal inflammation. Although it may seem as if these changes are random and entirely dependent upon UV exposure, photodamage is, in fact, a tightly controlled and regulated process that is facilitated by specialized skin cells. The ways in which these cells interact with each other and their extracellular matrix ultimately determines the morphological and histological consequences of light exposure in the skin. The cells and proteins involved in this process are numerous and the connections between them are complex. However, exposure of the skin to UVR initiates oxidative stress and triggers the synthesis of matrix metalloproteases (MMP) [37]. The production of MMP results in the degradation of dermal collagen and other matrix molecules thus reducing the elasticity and integrity of the skin[38].

### 6.2. Keratinocytes

Since keratinocytes in the upper layers of the epidermis are dead and do not underdo mitosis or metabolisis, they are relatively resistant to UVR induced damage. Younger, keratinocytes located deeper in the epidermis are much more susceptible to UVR damage and repeated UVR exposure results in accumulated DNA mutations that can lead to epidermal malignancies. Keratinocytes play a central role in elaborating innate responses that lead to inflammation and influence the generation of adaptive immune responses in skin.

Apart from the minor cellular constituents of the epidermis, specifically Langerhans cells and melanocytes, keratinocytes are the major source of cytokines. UV radiation exposure stimulates keratinocytes to secrete abundant pro-inflammatory IL-1-family proteins, IL-1$\alpha$, IL-1$\beta$, IL-18, and IL-33. Normal skin contains only low levels of inactive precursor forms of IL-1$\beta$ and IL-18, which require caspase 1-mediated proteolysis for their maturation and secretion. However, caspase-1 activation is not constitutive, but dependents on the UV-induced formation of an active inflammasome complex. IL-1 family cytokines can induce a secondary cascade of mediators and cytokines from keratinocytes and other cells resulting in wide range of innate processes including infiltration of inflammatory leukocytes, induction of immunosuppression, DNA repair or apoptosis. Thus, the ability of keratinocytes to produce a wide repertoire of proinflammatory cytokines can influence the immune response locally as well as systematically, and alter the host response to photodamaged cells.

### 6.3. Fibroblasts and langerhans cells

The most common type of cell found in the connective tissue of skin is fibroblast. They assist in producing and organizing the extracellular matrix of the dermis and also communicate with each other and other cells in the regulation of skin physiology. Dermal fibroblasts are a heterogeneous population and are identified by their location within the dermis. Fibroblasts that exist in the papillary dermis are generally in a more active state and divide at faster rates than those that exist in the reticular layer of the dermis[39]. Fibroblasts produce and secrete precursors of the extracellular matrix such as collagen, glycoproteins, and other molecules that support the structure of the skin.

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Studies have shown that when exposed to UVA, fibroblasts proliferative activity is slowed or stopped due to morphological and functional changes. When exposed to sublethal UVA radiation does *in vitro*, researchers observed mutations in the mitochondrial DNA (mtDNA) in fibroblasts[40]. Subsequent studies have shown that the initial mutations in the mtDNA induced by UVR render the mitochondria in the fibroblasts more susceptible to ROS damage due to an increase in common deletions and a persistence of the mutations event after the termination of UVR exposure[41].

Langerhans cells are a subset of dendritic cells and are found in the epidermis of the skin. Langerhans cells play a role in the initiation and regulation of immune response by acquiring antigens in the skin and migrating to lymph nodes where immune responses are initiated. When human skin is exposed to UVR it results in a depletion of Langerhans cells and reduces the expression of immunophenotypic markers associated with antigen presentation[42]-[44]. The lack of functional Langerhans cells ultimately results in an immunosuppressive state in the skin[45] which also involves regulatory T cells and cytokine production[46].

### 6.4. Mast cells

It has been recognized that photoaging and normal chronological aging differ in several morphological and biological aspects, one of which is distinct alterations in elastic fibers and microvasculature. Previous studies have shown that one of the main contributors to this phenomenon is activation of immune cells in the epidermis, one of which is the mast cell. Studies comparing photo-exposed and sun-protected skin from the same individual shows that sun-exposed skin is characterized by the presence of higher numbers of infiltrating leucocytes and mast cells than is found in sun-protected skin[47]. Mast cells are cutaneous cells that contain pro-fibrotic and pro-inflammatory mediators and can contribute to skin inflammation and disease. Recent evidence has suggested that mast cells maintain tissue homeostasis and that many of the responses observed in photoaging are an attempt of the mast cell to repair damaged tissue systems. Mast cell activation can initiate or exacerbate many of the alterations associated with chronically sun-exposed skin such as massive accumulation of abnormal elastic fibers, loss of collagen, increase in glycosaminoglycans and telangiectatic vessels[47]. For example, mast cells produce fibroblast growth factors that can activate fibroblasts to produce abnormal matrix molecules. Mast cells also produce matrix-degrading enzymes and can activate the production of matrix proteinases by other skin cells such as keratinocytes. Mast cell products can recruit and activate infiltrating inflammatory cells such as neutrophils, macrophages and lymphocytes that can further damage the local tissue (see Figure 4).

Although an increased number of mast cells have been associated with photoaging, very little is known about their role in this process. The interaction between mast cells and other skin cells such as keratinocytes in photoaging is poorly understood. In fact, the precise mechanisms of mast cell activation in photodamaged skin is not known although it does not appear to be through the more common pathways such as the high affinity IgE receptor (FcεRI) or pathogen-associated pattern recognition receptors. In photoaging, mast cell acti-

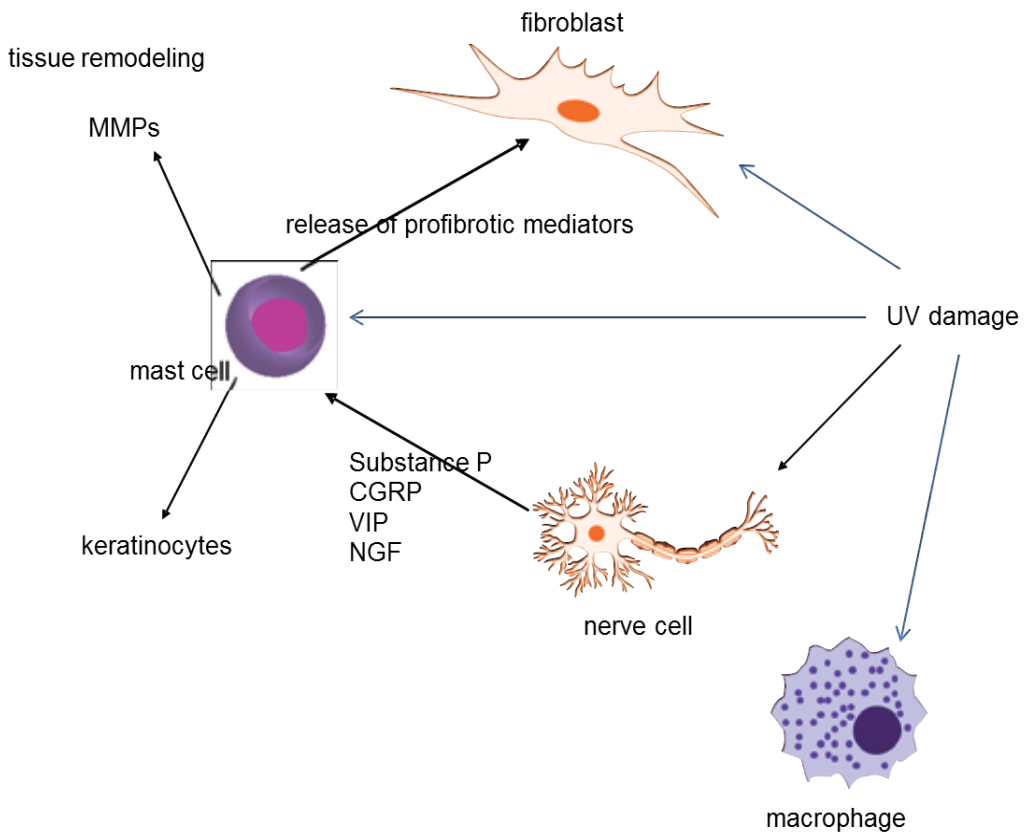


**Figure 4.** Role of skin cells in photoaging.

vation appears to be unique but our laboratory has shown that G protein coupled receptors (GPCR) may be responsible. Chronic exposure to UV causes sensory nerve fibers to release a series of neuropeptides including substance P (SP) and calcitonin gene-related peptide (CGRP)[48], both of which can activate mast cell degranulation and pro-inflammatory mediator release[49]. Furthermore, opiates that modify nerve function can also activate skin mast cells[50]. Interestingly, SP causes mast cells to downregulate their expression of allergen-activated receptors (FcεRI)[51] and primes them to respond to pathogen-activated (TLR) stimuli[52]. In skin that is chronically exposed to UV, mast cells may modulate a chronic inflammatory state, thus causing further damage to skin's structural integrity. Furthermore, mast cells appear to be important in the immunosuppressive environment of photodamaged skin since UVB causes a mast cell-dependent suppression of contact hypersensitivity responses[53]. This finding supports our hypothesis that human mast cells exposed to UV may undergo a phenotypic change from an allergic inflammatory effector cell to a more pathogen/structural response cell capable orchestrating structural changes in the skin. However, it is also possible that mast cell activation is a protective phenomenon and that further

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potentiating mast cell activation in photodamaged skin may, in fact, inhibit or reverse some of the immunosuppression associated with photodamage.

### 6.5. Inflammation during photodamage

Traditionally, photodamage has been characterized by the mechanical damage caused to the skin's constituents – i.e. the degradation of the proteins and structures that hold the skin together and give its integrity. However, recent evidence has shown that at least some of the damage associated with UV exposure is due to the initiation of low but chronic inflammation that ultimately results in impaired cellular responses. This has some major implications because it suggests that the skin is not only losing its ability to form a physical barrier but is also losing some of its immune and regenerative abilities. Recent studies have shown that photodamage reduces immune responses by inhibiting the pathways that activate immune cells and causes changes in biochemical processes that regulate wound healing.

Using histological analysis, Bosset et al showed that sun-exposed skin had an increase in infiltrating mononuclear cells than sun-protected skin[54]-[56]. Immunohistochemical analysis also showed an increased number of cells that one would normally associate with a Th2 inflammatory response – namely, mast cells, macrophages and CD4+ CD45RO+ T cells -- in sun-exposed dermis as well as a higher number of CD1a+ dendritic cells in sun-exposed epidermis, compared with the sun-protected samples. Although Bosset et al. found that photoaging displayed histological features of chronic skin inflammation they found a decreased expression of interleukin-1beta mRNA in sun-exposed compared with sun-protected suggesting that other pro-inflammatory mediators were mediating these effects. This hypothesis seems likely since these patients' skin looked clinically normal and would suggest an inflammatory pathology that is very different from those normally associated with acute Th2 inflammatory reactions such as atopic dermatitis or urticaria.

Inflammation is a complex process that has many different points of initiation and can be perpetuated by a diverse array of mediators. However, a study by Yan et al.[57] suggests that leukotrienes, potent lipid inflammatory mediators, may be responsible for this low but chronic inflammatory process associated with UV exposure. In their study, these investigators evaluated the protective effects of a nonsedative histamine H1-receptor antagonist, mizolastine, on UVB-exposed skin dermal fibroblasts. They found that relatively low levels of mizolastine (10 nM) inhibited UVB-induced $LTB_4$ production by skin fibroblasts. This decrease in $LTB_4$ production was associated with a down-regulation of 5-lipooxygenase ( 5-LO) mRNA expression and inhibition of 5-LO translocation suggesting that UV-induced leukotriene synthesis was dependent upon 5-LO expression.

The idea that chronic inflammation due to photodamage is a Th2 mediated response is possible, but some evidence suggests that it may be, in fact, a Th1 mediated event. UVB exposure of the skin results in a profound upregulation of the anti-inflammatory cytokine IL-10 and suppression of contact hypersensitivity (CHS). Furthermore, using an IL-10 transgenic mouse model (IL-10tg), Ma et al.[58] have shown that unexposed IL-10tg animals showed a diminished CHS response compared to wild-type. Yet when IL-10tg animals were exposed to UVB, the CHS response was not further suppressed, but rather was restored to the level

observed in unexposed wild-type animals suggesting a normalization of the hypersensitivity response. If one believes that Th1 and Th2 responses counter-balance one another, this data would suggest that chronic inflammation associated with photodamage is mediated by a Th1 controlled inflammatory response.

## 7. Natural ingredients as treatments for photodamage and photodamage

### 7.1. Targeting the skin's immune response

Natural products that are used to treat the symptoms associated with photoaging and photodamage are designed to either stimulate new skin cell formation or inhibit the biochemical processes that cause skin damage. Sometimes, compounds are promoted as having biological activity in both of these contexts. Natural health products (NHPs) used to treat photodamaged skin are often mixtures of many different molecules and the precise mechanism of their beneficial effects are not always easy to determine from the myriad of information (anecdotal and experimental) available. However, there are a few groups of compounds that are naturally-derived and that appear to have some efficacy in at least reducing some of the biochemical manifestations of photodamage.

Botanical extracts are the most popular sources of naturally-sourced bioactives in skin health clinics. Most often, these are mixed into topical creams and applied to the epidermis. These extracts can even provide some small measure of sun protective activity (SPF) as well as reducing the biochemical and cellular consequences of photodamage described previously[59]. Topical application of green and white tea extracts, can reduce some of the detrimental immunomodulatory effects of photodamage[60]. Topical administration of Vitamin A and its analogues inhibit the expression of MMP and stimulate collagen synthesis in both photodamaged and photoprotected aged skin[61]. The list of these compounds is extensive and there are many comprehensive reviews on these compounds and their effects on human skin. However, these compounds all share the ability to modify the cellular responses in the dermis, whether through the induction of regenerative processes associated with keratinocyte renewal or inhibition of enzymes that break down the structural integrity of the skin. These compounds are all tested for their direct effect on these systems in bioassays designed to tease out the mechanisms on a single or small group of skin cells.

Some of these extracts, however, have efficacy when taken orally so their mechanisms of much more difficult to determine. For example, taking a specific *Polypodium leucotomos* extract (Fernblock, *Cantabria Farmaceutica*) 7.5 mg/kg daily for two doses before UVA treatment can modestly reduce erythema, edema, and signs of epidermal damage and may decrease subsequent hyperpigmentation in some patients[62]. In fact, it has been suggested that a diet rich in green tea polyphenols, grape seed proanthocyanidins and silymarin may act as chemopreventative agents to protect skin from photocarcinogenesis[63]. Taking what we know about the skin immune system and the possible molecular targets of these compounds we can hypothesize that this process may involve the induction of interleukin-12 (IL-12), DNA repair mechanisms, protection of Langerhans cell depletion and stimulation of cytotoxic T

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cells. However, many questions remain. How much of these chemoprotective agents must we eat and how often? Are some people (i.e. different skin tones) better protected by these dietary compounds than others? What are the best sources of these compounds in our diet? These are all relevant questions that are difficult to answer experimentally.

In addition to prophylactic treatments to alleviate the symptoms of photoaging, there is a strong market for compounds that can either stop or delay the sigs of photoaging. Anti-aging products are used to reduce the various cellular processes associated with senescence – both intrinsic (chronoaging) and extrinsic (photoaging) ageing. Younger individuals seek to prevent or reduce the manifestations of the extrinsic aging process which is responsible for approximately 80 percent of facial aging. Older individuals are often concerned with preventing or reversing the signs of both intrinsic and extrinsic aging processes. As such, treatments used to prevent photoaging target the underlying inflammatory and molecular responses associated with damage of skin integrity. Treatments used to reverse or stop the appearance of photoaging often target the biomechanical properties of skin.

There are numerous compounds currently on the market and in development and it is beyond the scope of this chapter to cover all of them. However, alpha hydroxy acids serve as excellent example to illustrate their effects on the skin microenvironment. AHA include glycolic, citric, lactic, malic, tartaric and lactic acids and are used extensively to promote skin rejuvenation and renewal. They are sometimes marketed as "fruit acids" because citric acid is found in citric fruits, malic acid is present in apples and tartaric acid is sometimes isolated from grapes. However, other foods also contain these acids (sugarcane contains glycolic acid and sour milk contains lactic acid). AHA work mainly by exfoliating the stratum corneum thus improving the smoothness and texture of skin. Although the exact mechanism of this effect is unknown, it is believed that AHA inhibit the enzymes that form ionic sulfate and phosphate bonds in the stratum corneum[64]. In the dermis, AHA induce desquamation and promoting cell renewal as well as stimulate collagen synthesis and induce dermal thickness[65].

AHA peels have recently been recognized as important adjunctive therapy in a variety of conditions including photodamage, actinic damage, melasma, hyperpigmentation disorders, acne, and rosacea. Since AHA are considered to have a reasonable level of safety and efficacy in a variety of skin types, they have been used extensively in the cosmetic industry as well[66]. Although their exact mechanism of action is unknown, it has been suggested that AHA improve skin pathological disorders by thinning the stratum corneum, promoting epidermolysis, dispersing basal layer melanin, and increasing collagen synthesis within the dermis[67]. Specifically in patients with photodamaged skin, AHA peels and topical products are often combined with retinoids and other antioxidants to reduce the thickness of the stratum corneum and smooth out the skin's surface. It has been suggested that AHA may help increase the skin's barrier function, making it more resilient to chemical damage[68].

A new class of hydroxy acids called polyhydroxy acids (PHAs), have similar effects as AHA but irritate the skin the same way as classical AHAs. PHAs cam be used on clinically sensitive skin, including rosacea and atopic dermatitis, and are compatible with cosmetic procedures[69]. Compared to AHA, PHA formulations are more moisturizing and can enhance

stratum corneum barrier function. PHAs such as gluconolactone or lactobionic acid may be used in combination with other products, ingredients, or procedures such as laser and microdermabrasion to provide additional benefits to therapy or to enhance the therapeutic effect. PHAs used in combination with retinyl acetate (pro-vitamin A) in a cream base can have some anti-aging skin benefits such as skin smoothing and plumping although it is unclear if these also involve biochemical or immunomodulatory effects. In some formulations, PHA and hydroquinone together have demonstrated some superficial improvement in morphological changes associated with photoaging and have resulted in a decrease in skin pigmentation.

## 8. Conclusions

The skin is a complex and dynamic organ that is constantly bombarded with environmental trauma including UV radiation. UV light can cause major and often irreversible changes in the skin including damage to the three layers of skin. In the context of this constantly changing and sometimes harsh environment, the skin must maintain homeostasis and protect a delicate network of biochemical and physiological systems. There are a number of processes that are designed to protect the skin from photodamage, including the stratum corneum, melanin and underlying immunological processes to control inflammation. However, it is becoming increasingly clear that, over time, the skin's protective mechanisms can become overwhelmed by the damage caused by UV radiation, resulting in irreversible structural damage, chronic inflammation and, in some cases, photocarcinogenesis. Understanding the role that the microenvironment plays in this complex process is important to developing innovative new strategies for its treatment and possibly reversal.

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Chapter 10

# Asthma in the 21st Century — Unexpected Applications of Ancient Treatments

Priyanka Pundir, Xiaofeng Wang and
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Additional information is available at the end of the chapter

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## 1. Introduction

Asthma comes from the Greek word for "panting" and has been described as a pathological condition for centuries. It is a chronic inflammatory disorder of the airways in which many immunological cells play a role, including mast cells and eosinophils. In susceptible individuals, this inflammation causes symptoms which are usually associated with widespread variable airflow obstruction that is often reversible, either spontaneously or with treatment, and causes an associated airway hyperresponsiveness (AHR) to a variety of stimuli. The clinical features of asthma include dyspnea, wheezing and coughing.

During the last forty years there has been an increased understanding of the wide spectrum of this disease and as a result a number of effective treatments have been developed. Despite these advances, however, the mortality continues to increase and approximately 500 Canadians and 3500 Americans die each year from asthma. It remains a major cause of morbidity, as the leading cause of school absenteeism and the third leading cause of work absenteeism. The prevalence of asthma in North America has been on a constant rise over the last 25 years and it is estimated that currently over 3 million Canadians and 25 million Americans suffer from asthma. Worldwide the prevalence rates of asthma are rising on average by 50% each decade and developing a better understanding of the risk factors associated with this trend is critical. These may be broadly classified as either host genetic factors or environmental factors (Table 1).

One of the marked risk factors of asthma associated with the westernized lifestyle is our changing diet and/or nutritional status. It has been hypothesized that the significant change in our diet plays a dominant role in the etiology of asthma. Seaton *et al*. have proposed that

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| Genetic Factors | Environmental Factors |
|---|---|
| Atopy<br>Gender<br>Genetic predisposition<br>Race/ethnicity | Smoking<br>Allergens<br>Occupational sensitizers<br>Respiratory infections<br>Parasitic infections<br>Perinatal risk factors<br>Diet and nutrition |

**Table 1.** Risk factors associated with asthma

asthma prevalence has increased in UK because of an alteration in diet associated with industrialization [1]. This has lead to a substantial decline in the consumption of fresh fruits, green vegetables, fish and red meat, and as a result decrease in pulmonary antioxidant defences and an increase in susceptibility to inhaled irritants and allergens [1]. These foods are the main sources of antioxidants, substances that protect cells against the effects of free radicals generated during oxidative stress.

Oxidative stress is important in the pathophysiology of asthma [2] and development of AHR [3]. A large number of epidemiologic studies have reported the protective effects of dietary antioxidants such as micronutrients vitamin A, C, and E, polyphenol, and carotenoids against the development of asthma and decline of lung function. In a study on American children higher levels of antioxidants beta-carotene and Vitamin C, along with antioxidant trace mineral selenium is associated with a lower risk of asthma [4]. Dietary vitamin C intake is positively associated with 1 Second Forced Expiratory Volume ($FEV_1$) in children and adults [5-8] but less frequently with asthma or wheeze in children and adults [4, 9-11]. Dietary vitamin E intake is positively associated with ventilatory function [5, 6, 12] but negatively associated with asthma and wheeze in children [13], adult-onset wheeze [11] and the likelihood of atopic sensitization in adults [14]. Fresh fruits intake is inversely associated with wheeze [15] and chronic lung disease onset [16] and is positively associated with $FEV_1$ [17]. Total fruit and vegetable intake is inversely related to asthma prevalence [18] but not to $FEV_1$ [19] or airway obstruction [20]. Vegetables may protect against chronic bronchitis, asthma [21], and wheeze [22]. Moreover, dietary polyphenols intake are associated with lower disease risk with beneficial clinical outcomes attributed to both the antioxidants and anti-inflammatory properties of polyphenols [23]. Polyphenols consist of a large group of natural antioxidants extracted from plants and flavonoids comprise the most studied group.

In addition to antioxidants, intake of fats, particularly the changing composition of polyunsaturated fatty acids (PUFA) in westernized diets, has been implicated in the etiology of asthma. There has been a reduced intake of saturated fat accompanied by an increase in n-6 PUFA consumption, particularly linoleic acid and arachidonic acid. In addition, there has been a decrease in consumption of n-3 PUFA such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Thus, it has been postulated that the increased ratio of n-6:n-3 PUFA in diets of industrialized countries may also contributed to the increased asthma incidence.

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Ancient Egyptian papyrus writings contain prescriptions for asthma that include several herbs suggesting that naturally occurring bioactive compounds may been used to effectively treat asthma. This chapter summarizes the current knowledge on the effects of dietary compounds and nutrients on allergy and asthma, with a focus on the mechanisms involved, wherever possible.

## 2. Pathogenesis of asthma

Airway inflammation in asthma is a complex process involving the interactions between immunological mediators produced by inflammatory cells such as mast cells, eosinophils, basophils, neutrophils, dendritic cells and lymphocytes [24]. This inflammation leads to structural and architectural changes in the airways of asthmatic patients including collagen and fibronectin deposition, wall thickening, subepithelial fibrosis and hypertrophy, goblet and airway smooth muscle cell hyperplasia, and angiogenesis, all of which collectively contribute to the phenomenon known as airway remodeling [25].

Allergic inflammation is often classified into four phases [26]:

**a.** Induction of allergic reaction involving antigen uptake, processing and presentation (Figure 1),

**b.** Early-phase asthmatic reaction (EAR, Figure 2),

**c.** Late-phase asthmatic reactions (LAR, Figure 3), and

**d.** Chronic allergic inflammation (Figure 4)

## 3. Common molecular targets used in current asthma therapy

Inspite of the advances made in the field of asthma treatments, some patients remain less responsive to conventional therapies than others. Current treatment strategy includes the combinations of bronchodilators, particularly short or long acting $\beta_2$-adrenergic agonists (SABA, LABA), and inhaled and oral corticosteroids. The current approach to the management of asthma includes the addition of drugs in a stepwise fashion based on the severity of symptoms, however the stronger drugs include more severe side effects. The treatment aims to reverse airflow obstruction and reduces asthma exacerbations thus improving quality of life. However, long-term use of high dose inhaled corticosteroids therapy may lead to detrimental effects, such as cataracts [46], osteoporosis in elderly patients [47], and stunting of growth in children [48]. Moreover, the combination therapy may not modify the disease progression and are not curative.

The limited efficacy and side effects associated with conventional treatments has lead to the introduction of nutraceuticals as a “safer“alternative therapy and for those whom symptoms

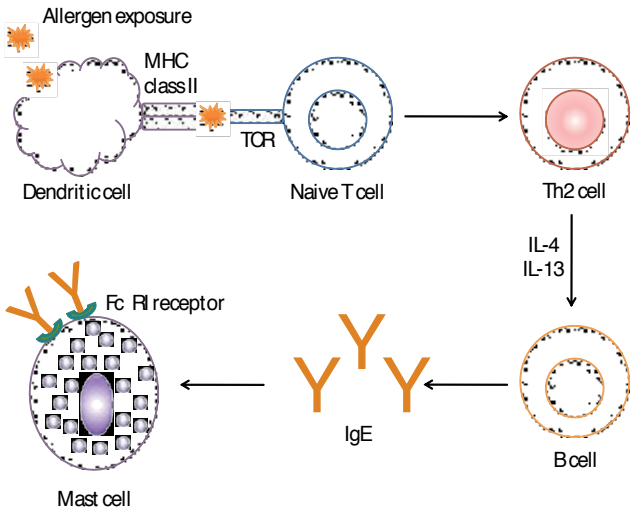


**Figure 1.** AHR and bronchial inflammation starts with the inhalation of an allergen. The allergen enters tissues through disrupted epithelium. It is then captured by antigen-presenting cells (APCs), usually dendritic cells (DCs), but also alveolar macrophages and B cells [27]. Allergen-loaded DCs migrate to regional lymph nodes where they present peptides to naïve T cells [28]. Presentation of processed allergen in the form of peptide fragments on MHC class II molecules to naïve T cells signals activation and clonal expansion of T cells [27]. In the presence of interleukin (IL)-4, naïve T cells acquire the characteristic of T helper 2 (Th2) cells [29]. Activated Th2 cells secrete greater amounts of IL-4 and IL-13 which triggers the isotype switch to immunoglobulin (Ig)E synthesis [29, 30]. IgE produced by B cells diffuses locally, enters the blood and is then distributed systematically. Allergen-specific or non-specific IgE binds to the high-affinity receptor for IgE (FcεRI) on the surface of tissue-resident mast cells and peripheral blood basophils, thereby sensitizing them to future allergen exposures [30].

are not improved with current therapies. Nutraceuticals is a very general term which encompasses many classifications of food products and derivatives that have the potential to either prevent or treat pathological conditions in humans or animals. For example, micronutrients such as vitamins and minerals and non-nutritive components of plant products such as polyphenols have some anti-inflammatory activity and have been used to supplement some foods to improve their health benefits. Table 3 summarizes some of the major nutraceuticals used to treat allergy and asthma currently.

The following sections discuss the current knowledge on the effects of nutraceuticals on inflammation associated with asthma with a focus on the cellular and molecular mechanism involved.

### 3.1. Anti-mediator agents

Anti-mediator agents are a group of drugs that antagonize the release of granule-associated preformed mediators, lipid mediators, cytokines, chemokines, and growth factors released by allergen-activated inflammatory cells. Several important groups of specific inhibitors against many of these inhibitors have been developed.

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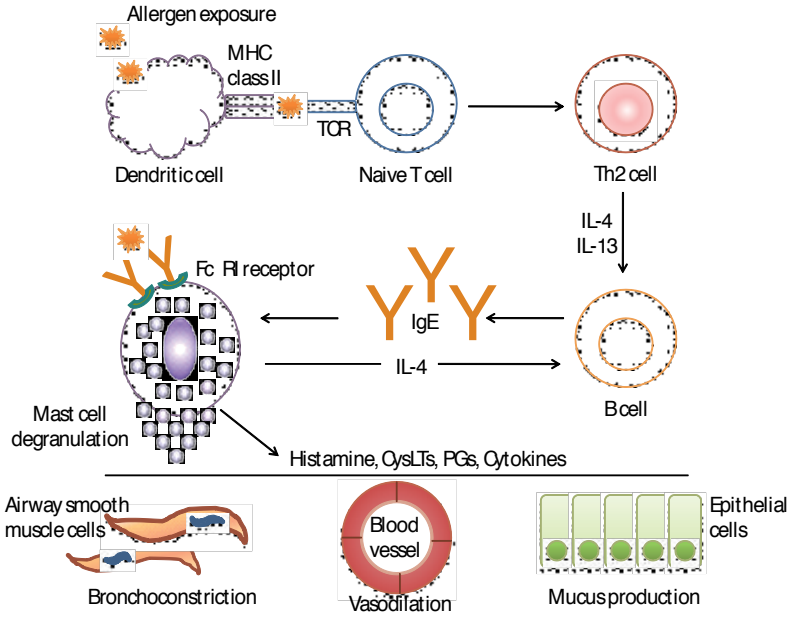


**Figure 2.** The EAR occurs within 30 min of allergen exposure and is principally initiated by mast cell and basophil activation [26]. Mast cells are widely distributed throughout the human respiratory tract and are found in large numbers in the walls of the alveoli and airways [31]. Asthmatics have allergen-specific IgE bound to the FcεRI receptors on mast cell surface. Upon cross-linking of adjacent IgE molecules by allergen, aggregation of FcεRI triggers a complex intracellular signaling process [32]. Activated mast cells release a diverse array of biologically active mediators: preformed granule-associated mediators, lipid-derived mediators, and *de novo* synthesized cytokines, chemokines, growth factors and other biologically active molecules (Table 2) [33]. The release of mast cell-derived mediators contributes to acute signs and symptoms associated with EAR that may range from mild rhinitis to anaphylactic shock. These mediators induce vasodilation, contraction of the bronchial smooth muscle (producing airflow obstruction and wheezing) and increased mucus secretion (exacerbating airflow obstruction in the lower airways) [26].

### *3.1.1. Lipid mediator blockers*

Montelukast is a current FDA approved drug used in asthma treatment and serves a prototypical drug for Lipid Mediator Blocking class of drugs. Its mechanism of actions works through the blocking of the CysLT receptor for leukotriene $D_4$ which reduces bronchoconstriction and inflammation. Zileuton, a related drug in the same class, is a 5-lipoxygenase inhibitor which blocks the synthesis of cysLTs and leukotriene $B_4$. These drugs while not natural products serve as a models in the search for nutraceuticals whom may share same or related mechanism of action and therefore may prove useful in asthma management. Antagonists of the prostaglandin $D_2$ receptors DP1 and CRTH2 reduce inflammation in a murine model of asthma, possibly by inhibiting prostaglandin synthesis [49, 50]. Antagonist of the leukotriene $B_4$ receptor BLT1 (R05101576) prevents airway inflammation and AHR in animal models and non-human primates [51]. Quercetin and luteolin, flavonoids found in fruits, vegetables and wine, inhibit the release of leukotrienes and PGD2 from human cultured mast cells [52]. Table 4 summarizes phytochemicals that act on pathways related to the synthesis of lipid mediators in allergic inflammation.

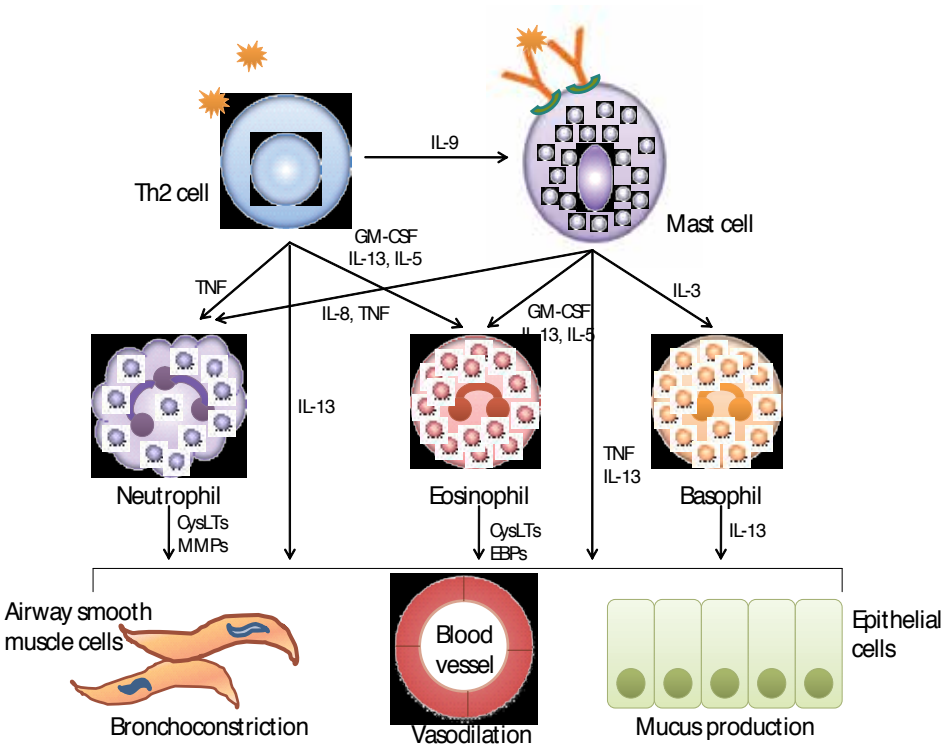


**Figure 3.** The LAR typically develops 2-6 hr following allergen challenge, often peaks after 6-9 hr, and has a more severe and prolonged phase. In general, allergen activated mast cells release various *de novo* synthesized cytokines, chemokines and growth factors, which are released more slowly than granule-associated mediators [34]. Thus, LAR is sustained by *de novo* synthesized mast cell-derived mediators which recruit inflammatory cells to the airways several hours after allergen challenge. These recruited cells include effector cells, such as eosinophils, basophils, neutrophils, macrophages, T cells, and DCs [34, 35]. These inflammatory cells are activated when they reach the airway and produce a vast array of inflammatory mediators that act on specific receptors and exacerbate airway inflammation and airway remodeling. Eosinophils are the central effector cells in the LAR [36], and are present not only in the airway wall [37] but are also found in large numbers in the sputum and bronchoalveolar lavage fluid (BALF) [38]. Eosinophils are a rich source of granule basic proteins (EBP), such as major basic proteins (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN), and can also generate lipid-mediators (prostaglandins and cysteinyl leukotrienes), cytokines (such as TNF, TGF-β, IL-4 and IL-13) and chemokines [39]. These eosinophil-derived products promote some of the pathophysiological hallmarks of asthma such as AHR [40]. The activation of peripheral blood neutrophils during allergen challenge results in their intravascular migration, adhesion to the endothelium, and migration to the site of inflammation and can be responsible for significant damage. Nocturnal asthma is associated with high levels of neutrophils, which correlate with the severity of the disease [41]. Furthermore, in a small number of patients who died of sudden-onset asthma, the predominant cell type in the sputum is the neutrophils, not eosinophils [42]. Neutrophils also predominate more frequently in the sputum of patients with acute exacerbations of asthma, mostly associated with respiratory tract infection [43]. T cells are not only important during the induction phase, but play also a very important role during ongoing inflammation. Th2 cells and their cytokines are crucial for promoting acute hypersensitivity responses, and for maintaining the state of chronic and relapsing eosinophil-predominant inflammation that is characteristic of chronic allergic inflammation. Elevated levels of $CD4^+$ T cells are observed in the bronchial mucosa of biopsy samples, BALF and sputum from patients with asthma [44]. In a majority of studies, T cells found in asthmatic patients express cytokines or transcription factors characteristic of Th2 cells, especially IL-4, IL-5, IL-9 and IL-13 [45].

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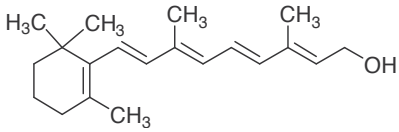
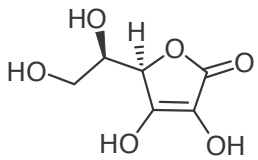
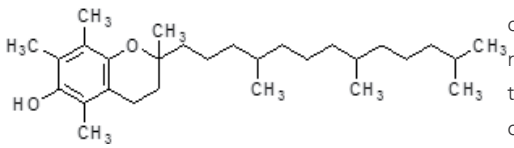
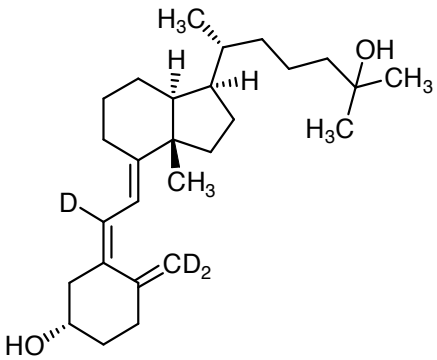
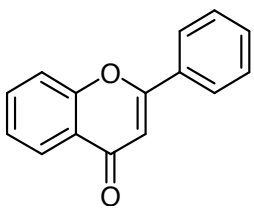
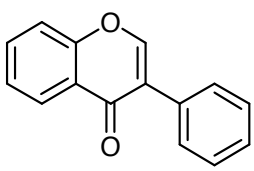
| Class of Product | Examples | Biological Effects |
|---|---|---|
| Preformed Mediators | Histamine, heparin | Increase vascular permeability, smooth muscle contraction |
| Enzymes | Tryptase, chymase, cathepsin G, carboxypeptidase | Remodel tissue matrix |
| Cytokines | IL-4, IL-13 | Stimulate Th2 cell response |
| | IL-3, IL-5, GM-CSF | Promote eosinophil production and activation |
| | Tumor necrosis factor (TNF) | Promotes inflammation and cytokine release by immune cells |
| Chemokines | CCL2, CCL3, CCL4, CXCL1, CXCL2, CXCL3, CXCL10 | Attract monocytes, macrophages and neutrophils |
| Lipid Mediators | Prostaglandin $D_2$, E2<br>Leukotriene B4, C4 | Cause smooth muscle contraction, increase vascular permeability, stimulate mucus secretion |
| | Platelet-activating factor | Attracts leukocytes, amplifies production of lipid mediators, activates neutrophils, eosinophils and platelets |

**Table 2.** Mast cell-derived proinflammatory mediators

| **Antioxidants** | | |
|---|---|---|
| *Vitamin A* |  | Vitamin A (retinoid) occurs in many foods, including carrots, broccoli, sweet potato, butter, spinach, pumpkin, and liver, cod liver oil. The form found in colorful fruits and vegetables is called provitamin A carotenoid. |
| *Vitamin C* |  | Vitamin C (ascorbic acid) is an essential nutrient for humans as it protects the body against oxidative stress and is a necessary in collagen synthesis. Fruits (kakadu plum, camu camu, rose hip, indian gooseberry, blackcurrant, orange, tangerine, and guava) and vegetables (green and red chilli pepper, red pepper, broccoli, Brussels sprout, spinach, and cabbage) are good sources of Vitamin C. |
| *Vitamin E* |  | Vitamin E is a fat-soluble vitamin that exists in eight different forms. It consists of a group of substances belonging to two closely related families, the tocopherols and tocotrienols, with each existing in a number of isomeric forms (α, β, γ, and δ). α-tocopherol is the most active form of Vitamin E in humans and is considered the major |

| **Antioxidants** | | |
|---|---|---|
| | | membrane-bound antioxidant employed by cells. Its main antioxidant function is protection against lipid peroxidation. There is an interaction between Vitamin E and other nutrients, particularly selenium and vitamin C in the antioxidant role.<br>Vitamin E is found in fruits (tomato, mango, and papaya), green leafy vegetables (lettuce, spinach, turnip, and beet), nuts and nut oils (almonds and hazelnuts), vegetable oils (wheat germ oil, sunflower oil, and safflower oil), meat, and poultry. |
| Vitamin D |  | |
| Flavonoids |  <br>Flavones<br>  <br>Isoflavones | Flavonoids constitute the most important single group of polyphenols of low molecular weight polyphenolic secondary plant metabolites, with more than 8,000 compounds described. They are found in fruits, vegetables, nuts, seeds, stems, flowers, roots, tea, wine, and coffee and are common substances in our daily diet. Their structure is a heterocyclic hydrocarbon, chromane, and substitution of its ring C in position 2 or 3 with a phenyl group (B-ring) results in flavans or isoflavans. An oxo-group in position 4 leads to flavanones and isoflavanones. The presence of a double bond between C2 and C3 provides flavones and isoflavones. An additional double bond in between C1 and C2 makes these compounds colourful anthocyanidins. Based on their structure, flavonoids are categorized into eight groups: |

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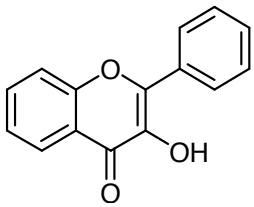
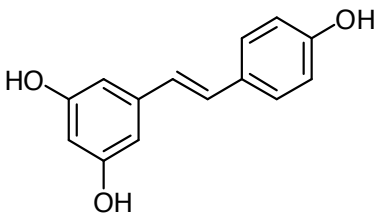
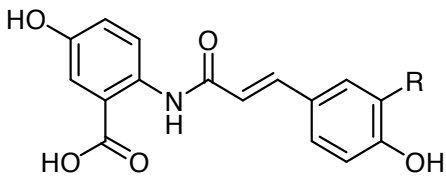
| **Antioxidants** | | |
|---|---|---|
| |  <br>Flavonols | flavans, flavanones, isoflavanones, flavones, isoflavones, anthocyanidins, chalcones, and flavonolignans (Table 11]. |
| Resveratrol |  | Resveratrol is a stilbenoid, a type of natural phenol, and a phytoalexin produced naturally by several plants when under attack by pathogens such as bacteria or fungi. Resveratrol is found in the skin of red grapes and in other fruits. It is sold as a nutritional supplement derived primarily from Japanese knotweed. |
| Selenium | | Selenium is derived from both vegetable and animal products, particularly seafood, liver, and cereals. As a member of the sulfur family of elements, it shares several chemical properties with sulfur, including valence states and the ability to form covalent bonds with carbon. It is unique among antioxidants in that it exerts its biological effects through direct incorporation into proteins (selenoproteins) as the amino acid selenocysteine. Some selenoproteins that have been characterized as important antioxidant enzymes include GPX-1, GPX-4, thioredoxin reductase-1 and thioredoxin reductase-2, and selenoprotein P. The selenium-dependent enzyme, GPX recycles glutathione, reducing lipid peroxidation by catalyzing the reduction of peroxides, including hydrogen peroxide. |
| Avenanthramide |  | Oats contain unique, low-molecular-weight, soluble phenolic compounds called avenanthramides (Avns), which are not present in other cereal grains. These compounds are antipathogens (phytoalexins), which are produced by the plant in response to exposure to pathogens |

| Antioxidants | |
|---|---|
| | such as fungi. Avns are conjugates of a phenylpropanoid with anthranilic acid or 5-hydroxy anthranilic acid. More than 20 different forms of Avns are present when extracted from oats, and the three major forms are A, B, and C. |

**Table 3.** Some of the dietary nutraceuticals indicated in asthma prevention.

| Phytochemicals<br>Flavonoids | Target/Function |
|---|---|
| 4,3′,5′-trihydroxystilbene | Inhibits Cyclooxygenase 1 |
| 4,3′-dihydoxy-5′methoxystilbene | inhibits Cyclooxygenase 1 |
| 4-hydoxy-3′5′-dimethoxystilbene | Inhibits Cyclooxygenase 1 |
| Acacetin | Inhibits Cyclooxygenase 1 |
| Andanthoflavone | Inhibits 12-Lipoxygenase, 15-Lipoxygenase |
| Apigenin | Inhibits Cyclooxygenase 2, 12-Lipoxygenase, 15-Lipoxygenase |
| Artonin E | Inhibits Cyclooxygenase 1, 5-Lipoxygenase, 12-Lipoxygenase, 15-Lipoxygenase |
| Baicalein | Inhibits Cyclooxygenase 1, Cyclooxygenase 2, 5-Lipoxygenase, 12-Lipoxygenase, 15-Lipoxygenase |
| Bicalin | Inhibits Cyclooxygenase 1, Cyclooxygenase 2 |
| Buddledin A | Inhibits Cyclooxygenase 1 |
| Chrysin | Inhibits Cyclooxygenase 1, 5-Lipoxygenase |
| Chrysol | Inhibits 5-Lipoxygenase |
| Cirsiliol | Inhibits 5-Lipoxygenase, 12-Lipoxygenase |
| Daidzein | Inhibits Cyclooxygenase 2 |
| Epicatechin | Inhibits 5-Lipoxygenase |
| Epigallocatechin | Inhibits 5-Lipoxygenase |
| Fisetin | Inhibits Phospholipase A2, 5-Lipoxygenase, 12-Lipoxygenase, 15-Lipoxygenase |
| Flavone | Inhibits 5-Lipoxygenase |
| Gambogenic acid | Inhibits Cyclooxygenase 2 |
| Genistein | Inhibits Cyclooxygenase 2 |

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| | |
|---|---|
| Ginkgetin | Inhibits 5-Lipoxygenase |
| Glycitein | Inhibits Cyclooxygenase 2 |
| Isoliquiritigenin | Inhibits Cyclooxygenase 1, Cyclooxygenase 2 |
| Kampferol | Inhibits Phospholipase A2 |
| Kaempferol | Inhibits 5-Lipoxygenase |
| Kenusanone A | Inhibits 5-Lipoxygenase |
| Kisetin | Inhibits Phospholipase A2 |
| Kuraridin | Inhibits Cyclooxygenase 1, 5-Lipoxygenase |
| Kurarinone | Inhibits Cyclooxygenase 1, 5-Lipoxygenase |
| Kuwanon C | Inhibits 5-Lipoxygenase, 12-Lipoxygenase |
| Luteolin | Inhibits 5-Lipoxygenase, 15-Lipoxygenase |
| Morelloflavone | Inhibits Phospholipase A2 |
| Morusin | Inhibits Cyclooxygenase 1, 12-Lipoxygenase |
| Mosuin | Inhibits 15-Lipoxygenase |
| Myricetin | Inhibits 5-Lipoxygenase |
| Narigenin | Inhibits Phospholipase A2, 5-Lipoxygenase |
| Oroxidin | Inhibits 5-Lipoxygenase |
| Pedalitin | Inhibits 5-Lipoxygenase |
| Quercetin | Inhibits Cyclooxygenase 1, Cyclooxygenase 2, 5-Lipoxygenase, 12-Lipoxygenase |
| Resveratrol | Inhibits Cyclooxygenase 1, Cyclooxygenase 2, 5-Lipoxygenase |
| Rhamnetin | Inhibits 5-Lipoxygenase |
| Sanggenon B | Inhibits Cyclooxygenase 1, 5-Lipoxygenase |
| Sanggenon D | Inhibits 5-Lipoxygenase |
| Scutellarein | Inhibits Phospholipase A2 |
| Silibinin | Inhibits 5-Lipoxygenase |
| Sophoflavanone A | Inhibits Cyclooxygenase 1 |
| Sophoflavanone G | Inhibits Cyclooxygenase 1, 5-Lipoxygenase, 12-Lipoxygenase |
| Tectorigenin | Inhibits Cyclooxygenase 2 |
| Wogonin | Inhibits Cyclooxygenase 2 |
| Polyphenols | |
| Anisic acid | Inhibits Phospholipase A2 |
| Caffeic acid | Inhibits 5-Lipoxygenase |
| Catechin | Inhibits Cyclooxygenase 1, 5-Lipoxygenase |

| | |
|---|---|
| Curcumin | Inhibits Phospholipase A2, Cyclooxygenase 1, Cyclooxygenase 2, 5-Lipoxygenase |
| Diphyllin acetapioside | Inhibits 5-Lipoxygenase |
| EGCG | Inhibits Cyclooxygenase 2 |
| Eugenol | Inhibits 5-Lipoxygenase |
| Gingerol | Inhibits 5-Lipoxygenase |
| Ginkgetin | Inhibits Phospholipase A2, 5-Lipoxygenase |
| Hydroxytyrosol | Inhibits 5-Lipoxygenase |
| Hyperforin | Inhibits 5-Lipoxygenase |
| Medicarpin | Inhibits 5-Lipoxygenase |
| Ohenethyl ferulate | Inhibits Cyclooxygenase 2 |
| Onosmins A and B | Inhibits 5-Lipoxygenase |
| Panaxynol | Inhibits 5-Lipoxygenase |
| Phenethyl ferulate | Inhibits 5-Lipoxygenase |
| Quercetagetin-7-O-beta-O-glucoside | Inhibits 5-Lipoxygenase |
| Rosmarinic acid | Inhibits 5-Lipoxygenase |
| Rosmarinic acid methylester | Inhibits 5-Lipoxygenase |
| Rosmarol | Inhibits Cyclooxygenase 20 |
| n-3 PUFA | Inhibits Cyclooxygenase 2 and 5-Lipoxygenase |

**Table 4.** Phytochemical inhibitors of lipid mediators

### *3.1.2. Cytokines blockers*

Cytokines exhibit pleiotropy and have overlapping functions in the pathogenesis of asthma, making them a major target for new asthma therapies. Allergic inflammation is driven by an imbalance between Th1 and Th2 cytokines, favoring the Th2 arm of the immune response and inhibition of Th2 cytokines IL-4, IL-5 and IL-13 prevents asthma progression in animal models. Anti-IL-4 administration in mice prevents development of acute and chronic allergic inflammation [53], therefore, natural products that specifically target cytokines or their receptors have the potential to be effective asthma treatments.

Our current pharmacological approach include the use humanized monoclonal antibodies against specific cytokine or receptor targets. This class of drugs, known as the biologics, has been approved for use in treatment of cancer, autoimmune and inflammatory diseases. Omalizumab is a drug currently approved for the management of asthma and is antibody targeting IgE. While not specifically a cytokine blocker it functions through the same mechanism of action. These drugs while very effective carry the risk of unforeseen side effects and under current production treatment costs remain very high ranging upwards from $15,000 to 60,000 per annum. Others examples including humanized IL-4-specific antibodies that block

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IL-4 receptor $\alpha$ that are under clinical trial [54]. Neutralizing antibodies against IL-5 (Mepolizumab and Reslizumab) and IL-5 receptor $\alpha$ (MEDI-563) remarkably inhibits IL-5 related pathways resulting in reduction of asthma exacerbations [55]. Tralokinumab, an anti-IL-13 monoclonal antibody, prevents the development of asthmatic phenotype, both in murine model as well asthmatic patients [56]. Suplatast tosilate inhibits IL-4 and IL-5 production from T cells and reduces AHR in asthmatic patients.

Some of the phytochemicals and potential treatments indicated against cytokines function are listed in Table 5. These products if proved to be effective, could be cost effective alternatives and being natural products have the potential to have less side effects.

| **Phytochemicals**<br>**Anti-oxidants** | **Target/Function and Effective Concentration(s)** | **References** |
|---|---|---|
| Vitamin A | Inhibits release of Th2 cytokines IL-4, IL-5 and IL-13 *in vitro* as well as *in vivo.* Suppresses production of IP-10, IL-6, TNF, GM-CSF and IFN-γ. | |
| Vitamin C | Effect on Th1/Th2 balance controversial. | |
| Vitamin E | Inhibits IL-1β, IL-6 and TNF response of human monocytes in asthmatic patients. Suppresses IL-4 levels in lungs of experimental allergic mice. | |
| **Flavonoids** | | |
| Apigenin | Reduces airway inflammation by down-regulating Th2 cytokines IL-4 and IL-13. Suppresses the expression of Th2 cytokines (IL-4, IL-13 and IL-5) in human basophils. Inhibits production of TNF, IL-6 and GM-CSF in HMC-1 cells. | [57, 58] |
| Baicalein | Decreases inflammatory cytokines such as TNF and IL-6 in allergic inflammation. Inhibits production of IL-6 in activated human mast cells, and GM-CSF from human cultured mast cells. | [52, 59] |
| Bicalin | Reduces TNF and IL-6 levels in plasma and BALF in cigarette smoke-induced COPD rat model. | [60] |
| Chrysin | Downregulates IL-4 and IL-13 expression and production in allergen-sensitized mice. Inhibits TNF, IL-1β, IL-4 and IL-6 expression in RBL-2H3 and HMC-1 cells. | [61] |
| Chrysol | Inhibits IL-4 production in antigen-stimulated RBL-2H3 cells. | [62] |
| Daidzein | Mast cell stabilizer; inhibits Th2 cytokines production. | |
| Fisetin | Inhibits IL-13 production in RBL-2H3 cells, and TNF, IL-6, IL-4 and IL-1β production in HMC-1 cells. Suppresses the expression of Th2 cytokines (IL-4, IL-13 and IL-5)in human basophils. Attenuates LPS-induced TNF, IL-6 and IL-10 release in leukocytes of patients with COPD. | [57, 63-65] |
| Genistein | Inhibits TNF production in PBMCs from asthmatic patients. | |
| Ginkgetin | Inhibits TNF expression in activated macrophages. | |

| Phytochemicals Anti-oxidants | Target/Function and Effective Concentration(s) | References |
|---|---|---|
| Isoliquiritigenin | Inhibits the release of TNF and IL-6 in activated inflammatory cells. | |
| Kaempferol | Impairs Th2 cytokines production (IL-5 and IL-13) in OVA-sensitized mice. Suppresses the release of IL-4 and TNF in RBL-2H3 cells and macrophages. Inhibits IgE-mediated TNF and IL-6 release in hCBMCs. | |
| Kuraridin | Suppresses expression of TNF and IL-1β in LPS-stimulated macrophages (40 μM). | [66] |
| Kurarinone | Suppresses expression of TNF and IL-1β in LPS-stimulated macrophages (40 μM). | [66] |
| Luteolin | Reduces the levels of TNF and IL-1β in LPS-stimulated macrophages (8 & 16 μM). Inhibits induction of TNF, IL-6 and GM-CSF in HMC-1 cells (10 & 50 μM). Inhibits Th2 cytokines (IL-4, IL-5 and IL-13) expression in murine asthma model (50 & 100 mg/kg body wt.). Inhibits myelin basic protein-induced IL-6, TGF-β1, and TNF release in hCBMCs (10 & 100 μM). Decreases TNF ($IC_{50}$ 7.9±4.6 μM) and IL-1β ($IC_{50}$ 5.1±0.4 μM) in PBMCs. Reduces IL-4 and IL-5 levels in BALF of murine asthma model (0.1 mg/kg body wt.). Inhibits antigen-IgE-mediated TNF ($IC_{50}$ 5.8 μM) and IL-4 ($IC_{50}$ 3.7 μM) production in RBL-2H3 cells. | [57, 67-73] |
| Morin | Inhibits IgE-mediated TNF and IL-6 release in hCBMCs (10 & 100 μM). Inhibits TNF secretion in LPS-activated macrophages. | [74, 75] |
| Myricetin | Inhibits TNF (30 μM) and IL-6 (30 μM) production in HMC-1 cells. Inhibits IgE-mediated TNF (10 & 100 μM) and IL-6 (1, 10 & 100 μM) release from hCBMCs. | [75, 76] |
| Naringenin | Suppresses Th2 cytokines production from CD4 T cells (0.8 mg/kg body wt.). Reduces IL-4 (25, 50 & 100 mg/kg body wt.) and IL-13 (50 & 100 mg/kg body wt.) levels in BALF of murine asthma model. Suppresses LPS-induced IL-1β (10, 25 & 50 μg/mL), IL-6 (5, 10, 25 & 50 μg/mL), and TNF (25 & 50 μg/mL) production in macrophages and human whole-blood samples. | [77-79] |
| Pedalitin | Inhibits TNF and IL-12 production in LPS-activated macrophages (40 μM). | [80] |
| Quercetin | Inhibits IgE-mediated TNF (10 & 100 μM) and IL-6 (1, 10 & 100 μM) release from hCBMCs. | [75] |
| Resveratrol | Inhibits increase in Th2 cytokines (IL-4 and IL-5) in plasma and BALF in asthmatic mouse model (30 mg/kg body wt.). Inhibits TNF induced GM-CSF and VEGF release in HASM cells. Inhibits PMA- and A23187-induced TNF and IL-6 release in HMC-1 cells. Decreases production of IL-1β in lung tissue of mice with LPS-induced acute lung injury (1 | [68, 81-86] |

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| Phytochemicals Anti-oxidants | Target/Function and Effective Concentration(s) | References |
|---|---|---|
| | mg/kg body wt.). Attenuates C5a-induced TNF, IL-1β and IL-6 production in mouse model (10 mg/kg body wt.). | |
| Scutellarein | Inhibits TNF and IL-12 production in LPS-activated macrophages (40 μM). | [80] |
| Silibinin | Polarizes Th1/Th2 balance towards Th1 by increasing IFN-γ (200 & 400 mg/kg body wt.) and decreasing IL-4 levels (200 & 400 mg/kg body wt.) in asthmatic mouse model. | [87] |
| **Polyphenols** | | |
| Caffeic acid | Inhibits increase in TNF and Th2 cytokines (IL-4 and IL-5) in BALF in asthmatic mouse model (10 mg/kg body wt. IP). Inhibits IL-10 expression in allergic patients' DCs (10 μM). Suppresses TNF and IL-6 levels in asthmatic patients (13% solution). | [88-90] |
| Curcumin | Attenuates the expression of IL-4 and IL-5 in BALF in asthmatic mouse model (20 mg/kg body wt.). Inhibits release of IL-10, TNF and IL-1β in HDM-activated eosinophils and bronchial epithelial cells (10 μM). Inhibits IL-5, GM-CSF and IL-4 production in lymphocytes from bronchial asthmatics (10 μM). Inhibits TNF secretion in activated HMC-1 cells (10 & 100 μM/L). Inhibits tryptase-induced IL-6 release in eosinophils (25 μM). | [91-95] |
| EGCG | Reduces TNF in BALF in asthmatic guinea pigs (25 mg/kg body wt. SC). Inhibits TNF and IL-6 production in HMC-1 cells (100 μM). Attenuates production of TNF in lungs of mice with LPS-induced acute lung injury (10 mg/kg body wt. IP). | [96-99] |
| Eugenol | Inhibits IL-1β, TNF and IL-6 release in macrophages. | [100, 101] |
| Gallic acid | Inhibits TNF and IL-6 in HMC-1 cells (10 μM). | [102] |
| Gingerol | Inhibits IL-1β and IL-12 release in peritoneal macrophages 100 ng/mL) | [103] |
| Hydroxytyrosol | Inhibits LPS-induced TNF production in THP-1 cells (25, 50 & 100 μM). Reduces TNF levels in LPS-treated mice. | [104, 105] |
| Rosmarinic acid | Reduces IL-4, IL-5 and IL-13 expression in lung of HDM-sensitized asthmatic mouse model (1.5 mg/day PO). Attenuates IL-1β, IL-6, and TNF increase in spleen and nasal mucosa of asthmatic mouse model (4 mg/kg body wt.). Inhibits IL-4 and IFN-γ release from $CD4^+$ T cells (1 & 5 μM). Inhibits diesel exhaust particles-induced IL-1β expression in mice lung (4.6 μg/kg body wt.). | [106-110] |
| **Terpenes** | | |
| Celastrol | Reduced mRNA expression of IL-5, IL-5, IL-13, TNF, and IFN-γ in BAL cells and lung tissue of asthmatic mouse model. | |

| Phytochemicals Anti-oxidants | Target/Function and Effective Concentration(s) | References |
|---|---|---|
| Costunolide | Inhibits production of TNF, IL-1β and IL-6 by LPS-stimulated macrophages (0.1, 0.5 & 1 μM). | [111, 112] |
| Helenalin | Inhibits TNF and IL-6 secretion by ASMCs (1 μM). | [113] |
| n-3 PUFA | EPA and DHA Lower BALF concentration of pro-inflammatory cytokines IL-1α, IL-2, IL-5, IL-9, IL-13, G-CSF and RANTES. EPA (120 μM) suppress TNF and IL-1β expression and production in LPS-stimulated alveolar macrophages from asthmatic patients | Ref? |

**Table 5.** Phytochemical inhibitors of proinflammatory cytokines

### *3.1.3. Chemokines and chemokine receptors blockers*

Chemokines (CC) and their receptors (CCR) play a crucial role in the recruitment of inflammatory cells into the airways and development of asthma. CC-chemokine receptor 3 CCR3, CCR4, and CRTH2 antagonists are being targets currently being evaluated for the treatment of asthma. A study found that treatment of asthmatic mice with an anti-CCR3 monoclonal antibody inhibits allergen-induced eosinophilia and $CD34^+$ progenitor cell infiltration into the lung, which is accompanied by reduced AHR [114, 115]. RS-1748, a CCR4 antagonist, inhibits OVA-induced airway inflammation in guinea pigs [116]. The number of CCR4-expressing Th2 cells is increased in the airways of asthmatic patients which can be blocked by a selective CCR4 antagonist [117], and therefore could be an effective therapy for asthma. Ramatroban and closely related TM30089 are antagonists for CRTH2, a chemokine receptor expressed on Th2 cells. They have been shown to attenuate allergen-induced EAR and LAR in animal models of asthma [118, 119]. Some of the phytochemicals indicated against chemokines function are listed in Table 6.

| Phytochemicals Flavonoids | Target/Function and Effective Concentration(s) | References |
|---|---|---|
| Apigenin | Suppresses the production of MDC and IP-10 in THP-1 cells ($10^{-6}$ and $10^{-5}$ M). Inhibits release of LPS-induced MCP-1 in J774.2 macrophages (10 & 30 μM). Inhibits production of IL-8 in HMC-1 cells. | [58, 120, 121] |
| Baicalein | Inhibits IL-8 and MCP-1 release in activated human mast cells. Inhibits eotaxin production in human dermal fibroblasts (10 μg/mL). | [59, 122] |
| Baicalin | Reduces IL-8 levels in plasma and BALF in cigarette smoke-induced COPD rat model. Inhibits eotaxin production in human dermal fibroblasts (10 μg/mL). | [60, 122] |

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| Phytochemicals Flavonoids | Target/Function and Effective Concentration(s) | References |
|---|---|---|
| Chrysin | Inhibits TNF-induced IL-8 expression in HEK 293 cells (20, 40 & 80 μM). | [123] |
| Chrysol | Inhibits MCP-1 production in antigen-stimulated RBL-2H3. | [62] |
| Fisetin | Inhibits TNF-induced IL-8 expression in HEK 293 (20, 40 & 80 μM). Inhibits IL-8 production in HMC-1 cells. | [65, 123] |
| Genistein | Blocks HDM-induced IL-8 release in human lung epithelial cells (50 μM). Inhibits IL-8 release in TNF-stimulated human keratinocytes (60 μM). Inhibits chemokine-stimulated eosinophil adherence ($10^{-7}$, $10^{-6}$ and $10^{-5}$ M). | [124-126] |
| Isoliquiritigenin | Inhibits eotaxin-1 secretion in human fetal lung fibroblasts ($IC_{50}$ 0.92±0.05 μg/mL). | [127] |
| Kaempferol | Suppresses LPS-induced production of MDC, IP-10 and IL-8 in THP-1. Inhibits MCP-1 production in antigen-stimulated RBL-2H3 and J774.2 macrophages (10 & 30 μM). Inhibits TNF-induced IL-8 expression in HEK 293 (20, 40 & 80 μM). | [62, 123, 128, 129] |
| Kuraridin | Suppresses expression of MCP-1 in LPS-stimulated macrophages (40 μM). | [66] |
| Kurarinone | Inhibits MCP-1-induced chemotaxis of THP-1 cells ($IC_{50}$ 19.2 μg/mL). Suppresses expression of MCP-1 in LPS-stimulated macrophages (40 μM). | [130] |
| Luteolin | Inhibits TNF-induced expression of MCP-1 (10, 20 & 30 μM) and CXCL-1 (20 & 30 μM) expression in keratinocytes. Inhibits induction of IL-8 in activated HMC-1 cells (50 μM) and hCBMCs. | [70, 131, 132] |
| Morin | Inhibits IL-8 production in antigen-stimulated hCBMCs (100 μM). | [75] |
| Myricetin | Inhibits IL-8 production in antigen-stimulated hCBMCs (100 μM). | [75] |
| Naringenin | Decreases secretion of PMA-induced IL-8 in HL-60 cells (20 μM). Suppresses LPS-induced IL-8 production by macrophages and human whole-blood samples (25 & 50 μg/mL). Inhibits expression of RANTES and eotaxin-1 in BALF and lungs in asthmatic mouse model (50 & 100 mg/kg body wt.). | [78, 79, 133] |
| Quercetin | Decreases production of IL-8 (3 & 30 μM) and MCP-1 ($10^{-4}$, $10^{-5}$ and $10^{-6}$ M) in activated HMC-1 and IL-8 in human bronchial epithelial cells (0.1, 10 & 25 μM). | [134-136] |
| Resveratrol | Decreases production of MIP-1α in lung tissue of mice with LPS-induced acute lung injury (10 mg/kg body wt.). Reduces TNF-induced IL-8 release in HASMCs in COPD. Inhibits IFNγ-induced production of IP-10 and MIG in macrophages and HMC-1 cells. | [68, 82, 83, 85, 86, 137] |

| Phytochemicals Flavonoids | Target/Function and Effective Concentration(s) | References |
|---|---|---|
| | Inhibits IL-8 production in THP-1 cells (0.1, 1, 10 & 100 µg/mL). Attenuates C5a-induced MIP-1α production in mouse model (1 mg/kg body wt.). | |
| Tectorigenin | Inhibits MCP-1 expression in endothelial cells. | [138] |
| Wogonin | Suppresses mite antigen-induced TARC expression in human keratinocytes (250 ng/mL). | [139] |
| **Polyphenols** | | |
| Caffeic acid | Decreases IL-8 release in chitinase-activated human airway epithelial cells (1 µM). Inhibits production of eotaxin in human lung fibroblast cells (1, 10 & 100 µM). Inhibits IP-10 expression in allergic patients' DCs (10 µM). Decreases expression of CXCR4 receptor on CD4 T cells (20 µg/mL). Suppresses IL-8 levels in asthmatic patients (13% soln.). | [89, 90, 140-143] |
| Curcumin | Inhibits tryptase-induced IL-8 release in eosinophils (25 µM). | |
| EGCG | Inhibits IL-8 release in TNF-stimulated human keratinocytes (0.5, 1, 5 & 10 µM). Reduces airway inflammation in asthmatic mouse model by binding to chemokines CXCL9, CXCL10 and CXCL11 (10 & 100 µM). Decreases MCP-1 and CCR2 expression on THP-1 cells (100 µM). Attenuates production of MIP-2 in lungs of mice with LPS-induced acute lung injury. Reduces expression of MCP-1 and IL-8 in HMC-1 cells (100 µM). Inhibits neutrophil migration by suppression of CINC-1 production (15, 50 & 150 µg/mL). Downregulates cigarette smoke-induced IL-8 release from bronchial epithelial cells. | [97, 99, 125, 144-147] |
| Gallic acid | Inhibits production of IL-8 and TARC (5 & 10 µg/mL) in neutrophils and keratinocytes respectively. Inhibits eotaxin and RANTES in pleural lavage fluid of allergen-challenged mouse model (100 mg/kg body wt.). Inhibits IL-8 release in HMC-1 cells (10 µM). | [148-150] |
| Rosmarinic acid | Inhibits LPS-induced production of MCP-1 and MIP-1α in bone-marrow derived DCs (100 µM). Reduces diesel exhaust particles-induced MIP-1α, MCP-1 and KC expression in mice lung (4.6 µg/kg body wt.). Inhibits expression of eotaxin in lungs of mite antigen-sensitized mice (1.5 mg/kg body wt. PO). Inhibits expression of CCL11 and CCR3 genes induced by TNF in human dermal fibroblast cells. | [102, 107, 110, 151] |
| Helenalin | Inhibits eotaxin and RANTES secretion in ASMCs (1 µM). | [113] |

**Table 6.** Phytochemical inhibitors of chemokines

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*3.1.4. Miscellaneous: IgE, histamine, enzymes*

The activation of mast cells involves the cross-linking of IgE bound to the $F_C\varepsilon RI$ surface receptor. Activation is measured in the laboratory by the release of the Beta-hexosaminidase enzyme (β-hex) from cytosolic granules into the interstitial fluid. The following compounds (Table 7) have been found to be inhibitors of β-Hex release in vitro and have to potential to be inhibitor of IgE-antigen activation.

| Phytochemicals | $IC_{50}$ | References |
|---|---|---|
| **Anti-oxidants** | | |
| Vitamin A/carotenoid | ? | [152] |
| **Flavonoids** | | |
| Apigenin | 4.5 μM | [153] |
| Baicalein | 17 μM | [153] |
| Chrysin | ? | [62] |
| Daidzein | ? | [83] |
| Fisetin | 3 μM | [73] |
| Genistein | 28.5 μg/mL | [83] |
| Ginkgetin | 6.52 μM | [154] |
| Glycitein | 28.5 μg/ml | [83] |
| Isoliquiritigenin | 24 μM | [155] |
| Kaempferol | 7.5 μM | [62] |
| Luteolin | 3 μM | [73] |
| Morin | 51 μM | [153] |
| Myricetin | 6.7 μM | [153] |
| Naringenin | 29 μM | [156] |
| Quercetin | 3 μM | [73] |
| Resveratrol | 14 μM | [157] |
| Sophoflavanone G | 20 μM | [158] |
| Tectorigenin | 0.193 mM | [159] |
| **Polyphenols** | | |
| Caffeic acid | ? | [160] |
| Curcumin | 5.3 μM | [161] |
| EGCG | ? | [162] |
| Ginkgetin | ? | |

| Phytochemicals | $IC_{50}$ | References |
|---|---|---|
| Medicarpin | >100 μM | [155] |
| Rosmarinic acid | ? | [160] |
| **Terpenes** | | |
| Celastrol | ? | [163] |
| Costunolide | 34 μM | [164] |

**Table 7.** Phytochemical inhibitors of beta-hexosaminidase enzyme

### 3.2. Inhibitors of intracellular signaling pathways

The previous section focused on compounds that affected the function of extracellular, cell surface/receptor and cell to cell interactions. The following group of compounds affect the intercellular functions of the cells particular the components of cell signalling pathways.

#### *3.2.1. Protein kinase inhibitor*

Protein kinases have a key role in the expression and activation of inflammatory mediators implicated in airway inflammation. Enhanced activation of p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), spleen tyrosine kinase (Syk), and phosphoinositol 3-kinase (PI3K) signaling pathways have all been proposed to have a role in the pathogenesis of asthma.

p38 MAPK is involved in the airway inflammation and remodeling. A selective synthetic p38 MAPK inhibitor SB2439063 reduces synthesis of Th2 cytokines [54] and thus has a potential application in asthma treatment. Inhaled p38 MAPK antisense oligonucleotide attenuates asthma in OVA-sensitized and –challenged mice [165]. The natural product limonene inhibits eosinophil migration in p38 MAPK dependent manner and was investigation in an *in vitro* bronchial asthma model [166].

JNK activity is increased in corticosteroid-resistant asthma and SP600125, a JNK inhibitor, reduces cytokines expression and inflammatory cells accumulation in BALF of asthmatic animal models. Celastrol, a natural compound, modulates the expression of JNK in asthma [167]. It supresses allergen-induced mouse asthma by decreasing expression of MAP kinases, ERK and JNK [168].

Syk is a protein kinase involved in signal transduction in many inflammatory cells, and its aberrant regulation is associated with asthma, thus is considered an interesting target for asthma therapies. BAY 61-3606, a synthetic Syk inhibitor, inhibits inflammatory mediator release from mast cells, basophils, eosinophils, and monocytes, and reduces allergic asthma in rats [169]. Eupatilin, a biological extract, inhibits Syk and blocks downstream signaling pathways in mast cell from guinea pig lung tissues, leading to inhibition of mediator release [170]. Thus, Syk inhibitors may have use clinically as a treatment for asthma.

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The PI3K pathway plays a major role in the pathogenesis of asthma by promoting eosinophil and neutrophil recruitment and degranulation [171]. Sorbus commixta water extract, an anti-inflammatory medicinal plant, remarkably blocks PI3K activity in antigen-activated macrophages, suggesting the usefulness of PI3K inhibitors in asthma [172].

### *3.2.2. Transcription factor inhibitors*

The increased expression of various inflammatory proteins seen in asthma is the result of enhanced gene transcription, since many of the genes are not expressed in normal cells but are selectively induced during inflammation. Changes in gene transcription are under the control of transcription factors which therefore play a key role in the pathogenesis of asthma.

Transcription factors such as nuclear factor-κB (NF-κB), GATA-3, signal transducers and activators of transcription protein (STAT)s, nuclear factor of activated T cells (NFAT), and peroxisome proliferator-activated receptors (PPAR) have been implicated in asthma and therefore represent therapeutic targets.

NF-κB is induced by many factors involved in asthmatic inflammation and is implicated in glucocorticoid-resistant asthma. Inhibition of IκB (inhibitor of NF-κB) by small molecule inhibitors suppresses inflammatory responses in mast cell [173], OVA-induced rat model of airway inflammation [174], and macrophages from BALF of asthmatic patients. A number of herbal preparations (i.e. andrographolide and narigenin) have been demonstrated to inhibit airway inflammation and AHR by inhibiting NF-κB activity in OVA-induced murine asthma [78, 175]. Many inhibitors of NF-κB have been identified belonging to the Flavonoids, Polyphenols and Terpenoids classes of compounds as well as n-3 PUFA (see Table 8).

| Flavonoid | Polyphenols | Terpenoids | PUFA |
|---|---|---|---|
| Apigenin | CAPE | Parthenolide | n-3 PUFA |
| Capsaicin | Curcumin | Costunolide | |
| Fisetin | Epigallocatechin-3-gallate | Helenalin | |
| Kuraridin | Gallic acid | Celastrol | |
| Kurarinone | Gingerol | Celaphanol A | |
| Luteolin | Rosmarinic acid | Kamebanin | |
| Myrecitin | | Kamebacetal A | |
| Naringenin | | Excisanin A | |
| Quercetin | | Orbiculin | |
| Resveratrol | | | |

**Table 8.** Naturally occurring NF-κB inhibitors

GATA-3 is a critical transcription factor that is specifically expressed by Th2 cells and is involved in their differentiation [176]. GATA-3 has been indicated in the development of airway eosinophilia and expression is increased in atopic asthma [177] and therefore is an obvious target for inhibition. Direct inhibition by using a specific antisense oligonucleotide or

interference RNA promises a novel approach for asthma treatment [178]. Polyphenols, such as apigenin and quercetin, ameliorate asthma symptoms, and suppress the translocation of GATA-3 in the cytosol of lung tissue of OVA-sensitized and -challenged mice [179].

NFAT transcription factor is mostly involved in the production of Th2 cytokines through its interaction with GATA-3 and activator protein-1 (AP-1) [180]. Immunosupressive drugs cyclosporin A and FK506 block NFAT activation [181]. The use of peptides known as inhibitors of NFAT-calcineurin association (INCA) represents an alternative asthma treatment strategy [182].

PPARs are transcription factors belonging to the nuclear receptor superfamily activated by PUFA derivatives, oxidized fatty acids and phospholipids. PPARγ activation might exhibit anti-inflammatory properties in different inflammatory processes. In a murine model of asthma, treatment with PPARγ ligand ciglitazone significantly reduces AHR and lung inflammation [183]. PPARα and PPARγ ligands also decreases allergen-induced AHR, lung inflammation as well as serum IgE levels in different asthma models [184]. Popular anti-asthmatic Traditional Chinese Medicine San-ao Decoction (SAD), comprising *Herba Ephedrae*, *Radix et Rhizoma Glycyrrhizae* and *Seneb Armeniacae Amarum*, has a significant effect on PPARγ activation [185].

### 3.3. Inhibitors of oxidative stress

Oxidative stress plays a critical role in the development of asthmatic conditions. Oxidative stress and its by-products drive a Th2-dependent immune response. A number of antioxidants have been explored for their anti-inflammatory and anti-asthmatic properties, and a number of natural products have emerged as promising candidates. Resveratrol, a component of red wine, possesses anti-inflammatory and antioxidant properties. It inhibits inflammatory cytokines release in patients with chronic obstructive pulmonary diseases (COPD) [186] and may be beneficial in asthma. Several other biological compounds such as *Sanguisorba offcinalis* [187], aqueous extract from the root of *Platycodi Radix* [188], stem and bark of *Ulmus davidiana* [189], and *Alpinia katsumadai* seed extracts [190] attenuate oxidative stress and asthmatic activity in OVA-induced murine asthma. The flavonoids and polyphenols are the main groups of compounds that display anti-oxidative properties as listed in table 9.

| Phytochemicals | Function and effective concentration(s) | References |
|---|---|---|
| **Flavonoids** | | |
| Apigenin | Suppresses LPS-induced NO production in RAW264.7 macrophages | [191] |
| Baicalein | Inhibits LPS-induced NO production and iNOS expression in RAW264.7 macrophages | [192] |
| Chrysin | Inhibits NO production ($IC_{50}$ 7.50±1.84 μM) in LPS-activated RAW264.7 macrophages | [74] |
| Fisetin | Inhibits TNF-induced ROS production in HEK cells | [123] |

| Phytochemicals | Function and effective concentration(s) | References |
|---|---|---|
| Kaempferol | Inhibits NO production ($IC_{50}$ 9.83±1.55 μM) in LPS-activated RAW264.7 macrophages | [74] |
| Kuraridin | Inhibits ROS, NO production (20 & 40 μM) and iNOS gene expression (40 μM) in LPS-activated RAW264.7 macrophages. | [66] |
| Kurarinone | Inhibits ROS, NO production (40 μM) and iNOS gene expression (40 μM) in LPS-activated RAW264.7 macrophages. | [66] |
| Luteolin | Inhibits NO production (4, 8 & 16 μM) in LPS-activated RAW264.7 macrophages. | [67] |
| Morin | Inhibits NO production ($IC_{50}$ 44.86±1.05 μM) in LPS-activated RAW264.7 macrophages | [74] |
| Naringenin | Inhibits lung iNOS expression in allergen-induced mouse asthma model | [78] |
| Quercetin | Inhibits NO production ($IC_{50}$ 36.9±1.24 μM) in LPS-activated RAW264.7 macrophages | [74] |
| Resveratrol | Inhibits NO production in mice with acute lung injury (1 mg/kg body wt.). | [85] |
| **Polyphenols** | | |
| Caffeic acid | Reduces ROS levels in BALF of OVA-sensitized and –challenged mice (10 mg/kg body wt. IP). | [88] |
| Curcumin | Reduces iNOS expression in lung tissue of OVA-sensitized asthmatic mice. | [91] |
| EGCG | Reduces NOS activity in lungs of OVA-sensitized asthmatic guinea pigs. | [96] |
| Hydroxytyrosol | Inhibits $NO_2$ production as well as iNOS expression in THP-1 cells (50 & 100 μM) | [104] |
| Rosmarinic acid | Inhibits formation of ROS and RNS in activates macrophages | [193] |
| Helenalin | Inhibits iNOS expression in LPS-stimulated macrophages (10 μM) | [137] |
| n-3 PUFA | Decreases exhaled NO from asthma patients challenged with mite allergen | [194] |

**Table 9.** Phytochemical inhibitors of oxidative stress

## 4. Experimental models of asthma

Understanding respiratory sensitization mechanisms is the first step to designing therapeutic agents that may relieve patients of their asthma symptoms. A number of *in vitro* and *in vivo* experimental models are able to reproduce one or more features of allergic response and have

been studied for a few decades. Animal models of asthma are the best characterized in terms of the inflammatory and remodeling processes. The use of gene knockout and transgenic animals and the therapeutic administration of antibodies or pharmacological antagonists/ inhibitors have helped to identify a range of pre-clinical targets for subsequent evaluation in humans. Small animal models of asthma, using mice, rats and guinea pigs, are most commonly used. Most of these models are based on active sensitization to an allergen such as OVA via the airways. *In vitro* model systems using inflammatory cells and airway-related cell types are widely used in studies on immuno-biological mechanisms of asthma. A more detailed description of the most commonly used models of asthma can be found in Table 10.

| **In Vivo Model** | **Route(s) of Administration** | **Primary Effects** |
|---|---|---|
| OVA-induced allergic asthma | Intranasal or aerosol challenge, intrathoracic inoculation, intradermal challenge | Increased serum IgE levels, histological changes in airways including cellular infiltration, mediator release, AHR, and remodeling. |
| LPS lung inflammation model | Intranasal | Leukocytes (mainly neutrophils) recruitment to lung within 4 hr of LPS treatment. |
| House dust mite exposure | Intraperitoneal sensitization followed by inhalational challenge | Increased serum IgE levels, histological changes in airways including cellular infiltration, mediator release, AHR, and remodeling. |
| Infection by Aspergillus fumigatus | Intraperitoneal sensitization followed by inhalational challenge | Increased serum IgE levels, histological changes in airways including cellular infiltration, mediator release, AHR, and remodeling. |
| Ragweed allergen exposure | Intraperitoneal sensitization followed by inhalational challenge | Airway inflammation and AHR. |
| Infection with Ascaris suum | Subcutaneous and intratracheal sensitization, aerosol challenge | Bronchoconstriction, AHR and cellular infiltration. |
| **In Vitro Cell Model** | **Cell Type** | **Primary Response(s)** |
| Mast cells: | | |
| Human mast cells | $CD34^+$-derived primary mast cells, cord blood mast cells, skin mast cells, lung mast cells, LAD2, LUVA , HMC-1, RBL-2H3 | Release of proinflammatory mediators such as histamine, tryptase, chymase, cytokines, chemokines, leukotrienes, and prostaglandins. |
| Rodent mast cells | Bone marrow-derived mast cells, peritoneal mast cells | Release of proinflammatory mediators such as histamine, tryptase, chymase, cytokines, chemokines, leukotrienes, and prostaglandins. |
| Eosinophils | Primary cells, EoL-1, AML14.3D10 | Release of proinflammatory mediators such as ECP, EPO, EDN, MBP, cytokines, and chemokines. |

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| In Vivo Model | Route(s) of Administration | Primary Effects |
|---|---|---|
| Bronchial epithelial cells | Primary cells, NHBE, BEAS-2B | Release of proinflammatory mediators such as cytokines and chemokines. Morphological changes. |
| Alveolar epithelial cells | Primary cells, A549 | Release of proinflammatory mediators such as cytokines and chemokines. Morphological changes. |
| Monocytes/ Macrophages | Primary cells, Mono-Mac-6, THP-1, RAW 264.7 | Release of proinflammatory mediators such as cytokines, chemokines, leukotrienes, prostaglandins, ROS, and RNS. |
| Dendritic cells | Primary cells, U-937, CD34-DC, Mo-DC, KG-1, MUTZ-3 | Release of proinflammatory mediators such as cytokines and chemokines. |

**Table 10.** *In vivo* and *in vitro* models for asthma studies

## 5. Evidence for the association between diet and asthma

### 5.1. Antioxidants

The airways are continuously exposed to oxidants, either generated endogenously by various metabolic reactions (e.g. from mitochondrial respiration or released from phagocytes) or derived from exogenous sources (e.g. air pollutants and cigarette smoke). Allergen-activated inflammatory cells from asthmatic patients produce more ROS than from healthy individuals. In addition, several inflammatory mediators including histamine, lipid mediators, cytokines, chemokines, ECP, and EPO are potential stimuli for ROS production in the airways, leading to asthma exacerbation.

Deficiency of endogenous antioxidant defenses has been reported in asthma [195]. Since a diet rich in vitamin A or carotenoids, vitamin C vitamin E, and flavonoids, has been associated with a decreased prevalence of asthma, understanding the relationship between dietary antioxidants and asthma-associated inflammatory responses has been a recent focus.

#### *5.1.1. Vitamin A and carotenoids*

A systemic review and meta-analysis by Allen *et al*. has shown that dietary vitamin A intake is significantly lower in asthmatic patients than in healthy subjects [196]. Asthmatic children have a lower serum vitamin A concentration than healthy controls [197]. Supplementation of the diet with lycopene, a carotene found in tomatoes and carrots, has a protective effect against asthma development in a murine model [198].

All-trans retinoic acid (ARTA), a derivative of vitamin A, inhibits airway inflammation in asthmatic rats. ARTA inhibits total cell counts and the proportion of inflammatory cells in BALF, suppresses the expression of NF-κB and intercellular adhering molecule-1 (ICAM-1), and increases the expression of iκB [199]. Retinoid acid also downregulates the expression of Th1 and Th2 chemokines in monocytes, including macrophage-derived chemokine and IP-10,

which are all important in the inflammatory process [200]. Airway smooth muscle cell migration, which contributes to the airway remodeling in chronic asthma is also inhibited by ARTA [201]. However, excessive intake of vitamin A exacerbates pulmonary hyperresponsiveness in murine asthma model, suggesting that excessive vitamin A may increase the risk and severity of asthma [202].

Mechanistically, vitamin A may regulate bronchial hyperreactivity by altering the function and abundance of the muscarinic M(2) receptors in bronchial tissue [203]. Moreover, carotenoids may regulate activation of a variety of transcription factors. Treatment of cells exposed to oxidative stress with β-carotene suppresses oxidative stress-induced activation of NF-κB and production of IL-6, TNF, and inflammatory cytokines. Carotenoids may influence the process of apoptosis in healthy cells. While the pro-apoptotic protein Bax is downregulated after induction of external stimuli, β-carotene is able to increase expression of the anti-apoptotic protein Bcl-2 in normal cells. In addition, β-carotene exhibits a pro-apoptotic effect in colon and leukemic cancer cells, and this effect occurs by a redox-dependent mechanism linked with NF-κB activity. These dual roles of vitamin A, including carotenoids, on apoptosis provide the capability of carotenoids as an effective anti-inflammatory agent in various diseases.

*5.1.2. Vitamin C*

Many observational studies have reported associations between reduced dietary/blood vitamin C levels and reduced lung functions. Asthmatic children undergoing an exacerbation have significant lower serum levels of vitamin C [204]. There is a positive correlation between serum vitamin C levels and asthma development in children (OR=0.72 per mg/dl, 95% CI=0.55, 0.95) [10]. Furthermore, asthma patients have significantly lower vitamin C level in both the cellular and fluid-phase fraction in induced sputum [205]. Higher maternal intake of citrus fruits rich in vitamin C during pregnancy is significantly associated with a reduced risk of allergic inflammation in the offspring [206]. Administration of vitamin C in OVA-challenged mice decreases AHR, influx of inflammatory cells in BALF and attenuates lung inflammation [207]. Similarly, high dose vitamin C supplementation significantly reduces eosinophilic infiltration in BALF and increases the Th1/Th2 cytokine secretion ratio; thus, skewing the Th1/Th2 balance toward non-allergic Th1 immune response in asthmatic mice [208].

A randomized, placebo controlled, double-blinded crossover trial has shown that vitamin C supplementation (1500 mg/day) attenuates asthma symptoms. Moreover, exhaled nitric oxide, urinary leukotriene $C_4$, $D_4$, $E_4$ and 9$\alpha$, 1β-prostaglandin $F_2$ after exercise are downregulated [209]. On the contrary, there are also studies showing no significant effect of vitamin C supplementation on asthma symptoms. For example, in a randomized, placebo-controlled, double-blind parallel group trial three hundred asthma patients provided with 1 g/day vitamin C or placebo for 6 weeks do not show any improvements of asthma symptoms [210], therefore, there is insufficient evidence from randomised-controlled trials to support the use of vitamin C for asthma treatment [211].

As its mechanism of action, vitamin C may regulate factors that can influence gene expression, apoptosis, and other cellular functions indicated in inflammation. In fact, vitamin C protects

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against cell death triggered by various stimuli, and major proportion of this protection is associated with its antioxidant ability [212]. Vitamin C inhibits the AP-1 activation by regulating MAPK-ERK pathway [213]. Treatment of cells exposed to UV-B irradiation with vitamin C results in a 50% decrease in JNK phosphorylation, which activates AP-1, therewith inhibiting the JNK/AP-1 signaling pathways [214]. At present, however, evidence from randomized controlled trials is insufficient to recommend a specific role for vitamin C in the treatment of asthma due to variable study design and generally poor reporting system.

### *5.1.3. Vitamin E*

The body of evidence from multiple studies suggests that a positive association between asthma outcomes and vitamin E intake or serum vitamin E levels. Asthmatic children have significantly lower serum levels of vitamin E than non-asthmatic children [204, 215]. A longitudinal birth cohort study has explored association between maternal plasma vitamin E, fetus and fetal lungs growth, and childhood asthma. The findings have shown that maternal vitamin E status has a positive effect on the growth of fetus and fetal lungs during early pregnancy and better asthma outcomes during childhood [216]. Moreover, high maternal vitamin E intake during pregnancy also reduces the risk of infantile wheeze [206]. Vitamin E intake is higher in control subjects than in asthma patients [217]. However there is no relationship found between serum vitamin E level and asthma [4, 218]. On the other hand, administration of vitamin E for 6 weeks does not have an effect on asthma features and serum immunoglobulin levels in adults [219].

Role of Vitamin E has been investigated in animal models of allergic asthma. Administration of Vitamin E to allergen-challenged mice reduces mitochondrial dysfunction, Th2 cytokines production, allergen-specific IgE, and expression of lipid mediators in lung leading to alleviation of asthmatic features [220]. Expression of IL-5 mRNA and protein in lung, and plasma IgE level are reduced after OVA sensitization and challenge compared to wild type mice in vitamin E transfer protein knockout mice [221]. Moreover, dietary supplementation with vitamin E affords variable degree of protection against ozone-induced enhanced airway response in allergen-sensitized guinea pigs [222]. However, oral $\alpha$-tocopherol has no protective effect on lung response in rat model of allergic asthma. There is no improvement in OVA-induced AHR, the inflammatory cell infiltrate and histological changes [223]. The observed opposite effects of vitamin E could be associated with the study design in an animal model of asthma. The effect of vitamin E deserves further evaluation.

Vitamin E may induce immunological effects via modulation of the functional activity of T cells and enhancing the phagocytic activity of peripheral granulocytes [224]. A derivative $\gamma$-tocopherol appears to be a more potent anti-inflammatory agent than $\alpha$-tocopherol. It decreases systemic oxidative stress, cytokine release from monocytes in asthmatic patients, and inhibits monocyte response to LPS and LPS-induced degradation of IκB and JNK activation [225]. There is a contradictory study demonstrating that $\gamma$-tocopherol elevates inflammation and ablates the anti-inflammatory benefit of the $\alpha$-tocopherol by regulation of endothelial cell signals during leukocyte recruitment in experimental asthma [226]. Dietary tocopherols are taken up from the intestine and transported via the lymph to the blood and then to the liver.

In the liver, α-tocopherol is transferred to plasma lipoproteins, resulting in retention of γ-tocopherol in tissues at 10% that of α-tocopherol. On interpreting these two contradictory results, one should consider their serum levels with caution since low plasma level of γ-tocopherol (1.2–7.0 μM) may act as prooxidant, while higher level of γ-tocopherol (19.5 μM at 8 days) exerts antioxidative and anti-inflammatory effects.

*5.1.4. Vitamin D*

Over the past several years, the role of vitamin D in immunomodulation has been studied and shown to have a significant impact on innate and adaptive immunity to infections, including the pathophysiology of allergic asthma. It has been proposed that the increase in allergy and asthma is a consequence of widespread vitamin D insufficiency which appears to be frequent in industrialized countries, reflecting the insufficient intake of diet-sourced vitamin D.

The serum vitamin D level is associated with asthma in children as well as adults. A randomized, placebo controlled clinical study with 1024 children suffering from mild-to-moderate persistent asthma has shown that Vitamin D deficiency ias associated with a higher rate of severe asthma [227]. There is a significant positive correlation between forced vital capacity percent predicted and serum vitamin D level children with asthma. Moreover, 91.6% of these asthmatic children are not sufficient in serum vitamin D level [228]. Low level of vitamin D in serum are also associated with increased hyperresponsiveness and reduced glucocorticoid response in adults with asthma [229]. These studies have indicated that the low serum vitamin D level is related to reduced lung function and higher risk of asthma. Reduced the risk of asthma exacerbation triggered by acute respiratory tract infection is observed in a vitamin D supplementation [230]. Higher consumption of vitamin D during pregnancy may reduce the risk of childhood wheeze and asthma.

One possible mechanism of vitamin D's protective effect against asthma can be that it inhibits the maturation process of dendritic cells by suppressing the expression of costimulatory molecules HLA-DR, CD86, CD80, the maturation marker CD83, and IL-12 which are important for the recruitment of Th1 cells [231]. Vitamin D also upregulates the expression of IL-10 receptor in dendritic cells, which is an anti-inflammatory cytokine. In addition, it can promote the production of FoxP3 positive and IL-10 positive regulatory T cells, and induce the release of IL-10, TGF-β and CTLA-4 [232]. Furthermore, it may reverse steroid-resistance in asthmatic patients through induction of IL-10 secreting T-regulatory cells [233], and vitamin D has been shown to regulate expression of many genes in ASM cells, including genes previously implicated in asthma predisposition and pathogenesis [234].

**5.2. Flavonoids**

Flavonoids interfere with oxidation of lipids and other molecules and this strong antioxidative property makes them protective against airway diseases linked to oxidative stress. In fact, several epidemiologic studies suggest the beneficial effects of flavonoids on asthma. A population-based case-control study has shown that apple consumption and red wine intake are inversely associated with asthma prevalence or severity, perhaps due to a protective effect

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of flavonoids [18]. Moreover, a 30-year longitudinal epidemiological study has reported that the incidence of asthma is lower in populations with higher intake of flavonoids [235].

| Flavonoid Subclass | Dietary Flavonoids | Some Common Food Sources |
|---|---|---|
| Anthocyanidins | Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin, Petunidin | Red, blue, and purple berries; red and purple grapes; red wine |
| Flavanols | Monomers (Catechins): Catechin, Epicatechin, Epigallocatechin Epicatechin gallate, Epigallocatechin gallate<br>Dimers and Polymers: Theaflavins, Thearubigins, Proanthocyanidins | **Catechins:** Teas (particularly green and white), chocolate, grapes, berries, apples<br>**Theaflavins, Thearubigins:** Teas (particularly black and oolong)<br>**Proanthocyanidins:** Chocolate, apples, berries, red grapes, red wine |
| Flavanones | Hesperetin, Naringenin, Eriodictyol | Citrus fruits and juices, e.g., oranges, grapefruits, lemons |
| Flavonols | Quercetin, Kaempferol, Myricetin, Isorhamnetin | Widely distributed: yellow onions, scallions, kale, broccoli, apples, berries, teas |
| Flavones | Apigenin, Luteolin | Parsley, thyme, celery, hot peppers |
| Isoflavones | Daidzein, Genistein, Glycitein | Soybeans, soy foods, legumes |

**Table 11.** Common dietary flavanoids.

Beyond antioxidative effects, flavonoids inhibit the release of histamine and other preformed granule associated mediators by inhibiting the activation of basophils and mast cells [66]. Flavonoids inhibit synthesis of IL-4, IL-13, and CD40 ligand but initiate generation of new phospholipid-derived mediators. One of the well-characterized flavonoids, quercetin, inhibits eosinophilic secretion of Charcot-Leyden crystal protein and ECP in a concentration-dependent manner. Very recently, Li *et al*. demonstrated that apigenin exhibits an anti-inflammatory activity in a murine asthma model and can switch the immune response to allergens toward the Th1 profile. These findings suggest that flavonoids are anti-allergenic and anti-inflammatory agents effective in treating/preventing asthma.

Vascular changes are one of the major components of asthmatic pathogenesis. These changes include an increase in vascular permeability, vascular dilation/engorgement, and vasculogenesis/angiogenesis. Flavonoids and their related compounds have been shown to modulate expression of HIF-1, VEGF, matrix metalloproteinases (MMPs), and epidermal growth factor receptor but also inhibit NF-κB, PI3K/Akt, and ERK1/2 signaling pathways [236]. These observations suggest that flavonoids as well as their related compounds inhibit certain steps of angiogenesis including cell migration, microcapillary tube formation, and MMP expression.

Many flavonoids have been tested for their anti-asthma effect. Quercetin decreases the eosinophil recruitment, reduces IL-5 and IL-4 levels, and inhibits NF-κB activation in BALF in OVA-induced mouse model [237]. It also regulates Th1/Th2 balance by enhancing IFN-γ and decreasing IL-4 levels in mouse asthma model [179]. Naringenin alleviates airway inflamma-

tion and reactivity by decreasing serum total IgE level and IL-4, IL-13 level in BALF and inhibiting NF-κB activity [78]. Licorice is a Traditional Chinese Medicine, which contain many flavonoids. Flavonoids extracted from licorice attenuates LPS-induced acute pulmonary inflammation by inhibiting inflammatory cells infiltration and inflammatory mediator release [238]. Neutrophils, macrophages and lymphocytes accumulation in BALF, lung TNF and IL-1β mRNA expression and lung myeloperoxidase activity are reduced; whereas BALF superoxide dismutase activity is increased [238]. Flavonoids from red algae decrease eosinophil infiltration, levels of TNF, IL-4 and IL-5 in BALF, airway luminal narrowing, AHR and level of allergen-specific IgE in the serum [239]. A complex mixture of bioflavonoids derived from purple passion fruit peel extract supplemented to asthma patients in a randomized, placebo-controlled, double-blinded trial alleviates asthma clinical symptoms, including FVC and FEV1 [240].

### 5.3. Resveratrol

Resveratrol scavenges intracellular ROS by inducing and stabilizing antioxidant enzymes such as catalase, SOD, and glutathione peroxidase hemoxygenase. In addition to its reducing properties, resveratrol has been shown to attenuate inflammation via inhibition of prostaglandin production [241] and to decrease the phosphorylation of ERK1/2, COX-2 activity, and activity of various transcription factors including NF-κB, STAT3, HIF-1α, and β-catenin [236]. Resveratrol also inhibits protein kinases (e.g. src, PI3K, JNK, and Akt) and the production of inflammatory mediators (e.g. IFN-γ, TNF, COX-2, iNOS, CRP and various interleukins). Recent studies have reported that resveratrol activates sirtuin1 (SIRT1) which is modulates apoptosis and has been shown to increase longevity in some experimental systems [242]. SIRT1 modulates poly (ADP-ribose) polymerase-1 (PARP-1) activity upon DNA damage. Activation of SIRT1 by resveratrol leads to a decrease in PARP-1 activity and promotes cell survival, which can attenuate the inflammatory reaction. We investigated the effects of resveratrol on human mast cell activation in comparison to the anti-allergic drug tranilast. The results show that resveratrol inhibits mast cell degranulation, cytokine, chemokine and leukotriene release, and is more efficacious than tranilast [316].

Resveratrol is able to modulate innate immune response by inhibiting expression of costimulatory molecules (CD80 and CD86) and major histocompatibility complex classes I and II in bone marrow-derived dendritic cells and inhibit angiogenesis pathway that is mediated through expression of MMPs, VEGF, cathepsin D, ICAM-1, and E-selectin [236]. These findings suggest that resveratrol can be a very attractive compound for preventing/treating asthma since this compound displays multiple therapeutic effects, showing antioxidative, anti-inflammatory, immune modulating, and vascular protective property.

Resveratrol has been shown to inhibit the airway inflammation and hyperresponsiveness in OVA-induced mouse asthma by reducing eosinophil/neutrophils infiltration, the levels of IL-4 and IL-5 in plasma and BALF [81]. It can modulate Th1/Th2 balance, polarization of naive $CD4^+$ T cells to the Th2 phenotype, and the expression of Th2 regulatory transcription factor, GATA-3 [81]. It also inhibits cytokine release in vitro by alveolar macrophages from patients with COPD, including IL-8 and GM-CSF [186].

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### 5.4. Selenium

Selenium is an important molecule in both innate and adaptive immune responses. It stabilizes activated platelets by inhibiting platelet aggregation and secretion of adenine nucleotide, thus possibly blocking the release of acrachidonic acid from platelet membrane [243]. In asthma, platelets participate by acting as inflammatory cells, by releasing mediators, spasmogens and/or by interacting with other inflammatory cell types [168]. Selenium affects the expression of endothelial cell adhesion molecules, E-selectin, P-selectin, ICAM-1, VCAM-1, and ELAM-1, which are crucial in the inflammatory process for recruitment of inflammatory cells into the target tissue [244].

Some studies have reported that asthma patients have lower selenium level in platelets and serum compared to healthy controls [245, 246]. While others studies have found no relationship between serum selenium level and asthma in Japanese and Europe populations [247, 248]. Selenium supplementation studies in mouse OVA-induced asthma models have shown that selenium has some protective effects on asthma-associated inflammation. Mice with decreased and increased levels of selenium intake show lower cytokine levels, airway inflammatory cell infiltration, serum anti-OVA IgE, airway hyperreactivity, and phosphorylated STAT-6 levels in the lung compared to medium selenium intake [249]. Selenium supplementation does not show any clinical benefit in adult asthma patients [250].

Despite the data showing positive effects of selenium on some of the pathologies associated with asthma, there are still some conflicting findings of selenium supplementation in animal and human studies. Thus the issue regarding selenium is not conclusive.

### 5.5. Avenanthramides

Avenanthramides (Avns) are extracted from oats and those synthetically prepared exhibit potent antioxidant properties *in vitro* and *in vivo*. The antioxidant activity of Avns is 10–30 times greater than that of oats' other phenolic antioxidants such as vanillin and caffeic acid. Avn-C, one of the three major Avns of oats, often comprises about one-third of the total concentration of Avns in oat grain (although the relative proportion of Avns is highly variable), it has the highest antioxidant activity *in vitro*. By far, these Avns constitute the major phenolic antioxidants present in the oat kernel. The antioxidant activity of Avn-enriched extract of oats has been investigated in laboratory animals. Supplementing the diet of rats at 100 mg/kg diet (providing about 20 mg Avns/kg body weight) has been reported to increase superoxide dismutase (SOD) activity in skeletal muscle, liver, and kidneys, and to enhance glutathione peroxidase activity in heart and skeletal muscles [251]. Supplementation at 200 mg/kg diet, which provides about 40 mg Avns/kg body weight in rats, attenuated the exercise-induced production of ROS [251].

In addition to demonstrating antioxidant activity, Avn compounds may also interact with cellular components, through their interactions with the molecular and signaling pathways that govern cellular responses during inflammation. Using the human aortic endothelial cell (HAEC) culture system, the potentially beneficial health effects of oat Avns was found to be mediated via modulation of the cellular and molecular processes that are known to play an

important role in the inflammation of arteries and the development of atherosclerosis [251]. They have been shown to inhibit vascular endothelial cell expression of adhesion molecules, including ICAM-1, VCAM-1, and E-selectin. Suppression of these adhesion molecules resulted in inhibition of monocyte adhesion to HAEC monolayers and reduced production of several inflammatory cytokines and chemokines, including IL-6, IL-8, and MCP-1, the inflammatory components involved in fatty streak formation in arteries. The production of proinflammatory cytokines, chemokines, and adhesion molecules by endothelial cells has been shown to be regulated by redox-sensitive signal transduction involving nuclear transcription factor NF-κB. The above-observed effects of Avns on HAEC and other cells are reported to be mediated through inhibition of NF-κB. More recently, dihydroavenanthramide (DHAv), a synthetic analog of Avn, has been shown to protect pancreatic β-cells from damage via inhibition of NF-κB. In a series of experiments, Guo *et al*. determined that suppression of the expression of NF-κB activity by Avns is mediated via inhibition of the phosphorylation of IKK and iκB, and by suppression of proteasome activity in endothelial cells [252]. A study by Sur *et al*. demonstrated anti-inflammatory activity of Avns in skin, inhibiting the degradation of IκB-$\alpha$ in human keratinocytes which correlates with decreased activation of NF-κB and subsequent reduction in IL-8 release [253]. Topical application mitigates skin inflammation in murine model of contact hypersensitivity and neurogenic inflammation and reduces pruritogen-induced scratching in murine itch model [253]. Taken together these observations suggest that Avns are potent anti-inflammatory agents with a potential application in asthma treatment.

### 5.6. Herbal preparations

Herbs have been used to treat airway diseases including asthma for thousands years in many nations, especially in Asian and African countries. In recent decades, some Chinese, Japanese, Indian, and African herbs have been tested for their anti-asthmatic effects.

#### *5.6.1. Boswellia serrata*

Boswellia serrata, Indian frankincense, is commonly found in many regions of the world, such as South Asia, Northern Africa, and Middle East. Traditional medicine using extract made from sap, has long been used to treat inflammatory diseases [204]. These extracts contain resin, amino acids, phenols, terpenes, polysaccharides [205] and β-boswellic acid the major active anti-inflammatory component [206].

Extract of Boswellia Serrata or β-boswellic acid has been reported to inhibit hypersensitivity reactions by regulating both the humoral and cellular immune systems They decrease primary antibody synthesis, inhibit polymorphonuclear leukocyte proliferation and infiltration, enhance the phagocytotic function of macrophages, and suppress the classical and alternate complement pathways [254, 255] and suppress the inflammation process, one of the critical pathological features in asthma. It has been shown that β-boswellic acid inhibits the production of proinflammatory cytokines, including TNF, IL-1, IL-2, IL-6, IL-12 and IFN-$\gamma$ by suppressing the activation of NF-κB [256]. It also inhibits histamine release from mast cells challenged with G protein stimulator c48/80 in a dose-dependent manner [257]. β-boswellic acid can down-regulate the synthesis of prostaglandins by inhibiting COX-1 in intact human platelets [258].

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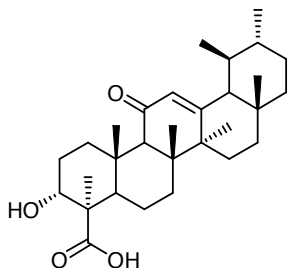
The synthesis of 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene $B_4$ from rat peritoneal polymorphonuclear leukocytes, which contribute to bronchoconstriction, and increased vascular permeability, are reduced by Boswellia Serrata extract as a result of 5-LO inhibition [259]. These results suggest that Boswellia Serrata might be effective in controlling the inflammation process and contraction of airway smooth muscle in asthmatic condition by inhibiting enzymes required for production of proinflammatory mediators and bronchoconstrictor.




Boswellia serrata

Preliminary clinical investigation has shown Boswellia Serrata's potential therapeutic effect on asthma. In a double-blind, placebo-controlled clinical study [260], 40 patients took 300 mg of extract daily for six weeks, while a control group received a lactose placebo for the same period of time. Lung and immune functions were recorded, including dyspnoea, rhonchi, frequency of attacks, FEV1, FVC, peak expiratory flow rate (PEFR), eosinophil count and erythrocyte sedimentation rate. In the treatment group 70% of patients and 27% in the control group showed improvement in terms of recorded physical symptoms and signs. These results suggest that Boswellia Serrata extract has potential benefit for asthma patients, although the age for control and treatment group was not perfectly matched. However, there is not enough evidence to draw a conclusion on the potential use of Boswellia Serrata for treating asthma in human.

### *5.6.2. Bromelain*

Bromelain is an extract from the pineapple stem, Ananas comosus, containing a mixture of cysteine proteases, peroxidase, acid phosphatase, protease inhibitors, and calcium, with cysteine proteases being the main functional components [214].

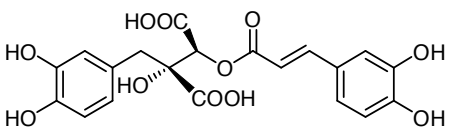
Bromelain modulates immune responses both *in vitro* and *in vivo*. In *vitro*, it downregulates mRNA expression of IL-2, IL-4, and IFN-$\gamma$ in T cells induced by phorbol myristate acetate (PMA), with the mechanism thought to be the inhibitionof p21ras and subsequent ERK-2 [261, 262]. In a study using peripheral blood mononuclear cells (PBMC), Bromelain decreases the expression of migration/activation related cell surface markers on leukocyte by proteolysis, including CD14, CD16, CD21, CD25, CD44, CD45RA, CD62L [263]. In addition, it can dose-dependently reduce CD25 expression in anti-CD3 antibody-stimulated $CD4^+$ T cells, which is upregulated when T cells are activated in inflammation, autoimmunity and allergy [264].

These results indicate that Bromelain may regulate inflammatory process by interfering the migration and activation of immune cells, primarily T cells. *In vivo*, Bromelain may inhibit IgG production and decrease IL-2 gene transcription in spleen, and significantly reduce blood $CD4^+$ T cell count [262]. In addition, it downregulates IFN-$\gamma$ mRNA expression in spleen [265]. These results indicate that Bromelain has regulatory effects on the adaptive immunity, principally by targeting T cell responses.

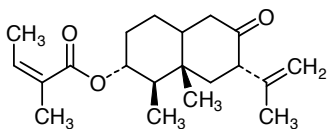
Bromelain administration via intraperitoneal injection alleviates some of the features of airway inflammation in the OVA-induced murine asthma model. It reduces the total numbers of leukocytes, eosinophils, $CD4^+$ and $CD8^+$ T cells in BALF, and decreases IL-13 concentration, which is a critical mediator for AHR in asthma [266]. In separate study, oral supplementation has been shown to suppress airway methacholine sensitivity, decrease IL-13 level, and eosinophils, $CD19^+$ B cells and $CD8^+$ T cells counts in BAL [267]. These results suggest that Bromelain modulates airway reactivity by altering the presence of leukocytes in airway, which is consistent with the *in vitro* results mentioned above. However, there is no clinical report available on the use of Bromelain against asthma so far.

### *5.6.3. Butterbur (Petasites hybridus)*

Butterbur is a member of the perennial sunflower family found in Europe and northern Asia. The ancient Greeks used butterbur roots to treat airway diseases and alleviate bronchial spasms [268].




Fukinolic acid




Petasin

Extract from the flower bud, leaves and root have been shown to inhibit β-hexosaminidase release, leukotriene C4/D4/E4 synthesis, and TNF production from IgE-sensitized RBL-2H3 cell [223]. A group of Japanese researchers reported that Japanese butterbur contains multiple active compounds including two eremophilane-type sesquiterpenes, six polyphenolic compounds, and two triterpene glycosides [223], and based on its inhibitory activity on mast cell degranulation, fukinolic acid is believed to be the most active component [269]. Another active component petasin, can reduce leukotriene and ECP production from eosinophils activated

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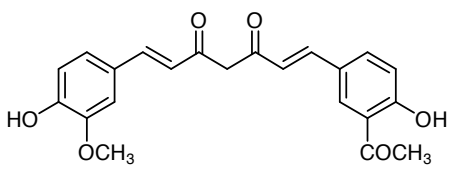
by platelet-activating factor (PAF) or C5a via suppression of cytosolic phospholipase A2 (cPLA2) activity, decreasing intracellular calcium concentration and inhibiting 5-LO translocation from the cytosol to nuclear membrane [270]. Pepsin inhibits leukotriene production from macrophages [271] and suppresses bronchial constriction induced by histamine, carbachol, KCl and leukotriene $D_4$ in isolated guinea pig trachea [272]. In the OVA murine model, butterbur extract given intranasally together with antigen challenge has been shown to inhibit airway inflammation induced by OVA and hyperresponsiveness to aerosolized methacholine, reduce eosinophil count and decrease Th2 cytokine production including IL-4, IL-5 and RANTES in BALF [227]. These results suggest that Butterbur may have inhibitory effects on proinflammatory mediator release from a broad range of immune cells.

In 2003, a prescription-based Butterbur extract was approved in Switzerland for the treatment of seasonal allergic rhinitis and in response some researchers have tested Butterbur extract for the treatment of asthma. Ziolo *et al* conducted an open clinical study on its effects on bronchial reactivity in asthma patients. Provided orally in a single dose for three time periods patients show significant improvement on FEV1, especially subjects those in longer treatment group[273]. In another randomized, double-blind, placebo-controlled clinical study, results have shown that the signs of asthma are significantly suppressed by Butterbur treatment including FEV1, exhaled NO, serum ECP and peripheral blood eosinophil count, suggesting Butterbur reduces some of the inflammatory markers associated with allergic respiratory inflammation [274], However, some long term adverse side effects have ben reported including abdominal pain, flatulence, and sneezing in pediatric patients and hair loss, cough, dyspnea, and severe depression for adult patients [230]. More studies with larger sample size are needed for the evaluation of Butterbur's clinical use on asthma.

### *5.6.4. Curcumin*

Curcumin is a yellow polyphenol compound, extracted from the rhizomes of *Curcuma longa* [231]. In ancient time, curcumin containing turmeric plants were widely used to treat swelling and wounds in Southern Asia [275].




Curcumin

Many pharmacological effects of curcumin have been reported, including antioxidative, anti-inflammatory and antimicrobial activities [276]. In terms of its antioxidative effects, curcumin is thought to be more potent than vitamin E [277] with the mechanism including downregulation of NO production, scavenging free radicals, and inducing heme oxygenase-1 to repair the oxidative damage caused by free radicals [278-280]. Curcumin can inhibit the production of proinflammatory cytokines such as IL-1β and IL-8, suppress inducible iNOS and NO

production, and modulate steroid activity. It's effect on steroid activity may be the result of inhibition of NF-κB through blocking IKK activity [281-284].

During allergic inflammation, curcumin may modulate both early and late phase responses by altering Th2 responses. In a murine latex-induced allergy model, characterized by an increased serum total IgE and latex specific $IgG_1$, elevated peripheral blood eosinophils count, and enhanced lung tissue IL-4, IL-5 and IL-13, intragastric curcumin administration reduces lung inflammation. Protein expression of costimulatory molecules CD80, CD86, and OX40-Ligand, and RNA expression of MMP-9, ornithine aminotransferase (OAT), and thymic stromal lymphopoietin (TSLP) in antigen-presenting cells are all decreased. These results suggest that curcumin may disrupt antigen presentation, so that has potential therapeutic value on allergen triggered airway inflammation [285].

Curcumin has been shown to have anti-asthmatic effects in both *in vivo* and *in vitro* studies. In OVA-induced asthma model in guinea pigs, curcumin treatment during OVA sensitization or following antigen challenge shows significant protective effects through attenuation of bronchial constriction and hyperreactivity [286]. This indicates curcumin has both preventive and therapeutic effects on asthma. In another study in an OVA-induced murine asthma model, curcumin's anti-asthmatic function is attributed to the suppression of iNOS and subsequent NO production, inhibition of inflammatory cytokine synthesis and downregulation of eosinophil recruitment to airway [91]. *In vitro*, curcumin supplementation inhibits IgE/antigen activation of mast cells through the principal activation pathway mediated by FcεRI directly inhibiting Syk kinase phosphorylation, which is critical for the propagation of signaling cascade. Subsequently, the phosphorylation of MAP kinases including p38, ERK 1/2 and JNK are supressed, which are crucial for gene transcription and production of proinflammatory cytokines [287]. In addition, curcumin inhibits HDM-induced lymphocyte proliferation and production of IL-2, IL-4, IL-5, and GM-CSF by lymphocytes from asthma patients [246]. These results indicate that curcumin may attenuate asthma symptom by inhibiting production of cytokines related to eosinophil function and IgE synthesis, and suppressing IgE-mediated reactions and hyperreactivity.

### *5.6.5. Licorice root (Glycyrrhiza glabra)*

Licorice root has been widely used around the world to treat cough since ancient time [247]. It contains the active compounds including glycyrrhizin, glycyrrhetinic acid, flavonoids, isoflavonoids, and chalcones [248]. Glycyrrhizin and glycyrrhetinic acid are considered to be the main active components [249] and are potent inhibitors of cortisol metabolism, due to their steroid like structures inhibiting the key steroid metabolic enzymes, delta 4-5-reductase, 11 beta-hydroxysteroid dehydrogenase and 20-hydroxysteroid dehydrogenase [250, 251]. Therefore, the benefits and side effects of steroid are both expected to be enhanced in the presence of glycyrrhetinic acid and glycyrrhizin.

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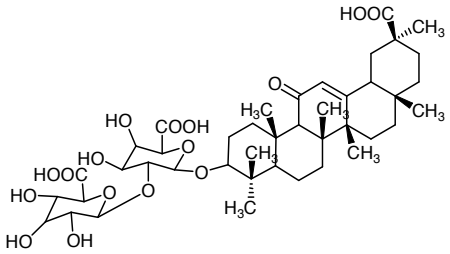
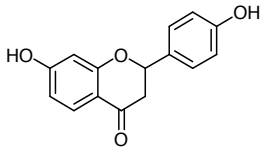
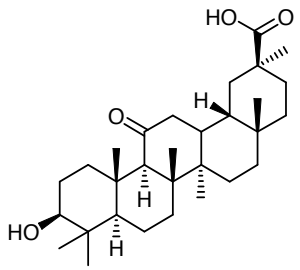



Glycyrrhizin




Liquiritige




Glycyrrhetinic acid

The anti-inflammatory effect of glycyrrhizin during virus infection has been well documented [288-290] and may alleviate allergic inflammation as well. In a contact skin hypersensitivity mouse model, glycyrrhizin and its metabolite 18 β-glycyrrhetinic acid-3-O-β-D-glucuronide show protective effects in terms of reduced passive cutaneous anaphylaxis and inflammation, with glycyrrhizin being more potent than 18 β-glycyrrhetinic acid-3-O-β-D-glucuronide [291]. In an OVA-induced murine asthma model, glycyrrhizin provided orally alleviates airway constriction and hyperreactivity, pulmonary inflammation. In BAL, IFNγ level is increased, while IL-4, IL-5 levels and eosinophil count are decreased. It also reduces OVA-specific IgE levels and upregulates total $IgG_{2a}$ in serum as well [292]. These results indicate that glycyrrhizin interfere the production of IgE by decreasing the IgE-stimulating cytokines.

The effects of glycyrrhetinic acid and liquiritigenin (a flavonoid of licorice root) on asthma have been tested both *in vivo* and *in vitro*. *In vitro*, glycyrrhetinic acid and liquiritigenin inhibits β-hexosaminidase release from RBL-2H3 cells induced by IgE/DNP, and from rat peritoneal mast cells challenged with c48/80. *In vivo*, they can suppress c48/80 induced passive cutaneous anaphylactic reaction in mice. In OVA-induced murine asthma model, glycyrrhetinic acid but

not liquiritigenin reduces the level of IgE in serum [293]. Flavonoids extracted from licorice root quench LPS-induced pulmonary inflammation by inhibiting the recruitment of neutrophils, macrophages and lymphocytes in BALF, and suppressing the mRNA expression of TNF and IL-1β in LPS-challenged lung tissue in mice [238]. The reported side effects of licorice root includes headache, hypertension, hypokalemia, premature birth, muscle weakness, and increase body weight, which were attributed to its function on inhibiting the steroid metabolism [294].

*5.6.6. Modified Mai-Men-Dong-Tang*

Mai-Men-Dong-Tang is an old Chinese herb formula commonly used for treating lung diseases, which contains Ophiopogon, Ginseng, Pinellia, Licorice, Jujube, and Oryza [260]. It is reported to increase the cough threshold to inhaled capsaicin in asthmatic patients. Also, the eosinophil count in peripheral blood, sputum eosinophil ratio, and serum eosinophil cationic protein level are significantly decreased, especially in patients with severe airway inflammation [261], which suggests that Mai-Men-Dong-Tang may alleviate asthma-related cough by inhibiting eosinophil function.

Modified Mai-Men-Dong-Tang (mMMDT) contains five herbs, Ophiopogon, American ginseng, Pinellia, Licorice root, and Lantern tridax [262]. The efficacy and safety of this formula to persistent, mild to moderate asthma has been evaluated in a double-blind, randomized clinical study of 100 patients with mild to moderate asthma. After 4 months, improvements in FEV1 and symptom scores has been reported in mMMDT treatment groups with decreased serum IgE and no drug-related adverse effects seen in terms of blood test, and liver, kidney functions [295]. Modified Mai-Men-Dong-Tang is a potential effective herb formula in treatment of childhood asthma for long time use. However, recommendation cannot be made because of small sample size used in the study.

*5.6.7. Ding-Chuan-Tang*

Ding-Chuan-Tang (DCT) is a traditional Chinese herb formula used for the treatment of cough, wheezing, and chest tightness, developed about four hundred years ago during the Ming dynasty. This formula contains nine herbs including *Radix glycyrrhizae, Tuber pinellia, Gingko bilboae, Herba ephedrae, Flos tussilaginis farfarae, Cortex mori albae radicis, Fructus perilla frutescens, Semen pruni armeniacae, Radix scutellariae baicalensis* [263]. In OVA-induced pig asthma model, DCT given orally to animals 30 min before antigen challenge inhibits the antigen induced immediate asthmatic responses. If it is given together with sensitization, immediate and late asthmatic responses are all suppressed. In addition, DCT relaxes trachea contracted with carbachol. The effects are attributed to decreased eosinophil infiltration to airway [263].

Randomized double-blind, placebo-controlled study to assess the effect of DCT on airway hyperreactivity in children with mild to moderate persistent asthma has shown that the FEV1 is significantly increased in DCT group (196%, p=0.034), but not in placebo control group. Compared to placebo control group, total clinical/medication score shows improvement in the DCT group (p=0.004). No side effects have been reported [296]. These results suggest that DCT

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might be effective in treating asthma in children. Larger sample size and wider population are required in further investigations.

### *5.6.8. STA-1 and STA-2*

STA is a combination of mMMDT and another Chinese herb formula Liu-Wei-Di-Huang-Wan (LWDHW), which is also used by Chinese as an anti-cough agent. LWDHW contains six herbs including *Rehmannia* root, *Alisma* rhizome, *Dioscorea* rhizome, *Poria, Hoelen, Moutan* root bark, *Shanzhu yu*. The formula for STA-1 and 2 are the same while the only difference being in the preparation of LWDHW is different [265]. In a mouse asthma model induced by intraperitoneally administrated dermatophagoides pteronyssinus group 5 allergen (Der p 5), oral STA-1 treatment during sensitization suppresses Der p 5-specific IgE production from animals in response to inhaled Der p 5 challenge. In addition, eosinophil and neutrophil airway infiltration, and airway hyperreactivity are all significantly reduced in STA-1 group compared to control animals [266]. The efficacy and side effects of STA-1 and STA-2 on childhood asthma treatment have been evaluated in a randomized, double-blind, placebo-controlled study. The herbs and placebo provided to pediatric patients with mild to moderate asthma reduces symptom scores, serum steroid concentration, total IgE, and allergen-specific IgE levels and improves FEV1 in the STA-1 group. STA-2 does not show protective effects. No severe side effects were reported [297]. These results indicate that STA-1 might be a valuable formula for childhood asthma, especially subjects induced by dust mite antigen. However, there is not enough evidence to draw a concrete conclusion. It is worthwhile to evaluate their potential use as immunotherapy as well.

### *5.6.9. Anti-Asthma Herbal Medicine Intervention (ASHMI)*

ASHMI is a relatively new formula developed by a group of Chinese researchers and physicians, which is an extract from three herbs: *Radix glycyrrhizaen prednisone, Radix sophorae flavescentis*, and *Ganoderma* [267]. In OVA-induced asthma, oral ASHMI treatment before and during OVA sensitization and challenge reduces AHR represented by time-integrated change in peak airway pressure. Eosinophil infiltration in BALF, lung inflammation, OVA-specific IgE production, and level of IL-4, IL-5, and IL-13 in lung and splenocyte cultures are significantly lower in ASHMI treated mice, whereas IFN-$\gamma$ production is increased [267, 268]. A 6-week treatment of ASHMI beginning 24 hr after the first OVA challenge in mice reduces early phase response by decreasing histamine, leukotriene C, and OVA-specific IgE levels, and suppresses late phase responses by decreasing eosinophil count and Th2 cytokines in BALF. In addition, it relieves contraction of murine tracheal rings by increasing the production of $PGI_2$ [269]. These results suggest that ASHMI inhibits asthmatic inflammation and airway muscle contraction, primarily by inhibiting Th2 cell function and might be suitable for treating antigen-induced asthma in both young and old subjects.

In clinical trial, ASHMI has been shown to improve lung function indicated by increased FEV1 and peak expiratory flow. Clinical symptom scores, use of $\beta$2-bronchodilators, serum IgE level, serum IL-5, IL-13 concentrations are all reduced, and some effects are even better than prednisone. During the study no adverse effect were recorded [298]. These results indicate the

effectiveness of ASHMI on treating asthma in both young and old adult patients. More adequately powered investigations are needed to evaluate ASHMI's effect on asthma.

### 5.7. n-3 polyunsaturated fatty acids

PUFA are a group of fatty acids with more than two carbon-carbon double bonds. There are three types of PUFA, n-3, n-6 and n-9, with their names based on the position of first double band from methyl end in their chemical structures. Currently, many studies have focused on n-3 and n-6 PUFA because EPA (20:4 n-3), Dihomo-γ-Linolenic acid (DGLA, 20:3 n-6) and Arachidonic acid (AA, 20:4 n-6) in cell membrane can be metabolized and become eicosanoid precursors, which are important modulatory autocrine molecules. Eicosanoids include prostaglandins, leukotrienes, thromboxanes, resolvins, lipoxins, are signal molecules that exert complex effects on health. They can modulate inflammation, fever, blood pressure, the immune system, etc. Eicosanoids can be made by oxidation of twenty carbon n-3 (EPA) and n-6 (DGLA, AA) PUFA. Eicosanoids from AA are proinflammatory, while those from EPA and DGLA are less so. There is competition between n-3 PUFA and n-6 PUFA in oxidation in terms of cyclooxygenase and lipoxygenase, which are critical enzymes for eicosanoid generation. AA is the predominant n-6 PUFA in body. In general, n-3 and n-6 are hypothesized to be beneficial and detrimental respectively [299, 300]. Fish, fish oil, krill, mussel and seal oil are natural sources of n-3 PUFA.

The major n-3 PUFA are listed in Table 12. In mammals, including humans, n-3 PUFA cannot be synthesized *de novo*. Therefore they must be absorbed through the diet or produced from α-Linolenic acid (ALA), which is an essential fatty acid. Among them, the health beneficial effects of EPA and DHA (22:6 n-3) are well documented in a broad range of health and disease conditions. The consumption of EPA and DHA are associated with lower risk of cancer, hyperlipidemia, and cardiovascular disease, high blood pressure, and neurodegenerative diseases [301-304]. Their regulating function on immune system was also well known and are involved in activation of immune cells like of T cells, B cells, mast cells and basophils [305, 306].

In recent decade, the relationship between n-3 PUFA and inflammatory diseases has been investigated in many studies. In a study conducted in rheumatoid arthritis patients, significant improvement in symptoms have been reported after 3 month fish oil supplementation in terms of tender joint count and duration of morning stiffness [307]. Besides reduction in the production of proinflammatory eicosanoids by competition with n-6 PUFA, n-3 PUFA has been found to be effective in inhibiting the synthesis of proinflammatory cytokines. In *fat-1* transgenic mice, which have a much lower n-6:n-3 PUFA in tissues because they are genetically modified to possess the ability to convert n-6 PUFA to n-3 PUFA, serum proinflammatory cytokines, including TNF, IL-1β, and IL-6 are lower. [280]. DHA and ALA also reduce the mRNA expression of IL-1β, IL-6 in a cerulein-induced pancreatitis model. They inhibit the activation of AP-1, suppress DNA fragmentation and decrease mRNA expression of apoptotic genes including p53, Bax and apoptosis-inducing factor in hydrogen peroxide-treated pancreatic acinar cells [308]. A randomized, double-blind human study has confirmed their suppressing effect on production of proinflammatory cytokines [309]. It has been shown that n-3 fatty acids

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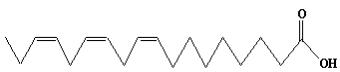
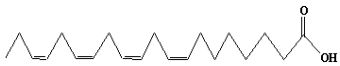
| Common name | Lipid name | Chemical name | Formula/MW | Structure |
|---|---|---|---|---|
| (HTA) | C16:3 (*n*–3) | all-cis-7,10,13-hexadecatrienoic acid | $C_{16}H_{26}O_2$/250.376 |  |
| α-Linolenic acid (ALA) | C18:3 (*n*–3) | all-cis-9,12,15-octadecatrienoic acid | $C_{18}H_{30}O_2$/278.430 |  |
| α -Parinaric acid | C18:4 (*n*–3) | all-cis-9,11,13,15-octadecatetraenoic acid | $C_{18}H_{28}O_2$/276.414 |  |
| Stearidonic acid (SDA) | C18:4 (*n*–3) | all-cis-6,9,12,15-octadecatetraenoic acid | $C_{18}H_{28}O_2$/276.414 |  |
| (ETE) | C20:3 (*n*–3) | all-cis-11,14,17-eicosatrienoic acid | $C_{20}H_{34}O_2$/306.483 |  |
| (ETA) | C20:4 (*n*–3) | all-cis-8,11,14,17-eicosatetraenoic acid | $C_{20}H_{32}O_2$/304.467 |  |
| (EPA) | C20:5 (*n*–3) | all-cis-5,8,11,14,17-eicosapentaenoic acid | $C_{20}H_{30}O_2$/302.451 |  |
| (DPA) | C22:5 (*n*–3) | all-cis-7,10,13,16,19-docosapentaenoic acid | $C_{22}H_{34}O_2$/330.504 |  |
| (DHA) | C22:6 (*n*–3) | all-cis-4,7,10,13,16,19-docosahexaenoic acid | $C_{22}H_{32}O_2$/328.488 |  |

**Table 12.** The common name, lipid name and chemical name of major n-3 PUFA

can alleviate inflammatory process by modulating cell signalling pathways in immune cells, such as T cell receptor pathway and cytokine receptor pathways. [310-312]

There have been a number of clinical studies that have shown n-3 PUFA's potentially protective effects on asthma, especially on childhood patients. There is a positive association between the n-6:n-3 PUFA in diet and risk for asthma [313]. A randomized, double-blind, placebo-controlled 3-year study on effect of n-3 PUFA supplementation on asthma has found that high n-3 PUFA diet intervention significantly reducesd the prevalence of cough in atopic children, suggesting that n-3 PUFA may be effective in preventing the development of asthma in early childhood [314]. In a cohort study on the relations between fish/cod oil intake and asthma,

results have shown that adults with low fish intake frequency (less than weekly) have increased risk to have asthma [315]. Another randomized double-blind study with 5-weeks n-3 PUFA supplementation has reported a significant decrease in exhaled NO from asthma patients challenged with mite allergen. Serum eosinophils count and ECP, and the production of CysLTs from isolated leukocyte stimulated with mite antigen are also reduced [194]. Overall, n-3 PUFA might be a promising remedy agents for allergic diseases like asthma but the mechanism remains to be elucidated.

## 6. Conclusion

The prevalence of asthma is becoming the mortality and morbidity pandemic of the 21st century. The cost of in quality of patient's lives and economic burden of treatment is continuing to grow at pace unmatched in our current health system. It is impossible to enter public classroom now without seeing a young sufferer of this condition and any trip to the emergency department will show how dangerous this disease can be. As the incidence and severity of the disease continues to rise, medical research is continuing to search new treatment strategies. While many treatments currently exists those reserve for the severest of conditions carry their own inherent risk which may match the severity of disease itself. It is for these reasons alone that health care professionals are now examining the traits of our ancestors in time when this epidemic was less severe to determine if their medicines and practices hold the answer for the next treatment strategy. By combining the scientific knowledge at the molecular and clinical level and the resources of past it might hold the answer to breathless pandemic of the 21st century.

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Chapter 11

# Plant Based Natural Products and Breast Cancer: Considering Multi-Faceted Disease Aspects, Past Successes, and Promising Future Interventions

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Additional information is available at the end of the chapter

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## 1. Introduction

### 1.1. Dealing with the burden

World-wide, approximately 410,000 of the estimated 1 million females diagnosed annually with breast cancer will die from the disease [1]. According to Canadian Cancer Statistics, breast cancer continues to be the most commonly diagnosed cancer among Canadian women over the age of 20 (excluding non-melanoma skin cancer) and is the second leading cause of cancer deaths [2]. In 2012, it was estimated that 22,700 Canadian women would be diagnosed with breast cancer and 5,200 would succumb to the disease [2]. Furthermore, the recently published Prince Edward Island (PEI) Cancer Trends Report outlining cancer cases diagnosed from 1980 to 2009 indicates that in PEI, breast cancer is the most commonly diagnosed cancer in women [3]. While the overall incidence rate of breast cancer has increased marginally (0.5% per annum) and mortality rates have stabilized since 1992, the long-term survival in PEI women is significantly lower than the Canadian rate [3]. In summary, breast cancer continues to be a world-wide issue requiring attention. Increased awareness and advanced screening, resulting in early detection, is attributed to the increased incidence of the disease and such factors have led to a high percentage (approximately 95%) of all breast cancer patients being initially diagnosed with curable disease [4]. However, 30-40% of patients diagnosed with curable breast cancer succumb to disease reoccurrence [5]. Although increased diagnosis and advances in the treatment of breast cancer are clearly contributing factors in disease progression, eradication of residual malignancies and metastatic tumors via a systemic approach is considered the key

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for success in treating cancer and increasing cancer patient survival [6]. There is an urgent need to explore agents that will be effective in preventing and treating metastasis of breast tumors.

### 1.2. The biology of breast cancer

In general, cancer occurs when a normal cell accumulates genetic and/or epigenetic changes caused by the activation or amplification of oncogenes and/or the mutation or loss of tumor suppressor function, resulting in the ability to proliferate indefinitely [7]. While these specific alterations lead to the transformation of the normal cell and partly determine the characterization of the tumor, the cell of origin and tumor (micro-) environment are also considered important factors contributing to tumor cell establishment, progression and therapeutic resistance [8].

## 2. Clinical characteristics and classification of breast cancer

### 2.1. Classifying breast cancer

Breast cancer classifications are largely explained by differences in tumor characteristics as determined by tumor appearance, histology, tumor marker expression (immunohistochemistry) or receptor status, and gene expression profiles. Alone, or in combination, these aspects can influence treatment, response and prognosis.

#### *2.1.1. Histology*

Breast cancer represents many different histologies, however the majority (estimated to be more than 85%) of breast cancers are collectively derived from the epithelium lining in the ducts or lobes, and are classified as mammary ductal or lobular carcinoma [9]. Furthermore, this classification can be defined as *in situ* (meaning the proliferation of cancer cells within the epithelial tissue without invasion of the surrounding tissue) or *invasive* whereby the surrounding tissue is affected [9]. Included in the histological description of breast cancer is the status of invasion to the perineural and/or lymphovascular space and the presence of such is associated with more aggressive disease [10].

#### *2.1.2. Grade*

Tumor grade is determined by comparing differentiation in normal and cancerous breast tissues. Normal cells in the breast become differentiated and acquire specific shapes and forms that reflect their function as part of the mammary system [9]. When cell division becomes uncontrolled, differentiation is lost. Breast cancers are either well differentiated (low grade), moderately differentiated (intermediate grade), or poorly differentiated (high grade) [9]. This progressive loss of features seen in normal breast cells is indicative of disease progression and poorly differentiated or high grade cancers have a worse prognosis than others [11].

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### *2.1.3. Stage*

Between 1943 and 1952, Pierre Denoix devised the TNM staging systems for all solid tumors to classify the progression of cancer [12]; the acronym can be explained by the fact that this model utilizes the size and extension of the primary tumor (T), its spread to the lymph nodes (N), and the presence of metastases (M). Although it is not utilized for all cancers (i.e.: brain and spinal cord cancer), the TNM system has progressed to the 7$^{th}$ textbook edition (TNM Classification of Malignant Tumors) [12] and remains the major system by which breast cancer is staged. An increase in stage number is based on larger tumor size, nodal spread (in particular, the sentinel node*) and metastasis and is positively correlated with a worse prognosis [13, 14].

* Certain cancers spread in a predictable manner, from the site of origin to nearby lymph nodes (lymph glands) and then to other parts of the body. The very first draining lymph node is termed the "sentinel node" and is important in the sentinel lymph node biopsy (SLNB) technique utilized to stage the spread of certain types of cancer. The absence of cancer within the sentinel lymph node indicates that there is a high likelihood that the cancer has not spread to any other area of the body. Lymph node metastasis is considered one of the most important predictive signs in breast cancer, and thus can serve to guide the surgeon/oncologist to the appropriate therapy.

## 2.2. Receptor status and gene expression profiles

Recent progress in molecular technologies has led to distinct breast cancer categories and five distinct tumor types and a normal breast-like group have been identified to date based on gene expression profiling [15], as summarized in Table 1.

| Molecular Sub-type based on Gene Expression Profiling | Receptor Status based on Immunohistochemistry | Reference |
|---|---|---|
| Luminal A | ER+ve and/or PR+ve, Her2–ve, any CK5/6/Her1 | [15-17] |
| Luminal B | ER+ve and/or PR+ve, Her2+ve, any CK5/6/Her1 | [15-17] |
| Basal-like (triple –ive) | ER–ve, PR–ve, Her2–ve, CK5,6+ve and/or Her1+ | [18] |
| Her-2+over-expressing (ERBB2) | ER–ve, PR–ve, Her2+ve, any CK5/6/Her1 | [19] |
| Normal Breast –like | "unclassified" (negative for all 5 markers), displays putative-initiating stem cell phenotype | [20, 21] |
| "claudin-low" group | "unclassified" (negative for all 5 markers), displays putative-initiating stem cell phenotype, Often triple negative, displays low expression of cell-cell junction proteins and e-cadherin, frequently infiltration with lymphocytes | [20, 22] |

Positive (+ve), negative (–ve), estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptors (EGFR) 1 and 2 (Her 1 and 2) and cytokeratin 5, 6 (CK5,6).

**Table 1.** Distinct Molecular Tumor Categories of Breast Cancer and their Receptor Status

However, most investigators use the presence (+) or absence (-) of immunohistochemical (IHS) markers or receptor combinations that are expressed by neoplastic cells. In this manner, distinct tumor categories to-date are identified by the expression of estrogen receptors (ER), progesterone receptors (PR), human epidermal growth factor receptors (EGFR) 1 and 2 (Her 1 and 2) and cytokeratin 5, 6 (CK5,6) [15-19, 23-25]. Generally, molecular sub-types correspond to IHC receptor status [26]. These subtypes are of great clinical and research importance as they are utilized to administer and target therapeutic regimes based on predictions of response. Furthermore, these subtypes have been shown to display a wide variety of responses to different treatments [27-30] and are associated with other clinical outcomes, such as patient relapse and overall survival. The most favorable outcomes are noted for the luminal A subtype, which are hormone sensitive [31]. The Her2+ and basal subtypes are noted as more aggressive and have fewer therapeutic options [31-33]. The normal-like and claudin-low are unclassified (negative for all major receptors), and associated with poor prognosis [20-22, 30, 34]. Several small studies support the concept that molecular subtype and tumor receptor status may change during ordinary disease progression and a major study completed by MacFarlane *et al*. in 2008 revealed that 21% of relapsed tumors had changes in either ER/PR or HER2 receptor status [35]. This significant proportion led the author to suggest that biopsies of relapsed/ metastatic breast cancers should be performed routinely. This also should be an important consideration in research. In summary, treatment options considered effective in primary stage cancer may no longer be optimal in later stages of the disease, including relapses and metastasis as determined by the current classification scheme.

### 2.3. Other classification approaches

Other breast cancer classification approaches are also used to assist in both prognostic and treatment decisions. These include computer models that are based on a combination of several factors and offer individual survival predictions and calculations of treatment benefits [36]. For example, patients undergoing systemic adjuvant therapy can determine optimal treatment through the commercially available computer model *Adjuvant* which has been successfully validated in several cohorts, including the United States and Canada [36, 37]. Other useful classification tools utilized for breast cancer treatment choices include prognostic assessments (such as USC/Van Nuys prognostic index (VNPI)) and general comorbidity assessments [38, 39]. Also consider the case of familial breast cancer (genetic classification) whereby a patient may opt to undergo preventative measures such as mastectomy. Additionally, immunohistochemistry testing, other than those mentioned earlier, continue to prove favorable as prognostic markers across various molecular subtypes [40]. For example, in human breast cancer epithelial cell proliferation is considered a significant prognostic marker [41] and could possibly be used as a prediction tool to measure different hormone treatment related risks [42]. Therefore, the immunohistochemical marker, Ki67 (a nuclear protein expressed by cells in all active phases of the cycle except for quiescent or resting cells) is utilized frequently to evaluate proliferation [43]. Such labeling indicates a significant association with higher carcinoma grade, clinical response to endocrine therapy, higher risk of relapse, and worse survival in patients with early breast cancer [44].

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## 3. Cell of origin

While scientists have predicted that specific breast cancer types may arise from different types of progenitor cells, it has been difficult to identify the cells of origin, and the topic remains controversial to date. Breast tumors represent a heterogeneous collection of cell populations with different biological properties [45]. Shared molecular features have made it difficult to distinguish the cell populations of breast cancer tumors and only recently have researchers been able to differentiate stem cells from other progenitor cells. Within this context, tumor evolution has been explained by two main theories. The traditional clonal evolution model is based on the premise that all tumor cells have the capacity to undergo self-renewal which is an indication of their potential to undergo tumor progression and drug resistance [46, 47]. The Cancer Stem Cell (CSC) theory emphasizes the ability of only a minor population of tumorigenic cells capable of self-renewal and differentiation [20, 48] and this specific sub-set of CSC's gives rise to new tumors which are phenotypically identical to the original tumors [49]. CSC's are believed to be a small population of cells with dysregulated self–renewal properties capable of continuous self-renewal and differentiation and responsible for tumor existence treatment resistance and relapse. Existence of CSC's in various types of tumours, including breast cancer [50],[51] has been identified. ALDH (aldehyde dehydrogenase)-1 is a marker of normal and malignant human mammary stem cells [52], and these cells can also be isolated using the cell surface markers epithelial-specific antigen (ESA), CD44 and the absence of the expression of CD24 [53]. When transformed cells undergo epithelial-mesenchymal transition (EMT), they have been noted to gain properties of stem cells [54]. Although evidence supporting the CSC model was initially obtained from acute myeloid leukemia [55], successive studies maintain that solid tumors, including breast cancer tumors, are also driven and sustained by CSCs [50].

Regardless of origin, an abundance of research has clearly confirmed the existence of cancer stem cells (CSCs) or tumor-initiating cells (TICs) in a variety of human cancers [55-58], including breast cancer [50]. Nonetheless, most of the therapeutic approaches available, inclusive of chemotherapy and radiation, lack the ability to effectively kill these populations [59-62]. This may explain the lack of progress in eliminating cancerous tumors and preventing metastasis and may help to rationalize therapeutic resistance; therefore, the CSC or TIC population has become a target for cancer prevention and therapy [63]. A general consensus exists in the literature that breast cancer re-occurrence is assumed to be caused by a subpopulation of tumor initiating cells possessing stem cell attributes of a tumor as well as resistance to chemotherapy, radiation and other forms of treatment [64-66]. Of great interest is the role of CSC's in tumor relapse and resistance to therapy and recent articles suggesting that such resistance can be overcome.

## 4. Breast cancer progression models

The combined research efforts of various scientific disciplines have resulted in the development of a disease progression model for breast cancer (See Figure 1) and include a continuum of lesions through to invasive carcinoma and eventually metastatic disease [30, 48, 67, 68]. For decades, it was thought that metastatic dissemination occurred as a final step in cancer and

was the responsibility of genetic changes of malignant cells in the primary tumor [69]. In 2008, Husemann *et al*, used transgenic mice to show systemic dissemination (specifically to lungs and bone marrow) of mammary tissue derived premalignant cells prior to the emergence of mammary tumors [70]. Additionally, this research reported that systemic dissemination of tumor cells can occur in pre-invasive stages of tumor progression as observed in female patients with ductal carcinoma *in situ*. A complementary accumulation of evidence supports the evolution of an early dissemination model, where malignant cells outside the primary lesion can also migrate to distal sites (such as lung and bone marrow) and cause tumors via various genetic programs [54, 71, 72]. Such a model is inclusive to "self-seeding", the term coined when cancer cells not only seeds regional (lymph nodes) and distant sites but also fuel the growth of the original tumor itself [73].

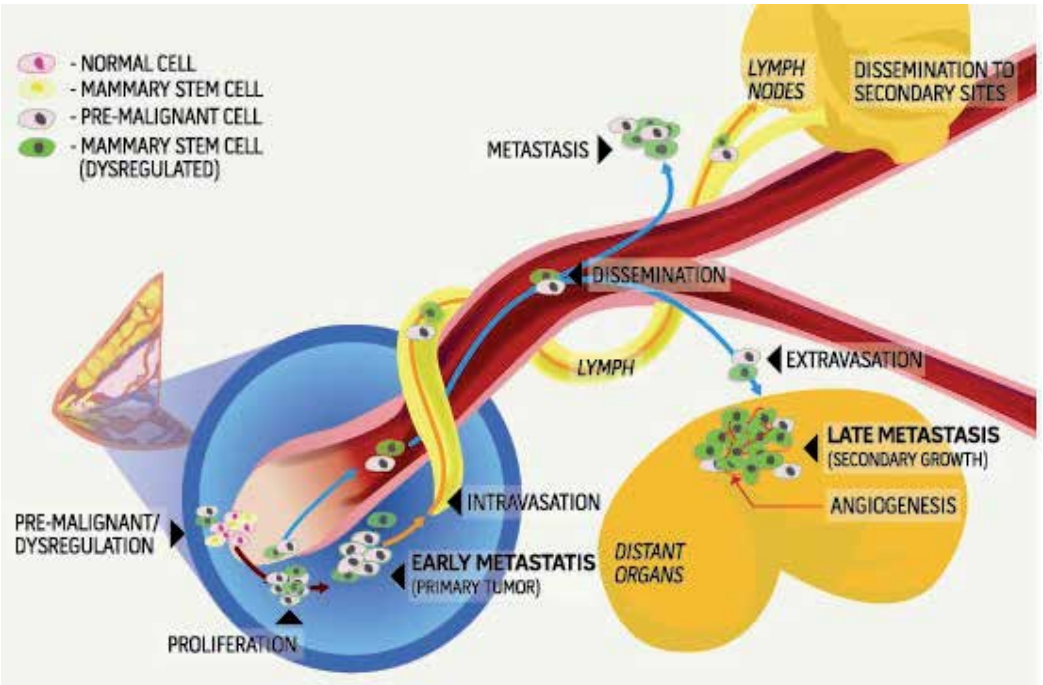


**Figure 1. (Tobin, GA, 2011) Physiological Aspects of Disease Progression in Breast Cancer:** The development of breast cancer has been proposed as a multi-step process. The most deadly aspect of breast cancer is metastasis and involves a cascade of reactions. Although the molecular mechanisms underlying this process are not fully understood, any disruption along the cascade could arrest disease progression.

Thus, breast cancer is not a single disease, but rather an assortment of diseases with diverse characteristics and clinical outcomes which will likely always require a variety and/or combination of treatments or alternatively, a broad spectrum application. Combine this with the fact that, despite major advances in our understanding of the biology of cancer, further research is required to improve our understanding of tumor establishment, progression and dissemination - the principal cause of mortality. Then, our goal in breast cancer research

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regarding treatment would be to identify novel therapies and maximize, based on our current understanding of the disease, the use of such in preventing and treating as many aspects of the disease as possible.

## 5. Molecular and endocrine controls

### 5.1. BRCA and other gene expression signatures associated with increased risk of breast cancer

Less time, low cost and new sequencing technologies are all accomplishments that yield impact on molecular biomedical research. Recently, for example, such advances (combined with some prospective epidemiology studies) have allowed researchers to compare the DNA from healthy breast tissue, initial tumor cells and then cells obtained nine years later when the breast cancer had metastasized [74]. The 32 DNA mutations reported in the metastasized cells are a prime example of how technological advances can serve to provide us with ample data regarding gene expression that may offer insights into the progression of breast cancer disease. However, consider that gene expression profiling / signatures of primary breast tumors can only be used as a predictor of susceptibility or disease progression in breast cancer, particularly metastasis. Currently, it is not possible to accurately predict the risk of metastasis or prevent it. As a result, more than ½ of the patients treated with adjuvant chemotherapy are needlessly exposed to harmful side effects [5]. This presents another compelling reason to further study drug targets that are specific to, and have potential for, treatment in metastatic breast cancer.

Nonetheless, based on current knowledge, and aside from the many identified factors that could impact the risk of developing breast cancer (including personal and environmental), genetic mutations in critical cancer genes (both tumor suppressor and oncogenes) have been identified for their increased or associated risk with breast cancer [75]. Of the many that have been reported throughout the history of cancer genetics, Table 2 below captures those that stand out principally for their research and/or clinical significance in relation to breast cancer and summarizes major function, encoded proteins and known disease associations or risk factors. Such genetic aberrations are acquired over a person's lifetime; or less commonly, are inherited. While it is estimated that only 5% to 10% of breast cancers are hereditary, some gene variations are associated with both hereditary and somatic mutations [75]. The tumor suppressor genes *BRCA1* and *BRCA2* are the major genes related to hereditary breast cancer [76], and mutations in these and other *BRCA* genes in women are associated with a 60-80% risk of developing breast cancer throughout their life span [77]. Other genes with inherited alterations, including *CDH1*, *PTEN*, *TP53*, *CHEK 2*, and *ATM* have been noted to increase or are associated with the risk of developing breast cancer [78]. Most notably, the latter three have presented the strongest evidence related to the risk of developing breast cancer [79]. Somatic mutations mostly reference *ERBB2/HER2(neu)* in breast cancer, however *TP53* genes and others have been associated with some cases of breast cancer in this manner [80]. It is noteworthy that not all people who inherit mutations in these genes will develop cancer.

| Gene | Major Function: Associated Proteins | Risk Factor | Ref. |
|---|---|---|---|
| BRCA1 (BReast CAncer gene one) | Encodes breast cancer type 1 susceptibility protein; responsible for DNA repair, transcriptional regulation and cell cycle check point control. | Strong evidence indicating a 60 – 80% risk of developing breast cancer for people with mutations. | [81 - 86] |
| BRCA2 (BReast CAncer gene two) | Encodes BRCA2 susceptibility protein involved in the repair of chromosomal damage, especially in the error-free repair of DNA double strand breaks. As with BRCA1, indicates a high degree of risk. | Reduced levels of the BRCA2 protein may cause Fanconi anemia. Patients with such are prone to several types of cancers, including reproductive system associated tumors. | [87] [88] |
| ATM (Ataxia telangiectasia mutation) | Encodes protein with a phosphatidylinositol 3-kinase (PI3K)–like domain which plays a central role in the complex processes that repair DNA double-strand breaks. Also involved in regulation of cell cycle progression and the maintenance of genomic stability. | Confers susceptibility and linked to an modest increase (up to 2 times) of breast cancer. | [89-93] |
| p53 (TP53) (Tumor Protein p53) | Encodes p53 protein and regulates cell cycle, preventing tumor growth. | Causes Li-Fraumeni syndrome: results in higher-than-average-risk of breast cancer and several other cancers. | [94-96] |
| CHEK2 (Checkpoint kinase 2) | Encodes a serine/threonine-protein kinase which plays a critical role in DNA damage signaling pathways. Phosphorylates and regulates the functions of p53 and BRCA1. | Causes Li-Fraumeni syndrome. Can double breast cancer risk. | [97-99] |
| PTEN (Phosphatase and tensin homolog) | Encodes phosphatase and tensin homolog protein, namely phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase, which is involved in the regulation of the cell cycle. | Causes Cowden syndrome which presents a higher risk of both benign and cancerous tumors in the breast, digestive tract, thyroid, uterus, and ovaries. | [100, 101] |
| CDH1 (Cadherin 1 Gene) or E-cadherin (epithelial cadherin) | Encodes E-cadherin protein. Down-regulation decreases the strength of cellular adhesion, increases cellular motility; allowing cell invasion. | Increased risk of breast cancer, particularly invasive lobular breast cancer. | [102-104] |
| ** ERBB2, (HER2/neu), or VEGF | Encodes a transmembrane receptor with constitutive tyrosine-kinase activity. | Amplification occurs in 15-30% of human breast cancers. | [34] [80] |

** ERBB2 (erythroblastosis oncogene B), HER2/neu (Human Epidermal growth factor Receptor2, neu: derived from a rodent (neu)ral tumor), VEGF (vascular endothelial growth factor).

**Table 2.** Familiar Genetic Mutations, Encoding Proteins and their Major Functions in Relation to the Associated and Increased Risk of Developing Breast Cancer.

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Those listed in Table 2, as mentioned, represent familiar genes associated with breast cancer. However, others genes that are applicable in breast cancer progression will be reviewed in terms of their associated cell signaling pathways. Considering the profound implications that the CSC theory has for cancer chemoprevention and therapy, combined with our interest in plant based molecules, we will also examine gene function in the context of opportunities for natural product compounds in CSC self-renewal.

### 5.2. Endocrine controls

Over the years, and more notably since the discovery of suitable breast cancer cell lines and animal models, the symbiotic relationship between lab research and clinical investigations have advanced our knowledge of endocrine action. Breast cancer is influenced and highly regulated by several sex and growth hormones (including estrogens, androgens, progesterone, prolactin and insulin-like growth factors) and each of the sub-types and gene expression patterns of breast cancer are characterized by both unique and specific endocrine controls [105]. Particularly, estrogens and progesterone have received the greatest attention likely because of their involvement in normal and neoplastic mammary tissue and the scale of their associated risk estimation for breast cancer. Research into the role of androgens has likely been a consideration in breast cancer research as androgens are necessary precursors to all endogenous estrogens. The role of prolactin in the pathogenesis of breast cancer remains unclear given the scarcity of studies to date. Similarly, although well studied, the role of Insulin-like growth factor (IGF) in breast cancer is inconsistent.

There are challenges in defining the role of progesterone in breast cancer and the role of progesterone receptor (PR) action in breast cancer remains divisive. In breast cancer, progesterone has biphasic effects (both proliferative and inhibitory) on breast cancer cell lines grown *in vitro* [106, 107]. Depending upon cellular context and/or the presence of secondary agents, there may be a role for progesterone as a priming agent with growth promoting activity [106].

While progesterone is found as a single hormone, the major endogenous estrogens in females include estrone (E1), estradiol (E2), and estriol (E3) and are primarily produced during menopause, in non-pregnant and pregnant females, respectively [108]. A variety of synthetic (xenoestrogens) and natural substances have been identified that also possess estrogenic activity including those derived from plant products (phytoestrogens) and fungi (mycoestrogens) [108]. The actions of estrogens are mediated by their respective receptors binding to specific DNA sequences to activate the transcription of estrogen receptor (ER)-regulated genes, including direct target genes [109]. Approximately 80% of breast cancers demonstrate expression of PR and/or ER [110]. Once established, these breast cancers which are classified as either hormone-sensitive or hormone-receptor-positive cancers are reliant on hormones to grow. Thus, treatment includes the suppression of hormone production in the body for these specific breast cancers. Therefore, this section will provide an overview of estrogens, their past indications in the treatment of breast cancer and their potential role in relation to naturally occurring dietary compounds.

*5.2.1. The role of estrogens and breast cancer*

The link involving estrogen and breast cancer can be traced back to as early as 1896 when a surgeon in England reported an improvement in condition in three young female breast cancer patients after the removal of their ovaries [111]. Since estrogen had not yet been discovered, the surgeon had unknowingly removed the source of estrogen that promotes the survival and division of cancer cells [111]. Since that time, a lot of evidence has shown that interactions between estrogens and their receptors influence the pathogenesis of breast cancer. Estrogen promotes proliferative effects on cultured human breast cancer cells [112]. Estradiol affects breast cancer risk by controlling the mitotic rate of breast epithelial cells and high levels of estradiol in post-menopausal women are also known to increase the risk of breast cancer [113]. Estradiol has also been shown to increase breast cancer risk via its metabolite, catechol estrogen 4-hydroxyestradiol, causing direct DNA damage through the formation of free radicals [114]. Estradiol has also been shown to modulate breast cancer cell apoptosis [115]. ER is a major determinant of the cellular response of estrogen and has been indicated in breast cancer promotion [116, 117]. The binding of estrogen to the ER modulates the transcription of a series of genes, including those coding for proliferation.

The close relationship between the etiology of breast cancer and exposure to estrogen warrants examination of key variables that may affect estrogen homeostasis, in particular those exhibiting anti-estrogen activity. Because of their impact on the primary and metastatic aspects of disease, anti-hormonal drugs are the mainstay breast cancer treatment. The goal in treating hormone receptor +ve breast cancers is to utilize drugs which suppress production of estrogen in the body. Estrogens in naturally occurring dietary compounds such as soy are used as an alternative to hormone therapy because of their anti-proliferative effects. This practice, in breast cancer treatment, is widely known as hormonal therapy, or anti-estrogen therapy, but is not representative of the term hormone replacement therapy [118].

## 6. *In vitro* and *in vivo* breast cancer models and their relevance to human disease

In 2007, Vargo-Gogola and Rosen summarized appropriately, within their title, the challenge of modelling breast cancer; “One size does not fit all”[31]. Considering the heterogeneity nature of this disease, these researchers rather addressed the reasonable question of the most powerful way to investigate breast cancer with respect to cell lines and animal models. We agree with their conclusion that an integrated and multi-systems approach is the strongest way to model this disease. Here, we briefly discuss the most commonly utilized *in vivo* and *in vitro* models and their relevance in human breast cancer.

### 6.1. Mammary carcinoma cell lines

Our current knowledge of breast cancer is mainly based on *in vivo* and *in vitro* studies completed with breast cancer cell (BCC) lines. The first BCC line, namely BT-20, was established in 1958 [119] and since then the number of permanent lines sustained are relatively low despite continuous research in breast cancer [120]. In fact, the number of commercially available BCC

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lines (ranging from 70 – 80 based on ATCC and similar commercial suppliers) represent mostly cell lines that were established more than 25 years ago. It has been suggested that this inefficiency in producing new and improved lines is likely due to technical difficulties in extracting viable tumor cells from their neighboring stroma [120]. In the past, it has been estimated that more than two-thirds of all scientific abstracts related to studies on mentioned BCC lines include the MCF-7, T-47D, and MDA-MB-231 lines [120]. Although no single cell line is entirely representative of human breast cancer, cell lines have been widely used. As a result, we have a greater understanding of breast cancer biology. Additionally, these cell lines have advanced the ability of pre-clinical models to predict pharmaceutical activity and therapeutic applications for further study. Despite this, the use of cell lines for application in human disease is limited. The single most significant question, considering the devastating effects or outcomes of metastasis, is the issue of whether these cell lines are representative of metastatic conditions, or contain tumor initiating subpopulations. There are studies to indicate distinctions between tumor and non-tumor initiating populations at the cellular and molecular level [31, 121]. Research indicates that the two luminal subsets (A and B) evident in tumors are not apparent in the cell lines, and the basal-like cell lines are actually representative of two distinctive clusters (A and B) that are not apparent in analyses of primary tumors [16]. The differences in cell culture may be due to the absence of stromal or physiological interactions and/or signaling [122] [123]. However, there is evidence that cell lines may be derived from subpopulations of tumor cells that are selected because they grow well. For example, it has been noted that differences between the genome aberration patterns for the basal-like and luminal clusters in the cell line system don't match differences in these subtypes in primary tumors. Furthermore, the highly invasive Basal B cells carry the distinctive phenotype associated with the subpopulation of tumorigenic stem cells identified in breast cancer [50, 124]. Also, one must consider the issue of variants of the same cell lines and the phenotypic changes that can occur due to environmental exposures, rate of passage and age of cells.

Perhaps the most highly debated topic regarding the use of breast cancer cell lines revolves around the MDA-MB-435 cell line, and by extension MDA-MB-435S (ATCC® HTB-129™) and derivatives. Spontaneously metastatic and originally designated as Basal B and being derived from a breast carcinoma, the MDA-MB-435 cell line has subsequently been questioned because of recent evidence indicating that it might originate from melanoma or may have been cross-contaminated with the M14 melanoma cell line [125-130]. While some researchers continue to use this cell line as a breast cancer mouse model [131] and conclude that this cell line is in fact breast cancer cells [132], others insist that the MDA-MB-425 cell line is a melanoma cell line and renders its use as improper in breast cancer studies [133]. Later in the chapter, we will describe some research that has been conducted using MDA-MB435 cell lines and/or its derivatives as breast cancer models in research involving natural, despite this controversy. This may not be all bad news for natural product researchers. On the bright side, natural product therapies indicated in the controversial cells line MDA-MB-435 and its derivatives then point toward activity in melanoma as opposed to breast cancer.

Nonetheless, there is clearly a need for new cell lines that are representative of all the known sub-types of breast cancer. Currently, researchers have the option of using a group (or panel)

of subtype cell lines to increase power in their research, as proposed by Neve [2006] [129]. To date, and based on our knowledge, there has been no evidence of a male breast cancer derived cell line. With the incidence of male breast cancer rising [2], relevant models require further development.

Cell lines are easy to culture, inexpensive in comparison to animal models, and provide an unlimited source of homogenous material to work with. Comparisons between researcher and studies are usually fairly consistent and reproducible. A limitation of cell cultures is their inability to measure tumor-stromal cell interactions, however the growth of cell lines for transplantation allows for such *in vivo*.

### 6.2. Animal models

Animal models have allowed researchers to gain much insight into the disease progression, in particular metastasis. In addition to advancing the concept of metastasis [134, 135], our knowledge of the biological function of genes and signaling pathways has progressed as a result of the vast amount of information generated from animal models. Experimental systems based on mouse models that are currently used to study breast cancer can be categorized as tumor transplantation and genetically engineered mice (GEM) – often termed as transgenic. Here we will focus on commonly utilized models within each of these categories and discuss some of the advantages and limitations of each in relation to human breast cancer disease relevance.

#### *6.2.1. Transgenic or (GEM) models*

Different GEM breast cancer models are useful for studying distinct signaling interactions and for testing therapies that target pathways involved. Genomic deletion of tumor suppressor genes or the transgenic insertion of oncogenes allow these mice to be a relevant tool to investigate the spontaneous initiation of breast tumors in each step of metastasis [136]. Specific to breast cancer, the expression of oncogenes in explicit breast regions are restricted via a mammary gland specific promoter [136]. MMTV (mouse mammary tumor virus) or WAP (Whey Acidic Protein) promoter control the expression of oncogenes (i.e: PyMT, ErbB2, Wnt1, or Ras) in transgenic mice and initiate mammary gland tumors which lead to metastasis in various organs [137]. The MMTV-PyMT transgenic mouse model represents hyperplasia, adenoma, and early or late carcinoma as seen in human cancer stages. In breast cancer, this model demonstrates short latency tumor development specific to the mammary gland with a high incidence pulmonary metastasis [138]. The deletion of mammary epithelial cell-specific tumor suppressor genes results in similar conditions observed in human cancer patients, including spontaneous tumors, bone metastasis, loss of estrogen receptor (ER) expression, and hormone-responsiveness [139, 140]. Crossing GEM mice with other transgenic mice has allowed researchers to investigate the role of genes and signaling pathways in tumorigenesis and metastasis. While tumor progression was delayed in MMTV-PyMT or MMTV-ErbB2/neu mice crossed into an Akt or PTP1B-deleted genetic background [141, 142], increased metastasis was observed in the MMTV-PyMT mouse in a CD44_/_ background; the later indicates the importance of receptors and other secreted factors in epithelial–stromal interactions or overall

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tumor environment [139]. In this regard, much insight has been gained through the use of GEM models to indicate the role of both innate and adaptive immune responses in the progression of breast cancer. For example, pulmonary metastasis is notably diminished via the selective loss of the interleukin (IL-) 4 cytokine in PyMT/IL-4_/_ mice through inhibition of IL-4 mediated EGFR signaling of mammary tumors [143]. The understanding of such interactions are important if we consider that tumor-associated immune cells release numerous growth factors, including cytokines, chemokines, and enzymes that promote tumor growth, angiogenesis, and metastasis, and thus are associated with poor prognosis [136]. Inducible GEM mouse models (those capable of expression or repression of certain genes) have advanced knowledge with respect to the association of multiple genetic mutations of oncogenes in relation to tumor establishment and maintenance [144]. There are several drawbacks to consider in the use of GEM models. There are inherent differences in mouse and human, for example: BRCA1 and p53 are on the same chromosome in mice, but not in humans [136, 144]. Technical discrepancies, depending on methods used to generate the model and the interpretation of results can present varying outcomes. Also, temporal and spatial tumor development and metastasis differ depending on the mouse strain used. For example the percentage of ER/progesterone receptor (PR)+ve tumors in Wap-Cre/Trp53_/_ mice are high while MMTV-Cre/Trp53_/_ mice report a low incidence of such [145]. The molecular profile of mammary tumors from GEM models represent the subtypes of human breast cancers, including those included in Table 1, however no single model to date is representative of all expression patterns and characteristics of human cancer.

### *6.2.2. Tumor transplantation model*

Syngeneic transplantation refers to the transfer of cancer cells from one mouse into another with identical genetic background [146] and such models are used to establish organ specific metastasis [147]. The 4T1 breast cancer tumor model (a syngeneic mouse model) is considered excellent for testing experimental cell-based immunotherapy strategies and is comparable with stage IV human breast cancer [148]. The injection of 4T1 cells, originally derived from a spontaneous mouse mammary tumor are injected into the mammary fat pad of a syngeneic animal and the rapid proliferation of cells forms tumors and eventually metastasizes to the lungs, liver, bone and brain [146, 147, 149]. Furthermore, this model has been refined through the development of 4T1 cell lines to employ varying degrees of metastasis that are location specific and representative of distinct gene expression signatures [150]. Xenograft transplantation encompasses the use of human cancer cells into immuno-compromised mice via intravenous, intraperitoneal or subcutaneous injection, orthptopically or ectopically [136, 151]. First discovered in 1962, nude mice have been commonly used for xenotransplantation because they lack a thymus and are unable to mount most types of immune responses, including rejection of allografts and xenografts [152]. Severe Combined Immunodeficiency (SCID) mice present with impaired ability to make T or B lymphocytes and are used as model organisms for research into transplantation strategies as they cannot reject tumors and transplants [153]. In general, rodents with immunological defects are typically resistant to growth of mammary carcinomas, and despite improvements these approaches have yielded low percentage of successful breast tumor engraftment compared to other types of cancers

[154]. Recent mouse models have contributed significantly to the understanding of breast cancer, however further research into suitable animal models will be required to advance development of new therapies for breast cancer. The growth and metastasis of human breast cancer cell lines *in vivo* allows the measurement of gene function relative to disease progression and has provided much insight into the use of investigational drugs intended interrupt or interfere with tumor growth [155].

Table 3 provides an overview of the defining characteristics of established breast cancer cell lines (mouse and human) commonly used in both *in vitro* and transplantation models.

| Cell line | Species | ER Status | PR Status | Metastasis Location | Ref. |
|---|---|---|---|---|---|
| 4T1 | Mouse | + | + | Lymph node, blood, liver, lung, brain, bone | [149] |
| BT-474 | Human | + | + | Bone | [156] |
| FII3 | Mouse | + | + | Lung | [157, 158] |
| MCF-7 | Human | + | + | Lymph node, lymphatic vessel | [159, 160, 161] |
| MDA-MB-231 | Human | – | – | Lung, liver, brain and bone | [162, 163] |
| MDA-MB-435 | Human | – | – | Lung | [164] |
| MDA-MB-453 | Human | – | – | Bone | [165] |
| SUM1315 | Human | – | – | Lung, bone | [166, 167] |
| SUM149 | Human | – | – | Lung | [167] |
| T47D | Human | + | + | Lymph node, lymphatic vessel | [168] |

**Table 3.** Breast Cancer Cell Lines for *in vitro* and transplantation models

It is unlikely that any one transplantation model will ever replicate the complexity of the whole cancer process; however studies to date demonstrate that xenographs are relevant in human breast cancer. Treatment with Herceptin was shown to improve the anti-tumor activity of paclitaxel and doxorubicin against HER2/neu-overexpressing human breast cancer xenografts leading to consecutive favorable clinical trials [169, 170]. Davis *et al.* [2004] reported the effective inhibition of tumor growth and metastasis in an orthotopic xenograft model by the use of combination therapy of paclitaxel and neutralizing antibodies targeting vascular endothelial growth factor receptor 2 (VEGFR2) [171]. The results of this research likely led to the development of bevacizumab, a humanized monoclonal antibody that targets vascular endothelial growth factor A (VEGF-A) [172]. Although, bevacizumab was removed as a breast cancer indication by the FDA [173], this is yet another example of how transplantation models lead to further development in the clinic. While these are merely a few cases for breast cancer and the use of xenograft studies, the information obtained from such has been translated into successful clinical trials for a variety of cancers [174-178]. Furthermore, useful information has been gained from transplantation models with respect to toxicity and in the identification of predictive biomarkers.

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In spite of these successes, the xenograft model presents disadvantages in its ability to predict clinical response to therapy. Most animal tumors do not accurately model clinical metastatic disease. The use of immuno-compromised mice prevents an immune rejection response which is fundamentally different from the human system where the immune response promotes primary growth of tumor cells and their migration to secondary organs [136, 179]. Most xenograph models do not metastasize at the common sites of human breast cancer (such as lymph nodes, liver, bone and brain) as they prefer to colonize in the lungs [5]. However, improved models that have been created to represent human breast cancer metastasis from a primary orthotopic site to human bone, such as initially published in 2005 by Kuperwasser *et al* [167]. Consider that some xenograph models which utilize subcutaneous injection of tumor cells into the flank and mammary fat pad are not as representative of clinical disease as those that use orthotopic transplantation of cells into the mammary gland. Inherent discrepancies in the background of mice and humans should be considered, and this is of particular importance in predicting side effects for cancer therapy targets [180]. Additionally, the area of metastasis can change depending on the cells line utilized or methods of inoculation [167]; thus, there are considerable technical issues. One could overcome such restrictions by use of clinical isolates. However the utility of primary xerographs is inefficient; access is limited, results are restricted by sample size and thus, studies to date indicate only some degree of success [181]. Current research with aims to generate partial human immune systems or intact populations of human cells in mice systems may also overcome such limitations. We must consider that despite all advances to date, animals do not represent a complete model of human disease. For example, tumor relapse would be especially problematic to study as the usual life span in the majority of mice does not exceed two years. Aside from these underlying basic issues, there have been issues associated with the testing of natural products within these models. Further clarity on the fundamental mechanisms of tumor progression and metastasis and new drug targets will be unveiled as mouse models advance. Until then, we will continue to rely on current disease models and choose such based on disease state and therapeutic targets.

## 7. Breast cancer signaling pathways

Each of the identified breast cancer subtypes and gene expression patterns are dependent on different oncogenic pathways [105, 182]. The maintenance and differentiation of normal breast tissue is controlled by many signaling pathways and involves cytokines and chemokines, growth factors, steroid hormones, integrins, adhesion molecules and their respective receptors [183]. The regulation of such by single or combined components of the tumor microenvironment (such as fibroblasts, macrophages / lymphocytes, endothelial cells, vessels and proteins of the extracellular matrix (ECM) and stroma) have been implicated in various ways in the promotion, growth invasion and metastasis of breast cancer [184]. Cross talk or communication between the cancer cells and the factors within the tumor environment, including secretion factors from the tumor itself, can modify expression and signaling [184]. Of the several pathways indicated to play a role in cancer and CSC self-renewal, Notch, Wnt/Beta(β)-catenin,

and Hedgehog (Hh) have been identified in human mammary cancer [124, 185, 186]. Additionally, evidence has mounted to strengthen the link between nuclear factor kappa-B (NF-κB), stem cells and breast cancer as elegantly reviewed in a recent paper by Shostak and Chariot (2011) [187].

### 7.1. Notch pathway

Four Notch proteins, namely Notch-1 to Notch-4, are expressed as transmembrane receptors in a variety of stem/progenitor cells [188, 189]. The binding of specific surface-bound ligands are responsible for triggering cleavage events at the Notch proteins by ADAM (A Disintegrin and metalloproteinase domain-containing protein) protease family and γ-secretase [188-191] causing the intracellular domain of Notch to be released and translocate to the nucleus. Once in the nucleus, downstream target genes (including c-Myc, cyclin D1,p21, NF-κB) are activated [190, 192-197]. Known for their ability to modulate the development of various organs and control cell proliferation [198], the notch activated genes and pathways have been reported to drive tumor control through the expansion of CSCs [198-202]. This associated role in self-renewal function of malignant breast cancers CSCs [198], combined with the fact that Notch inhibitors can kill breast cancer cells *in vitro* and *in vivo*, may partially explain why Notch expression and activation has been associated with a poor prognosis in mammary carcinomas [203-205]. In fact, research findings in breast cancer have presented compelling reasons to target Notch as a therapeutic target in solid tumors. In addition to its ability to regulate survival and proliferation in bulk cancer cells [205] and CSCs [206-209], notch plays a pro-angiogenic role in tumor endothelial cells [210, 211]. Farnie *et al* reported that activated Notch-1, Notch-4, and Notch target Her-1 expression in ductal carcinoma mammospheres *in situ* samples, but not from normal breast tissue [206, 212, 213]. Inhibition of Notch with a gamma-secretase inhibitor (GSI) or a neutralizing Notch-4 antibody has been reported to reduce the ability of ductal carcinoma in situ-derived cells to form mammospheres [207, 214]. Such results suggest that Notch inhibition may have significant therapeutic effects in primary lesions, may be able to preferentially target breast CSCs (responsible for reoccurrence and metastatic disease) and counteract angiogenesis [213]. Cross talk with the NF-κB pathway and Notch1 have been reported in a variety of cell interactions [192, 215-219], including the stimulation of NF-κB promoters[217] and the expression of several NF-κB subunits [192, 215-220].

### 7.2. Wnt/β-catenin pathway

The canonical (Wnt/β-catenin) pathway, including Wnt-1, -3A and -8 is likely the best characterized and traditionally defines Wnt signaling, however other pathways have been described including a non-canonical (planar cell polarity) pathway (including Wnt-5A, - 11) and the Wnt/Ca2+ pathway (protein kinase A pathway) [221-224]. Although it has been more than 25 years since the discovery of the Wnt gene, its structure remains unknown and signaling pathways are not well defined, especially those independent of β-catenin. Perhaps this challenge can be somewhat explained by the recent discovery that differences in cell signaling outcomes may be attributable to precise pairings of Wnt ligands with analogous cellular receptors [225]. For example, if we consider that the mammalian genome codes for 19 Wnt proteins and 10 Fzd

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receptors, there are potentially 190 Wnt/Fzd pairing combinations [226]. Although all of these ligand/receptor pairings have not been unveiled, we already know that the Wnt/β-catenin pathway has been established for its ability to alter cell proliferation, migration, apoptosis, differentiation and stem cell self-renewal [224, 227-230]. The essential mediator of the canonical pathway is β-catenin, and its two known distinct functions are based on cell specific locations. Accumulation of β-catenin within the cytoplasm leads to activation of Wnt target genes such as c-Jun, c-Myc, fibronectin and cyclin D1 [186, 231-236]. Prior to nuclear translocation, β-catenin operates in the membrane to maintain cell–cell adhesion via cooperation with the epithelial cell–cell adhesion protein E-cadherin [223]. The Wnt signaling pathway is activated via the binding of ligands to transmembrane receptors encoded by the Frizzled (Fzd) gene family and in conjunction with co-receptors, such as low-density lipoprotein receptors (protein 5 and 6) [237] This Wnt-Fzd interaction results in dephosphorylation, accompanied by decreased levels of degradation and causes the accumulation of β-catenin in the nucleus [231]. In the absence of Wnt signaling, β-catenin is quickly degraded in the cytoplasm. Without Wnt signaling, phosphorylation of adenomatous polyposis coli (APC) [238] via a cytoplasmic destruction complex results in ubiquitination of β-catenin which is then prone to proteasomal degradation [231]. Additionally, nuclear levels of β-catenin are lessened by their interaction with APC and Axin, both known for their function in transporting β-catenin back to the cytoplasm. In the nucleus, transcriptional corepressors interact with DNA-binding T-cell factor/lymphoid-enhancer factor (Tcf/Lef) proteins, such as Groucho/TLE, and are enabled to block target-gene expression when β-catenin is held at low levels. [239-242]. Wnt binding to the Fzd or low-density lipoprotein receptor protein-membrane receptors results in the accumulation and stabilization of translocated (from cytoplasm to nucleus) β-catenin [237]. Inhibition of such interactions has been noted by secreted Fzd-related proteins, Dickkopfs, and Wnt inhibitory factor-1 (WIF-1) [243, 244].

Since the initial observation that Wnt overexpression results in malignant transformation of mouse mammary tissue [245], aberrant regulation of the Wnt signaling pathway has emerged as a prevalent theme and continues to develop as a fundamental mechanism in broad cancer biology [246]. While Wnt pathway mutations (genetic and epigenetic) are rare in mammary carcinoma, overactive Wnt signaling has been noted in the majority of breast cancers, including rare classes (i.e.: triple -ve type) via several potential mechanisms [145, 233, 247-256]. Several studies to date have indicated that the expression of both Wnt receptors and their ligands are characteristic of breast cancer and furthermore certain receptors and ligands may be breast cancer type specific. In 2004, Bafico *et al*. reported autocrine Wnt signaling in a panel of breast cancer cell lines, including MDA-MB-231, which were identified by the presence of unstabilized β-catenin and then subsequently reduced upon expression or by the addition of the soluble Wnt inhibitors sFRP1 or DKK1 [257]. The expression of the Wnt receptor FZD7 is characteristic of certain rare types of breast cancer [258]. Additionally, the knockdown of FZD7 in cell lines representative of triple -ve breast cancer reduced the expression of Wnt target genes, inhibited tumorigenesis *in vitro* and greatly retarded the capacity of the MDA-MD-231 cell line to form tumors in mice [246, 259]. With respect to Wnt ligands, secreted frizzled related protein (sFRP)-1, an effective competitor and binding site with FZD receptors for Wnt ligands, has been shown to be ectopically expressed in the MDA-MB-231 cell line [260]. This same study showed that the

sFRP1 expressing cells struggled to form tumors upon inoculation into the mammary fat pads of mice and their propensity to metastasize to lung was greatly impaired [260].

Specific to the maintenance of CSCs, Wnt/β-catenin signaling is implicated in many cancers [223, 224, 261-268], including breast cancer [269]. For example, radiation resistance of mouse mammary stem/progenitor cells has been correlated with overexpression of β-catenin in the stem cell survival pathway [266]. Additionally, overexpression of Wnt/β-catenin signaling was reported to promote expansion of the hepatic progenitor cell population in animal studies [267] and the elimination of β-catenin abrogates chemoresistant cell populations endowed with progenitor-like features [57]. Of great interest is the link between Wnt/β-catenin and PI3K (phosphoinositide 3 kinase) /Akt (protein kinase B) pathway as established by several studies. Korkaya *et al*. demonstrated that PI3K/Akt pathway is important in regulating the mammary stem/progenitor cells by promoting β-catenin downstream events through phosphorylation of GSK3β [60, 189]. Other studies have revealed the ability of activated Akt, such as phospho-Akt Ser473 to phosphorylate Ser9 on GSK3β, thereby decreasing the activity of GSK3β, and potentially stabilizing β-catenin [270-272].

In summary, the proof of concept for inhibiting Wnt signaling in cancer is in place. Furthermore there is an increasing amount of evidence to support a role for Wnt signaling in breast cancer; thus, a target has been created for future studies. Specific to breast cancer, the emphasis on target development ranges from antagonizing Wnt ligand secretion or binding to promote β-catenin degradation to specifically blocking β-catenin-mediated transcriptional activity [222]. Nonetheless, as noted several times throughout this chapter, the cooperation of Wnt pathway with other signaling pathways in cancer is an important consideration. Aside from the challenge of determining the most efficacious way to inhibit Wnt related factors, possible safety concerns should be considered; another compelling reason to explore specific targets in the Wnt pathways for all breast cancer sub-types.

### 7.3. Hh pathway

A crucial mediator of normal tissue development, with recent indications as a regulator of tumor-related vascular formation and function [273], the Hh signaling pathway in cancer is activated by ligand independent mutations in the pathway or through Hh overexpression (ligand-dependent) [189, 274, 275]. In the absence of Hh ligands, (Sonic Hh, Desert Hh and Indian Hh), their transmembrane receptor Patched (Ptch) associates with and blocks the G-protein-coupled phosphoprotein receptor Smoothened (Smo) and is only released when secreted Hh ligands bind to Ptch [189, 276, 277]. This binding triggers the dissociation of glioma-associated (Gli) family of zinc finger transcription factors. The three Gli proteins found in vertebrates include Gli1 and Gli2 (thought to activate Hh target genes) and Gli3 (known to act primarily as a repressor) which lead to the transcription of an assortment of genes including cyclin D, cyclin E, myc and elements of EGF pathway effectors through complex interactions with Costal2 (Cos2), Fused (Fu) and Suppressor of Fu (SuFu) [276-278]. Somatic mutations which activate Hh pathway have been implicated in a variety of human malignancies[278] including basal cell carcinomas, pancreatic cancer, medulloblastomas, leukemia, gastrointestinal, lung, ovarian, breast and prostate cancers [274, 275, 279]. Both *in vitro* and mouse model

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systems have demonstrated that the Hh signaling pathway plays a crucial role in regulating self-renewal of normal and malignant human mammary stem cells [51, 189]. Hh pathway inhibition has been shown to result in tumor growth inhibition mediated through the stromal microenvironment; as demonstrated in a xenograft model using a tumor and stromal cell co-injection procedure, and consistent with a paracrine signaling mechanism [280]. Although data describing the genetic alteration and the modulation of the expression pattern of Hh pathway components in mammary gland are limited, possible indications for the Hh pathway in development and maintenance of mammary cancer have been proposed [281]. However, a more significant role of Hh signaling has been revealed in prostate cancer studies, demonstrating that autocrine Hh signaling by tumor cells is a requirement for proliferation, viability and invasive behavior [282]. Additionally, the association of accelerated prostate cancer growth and progression with increased Hh signaling has been reported [283]. The Hh signaling pathway has been demonstrated as a critical pathway involved in stem cell self-renewal [276] including the essential role of Hh-Gli signaling in controlling the self-renewal behavior of human glioma CSCs and tumorigenicity [189, 284]. Known for its central role in the control of proliferation and differentiation of both embryonic stem cells and adult stem cells, aberrant activation of Hh signaling could be involved in the generation of CSCs and the development of cancer [278, 285]. In this regard, the development of Hh inhibitors may be a solution in the treatment of human cancers, including prevention of tumor progression. Essential similarities have been noted between Wnt and Hh signaling pathways [286] and their key roles in the physiological and pathological development of both embryonic and stem cells [278] [278]gives rise to the fact that crosstalk exists between the two. Signaling for both are activated by G-protein-coupled receptors [287, 288] and prevents phosphorylation-dependent proteolysis of key effectors (*Cubitus interruptus* or β-catenin) responsible for the conversion of a DNA-binding protein from a repressor to an activator of transcription [278, 289]. Considering the progression model of many cancers, specifically metastasis to bone, it is interesting to note that Wnt signaling has been reported to be downstream of Hh signaling, participating in bone development [278, 290]. Further proof proposing that Wnt signaling is downstream of Hh includes the ability of activated Gli1 to stimulate the transcription of Wnt ligands [276, 278]. It has been noted that molecules involved in Wnt signaling (i.e: GSK-3β) also play a regulatory role in Hh signaling [278, 286]. Furthermore, canonical Wnt/β-catenin signaling is required for the pathological response to oncogenic Hh signaling [278, 291].

### 7.4. NFκB Pathway

Along with their hallmark roles in cell survival, proliferation, inflammation and immunity, the NF-κB family of transcription factors are often constitutively expressed in breast cancer tumors [292]. Early studies on NF-κB pathway determined its key role in mammary epithelial proliferation, architecture and branching during early post-natal development [293, 294]. However, independent of its effects on mammary development, evidence exists to suggest that NF-κB regulates breast tumor progression [293, 294]. Constitutive activation of NF-κB in several breast tumor cell lines has been shown to profoundly affect the initiation and progression of breast cancer [295]. NF-κB is also required for the induction and maintenance of the EMT a process that critically controls breast cancer progression [296, 297]. Additionally, it is

evident that NF-κB mostly acts in specific breast cancer sub-types, namely estrogen receptor (ER)-ve and ErbB2+ve tumors [298, 299] and has been implicated in stem cell expansion in breast cancer studies [187].

Activation of NF-κB results in the constant nuclear localization of proteins including p50, p52, p65, cRel and RelB which subsequently up-regulate anti-apoptotic proteins causing an imbalance between normal cell growth and apoptotic cell death [300]. NF-κB-activation occurs mainly through two well characterized pathways, namely the canonical (classical) and the non-canonical (alternative). Both pathways systematically work in a similar fashion in that they are reliant on signal-induced phosphorylation and degradation of an inhibitory molecule to release and transport nuclear NF-κB proteins. However, they differ in the types of trigger signals, activated kinases, inhibitory molecules and NF-κB proteins utilized in each system. In addition to each of these aforementioned pathways, other NF-κB activating pathways exist and have been indicated in the initiation and progression of breast cancer, however we will not discuss these fully in this chapter other than in the context that they appear in the described and relevant research experiments.

Specifically, the canonical pathway involves translocation of a p50/p65 heterodimer to prompt the expression of genes intricated in cell proliferation as well as their survival, inflammatory properties and role in innate immunity [292]. This process occurs through a transforming growth factor beta activated kinase-1 (TAK1)-dependent pathway and is normally dependent on members of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF$\alpha$) or IL-1$\beta$ and other pro-inflammatory cytokines to degrade the inhibitor (IκB$\alpha$) by the NF-κB essential modulator ((NEMO)/IκB kinase (IKK))$\gamma$-containing IKK complex [292]. In 2004, Biswas *et al.* published results regarding the activation of NF-κB in human breast tumors and in carcinoma cell lines indicating that the canonical pathway contributes to tumor development [298]. The resulting highlight of this experiment gave rise to activated NF-κB as a therapeutic target for distinctive subclasses of ER-ve breast cancers [298]. Specifically, the (NEMO)-binding domain (NBD) peptide (a selective inhibitor of IKK) blocked heregulin-mediated activation of NF-κB and cell proliferation while inducing apoptosis on proliferating cells substantiating the hypothesis that certain breast cancer cells rely on NF-κB for aberrant cell proliferation and simultaneously avoid apoptosis [298]. More recently, Connelly *et al*. [2011] showed, via genetic approaches, that the canonical NF-κB-activating pathway is inhibited in defined frames during polyoma middle T oncogene (PyVT) tumorigenesis and that interruption of this pathway in the mammary epithelium increases the latency of tumors and decreases tumor burden [301].

The non-canonical pathway, considered to be critical in adaptive immunity, is similar to the canonical cascade as it also relies on an IKK$\alpha$ heterodimer, but not on NEMO/IKK$\gamma$ [302]. Prior to nuclear shuttling of 52/RelB dimers, the inhibitory molecule p100 is partially degraded through an NF-κB-inducing kinase (NIK)-dependent pathway [292]. Early studies revealed the enhanced expression of the NF-κB protein p52 in breast cancer samples giving rise to the involvement of the non-canonical pathway [303],[304]. The NF-κB protein RelB is increased in ER$\alpha$-ve breast cancer cells and is required for the maintenance of mesenchymal ER$\alpha$-ve breast cancer cells partially through the transcriptional induction of BCL2 [305]. Furthermore, RelB/p52 complexes have since been implicated in mammary carcinogenesis. For example, mouse

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mammary tumors induced by 7,12-dimethylbenz(a)anthracene treatment have been shown to increase RelB/p52 activity and the inhibition of RelB in breast cancer cells repressed cyclin D1 and c-Myc levels and growth in soft agar [306]. Perhaps the most conclusive proof of non-canonical NF-κB-activating pathway involvement occurred in studies employing a novel transgenic mouse model to consider the role of involved mediators (downstream of p100/p52] in both mammary development and tumorigenesis [307]. The results of this study indicated an increase in p100/p52 expression in tumors from mice expressing PyVT in the mammary gland, [307] with no change of nuclear p65 detected; an indication that the observation was limited to a deregulated non-canonical NF-κB-activating pathway [307].

*7.4.1. NF-κB and breast cancer stem cell renewal*

Recently, studies have strengthened the association between stem cells, breast cancer and NF-κB. Such have been captured in a review by Shostak and Chariot highlighting experiments to date complemented by a compelling rationale for targeting NF-κB and other developmental pathways involved in the self-renewal of normal stem cells [187]. The involvement of NF-κB in various signaling cascades has proven critical in several studies involving breast cancer stem cell expansion.

Cao *et al*. [2007] showed that IKKα is both a regulator of mammary epithelial proliferation and a contributor to ErbB2-induced oncogenesis [308]. Specifically, breast cancer cells from IKKα (AA/AA) knock-in mice (whereby IKKα activation is disrupted) crossed with the Her2 murine breast cancer model, exhibited diminished self-renewal capacity and resulted in the inability to establish secondary tumors [308]. Breast cancers that generate primary, as opposed to secondary mammospheres such as seen in mice used in these experiments, suggests that IKKα is likewise required for the self-renewal of tumor-initiating cells from the Her2 breast cancer model [187]. Additionally, mutated IKKα slowed tumor development following exposure to 7,12-dimethylbenzaanthracene or the MMTV-c-neu (ErbB2/Her2) transgene; however there was no effect on MMTV-v-Ha-ras-induced cancer despite the fact that both of these oncogenes rely on cyclin D1 [308]. In this same series of studies, carcinoma cells from another mouse model (IKKα(AA/AA)/MMTV-c-neu) underwent premature senescence when cultured under conditions used for propagation of mammary gland stem cells. Altogether, these mouse models of breast cancer show that IKKα seems to act as a central protein in the activation of NF-κB during breast cancer stem cell self-renewal [308]. Therefore, the researchers concluded that IKKα may represent a novel and specific target for treatment of ErbB2+ve breast cancer.

While NF-κB appears to be activated in luminal progenitor cells during differentiation of mammary colony-forming cells [309], the mammary stem-like basally located cells are devoid of NF-κB activity [309, 310]. Taken together, these studies suggest that only the canonical NF-κB pathway is active in normal luminal progenitor cells before transformation and is required for the formation of mammary luminal-type epithelial neoplasias [309]; a reminder of the importance of understanding the cellular etiology underpinning breast tumor heterogeneity [310].

Another interesting role for NF-κB signaling involves the link between inflammation and cancer. While the mechanism linking inflammation and cancer has yet to be explained, we do know that the inflammatory cytokine, interleukin(IL)-6 is up-regulated in epithelial cancers, including breast cancer [311]. We also know that NF-κB regulates the expression of anti-apoptotic genes and activates different pro-inflammatory cytokines and chemokines, including IL-6 [312, 313]. Further clarity on the interactions of NF-κB signaling, inflammation and cancer has been gained through a study showing that the temporary activation of Src oncoprotein mediates an epigenetic event whereby immortalized breast cells are stably transformed to a cell lines that represent self-renewing mammospheres containing cancer stem cells [313]. The inflammatory response triggered by the activation of Src and further downstream signaling which inhibits IL-6 expression is mediated by NF-κB [313]. It has been shown that the transformation of cells utilized within this experiment occurs via a positive feedback loop whereby IL-6 mediated STAT3 transcription factor stimulation activates NF-κB [313]. These authors have demonstrated that Src activation triggers a rapid inflammatory response mediated by NF-κB that is critical for cellular transformation. While this study defines Src's role as an oncogenic kinase promoting the expansion of breast cancer stem cells, it also demonstrated the critical involvement of NF-κB in the process [313].

It is known that the onset of progestin-driven breast cancer is affected by the deletion of IKK$\alpha$ in mammary-gland epithelial cells [314]. Such studies are relevant in breast cancers as they consider the importance of associated risk factors between hormone replacement therapy (i.e.: progesterones or synthetic derivatives) and the increased risk of incident of fatal breast cancer [314]. The expression of both receptor activator of NF-κB (RANK) and RANK ligand (RANKL) have been observed in primary breast cancers in humans and breast cancer cell lines [315]. Studies to date indicate that the RANKL/ RANK system is mediated in part by IKK-$\alpha$–NF-κB signaling and controls the incidence and onset of progestin-driven breast cancer; more specifically a loss of RANK expression significantly impairs the self-renewal capacity of cancer stem cells [314, 316]. Thus, because of the link between the RANKL/RANK system and progestin-driven epithelial carcinogenesis, RANKL inhibition could be considered as a novel approach to the prevention and/or treatment of breast cancer [314].

More recently, a model of Her2-dependent tumorigenesis indicated that breast cancer stem cell renewal is regulated by epithelial NF-κB through a reduction in the expression of key embryonic stem cell regulators, namely Sox2 and Nanog [317]. Specifically, NF-κB was required for both proliferation and colony formation of Her2-derived murine mammary tumor cell lines [317]. Additionally, the rate of initiation of Her2 tumors was governed by NF-κB [317].

### 7.5. Summarizing pathway interruptions/targets

In many human breast cancers, all three developmental pathways (Wnt, Notch and Hh) appear to be deregulated and control the self-renewal of normal stem cells from a molecular perspective [186]. Additionally, the involvement of NF-κB has emerged as another involved pathway in breast cancer based on what we know about Her2, a membrane bound receptor tyrosine kinase. Her 2 is overexpressed in 30% of breast cancers and critically controls the cancer stem-cell population [318]. Since Her2 activates NF-κB through the canonical pathway [319] [27],

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the hypothesis exists that the NF-κB pathway may be involved in the biology of breast cancer stem cells. It is also obvious that these interrupted pathways, and likely others unknown at this time, are responsible for certain stages of cancer progression or cancer cell aggression. If so, then we are in agreement with others who have noted that the development and identification of selective inhibitors of specific signaling pathways is an attractive approach for the prevention of tumor progression and/or treatment of cancers [278]. However, we have also mentioned several examples of cross-talk between the components of different cell signaling pathways. This concept then introduces the task of targeting multiple pathways in an effort to prevent progression and metastasis of cancers. Alternatively, and based on the premise that certain pathways operate downstream of others, it would be more reasonable to focus on inhibitors of overarching pathways, such as NF-κB. In summary, such oncogenic pathway signatures are fundamental in natural product testing. Therapeutic approaches involving natural products may provide a link between pathway deregulation and therapeutic sensitivity indicating an opportunity for the development of target compound(s).

## 8. Prevention and treatment of breast cancer with natural products: Past successes and promising future treatments

Currently, hormones and cytotoxic drugs remain the standard treatment for metastatic breast cancers. Development of a therapeutic approach for treating tumors, tumor reoccurrence and metastatic tumors is crucial for reducing mortality in cancer patients [6]. There is an urgent need to explore agents that will be effective in preventing and treating metastasis of breast cancer. For centuries, nature has provided us with a rich source of compounds for various disease treatments. Such naturally-derived molecules have been utilized in formal drug discovery platforms of the pharmaceutical industry. Greater than 60% of new chemical entries at the National Cancer Institute from 1981-2002 were either natural products or were derived from natural products [320]. Such naturally-occurring sources can be defined by their origin and include biotic (i.e.: forests, plants, animals, birds and marine organisms) and abiotic (i.e.: land, water, air and minerals such as gold, iron, copper, and silver) components [321]. Within these categories, plants have proven to be a rich source of lead compounds (i.e: alkaloids, morphine, cocaine) or the basis for synthetic drugs (i.e: anesthetics from cocaine) [320, 321]. The complexity and variation of plant structures indicates that their evolution has naturally completed the screening process and that the creation of potent compounds makes them more likely to survive. Plants offer the advantage of abundance, and even with such clinical successes as paclitaxel (Taxol) from the yew tree, and the antimalarial agent artemisinin from *Artemisia annua*, the vast majority have not been studied [320]. Due to promising bioactivity and diversity, the plant environment offers a potential source of natural products. A vast number of studies in the discipline of epidemiology have confirmed an association between fruit and vegetable consumption and the reduced risk of several cancers resulting in an increased interest in the role of naturally occurring dietary compounds in the efficacy of cancer chemoprevention [322]. Thus, the exploration of plant-based molecules as anti-cancer drugs is appropriate.

The history of Tamoxifen and its derivatives in the successful treatment of estrogen receptor (ER)+ve breast cancers are well documented. In the past Tamoxifen was successful in reducing breast cancer mortality rate in hormone receptor+ve breast cancer patients by up to a third and thus was the stronghold of endocrine treatment [323]. Clinical trials have indicated that aromatase inhibitors (AI) have improved efficacy compared with Tamoxifen for the treatment of post-menopausal hormone receptor+ve patients [324-327]. Additionally, the response rate for third generation AIs as first-line agents range from 30%–50% in ER+ve advanced breast cancer. [323]. Leading to these discoveries, and in an effort to capitalize on the advantages of both anti-aromatase and anti-estrogenic activity, many natural products have been tested for their ability to prevent and treat breast cancer, *in vivo* and *in vitro*. Table 4 summarizes plant based compounds that have been indicated for their potential as a prevention or treatment in breast cancer. This list is not exhaustive; however it does capture the extracts studied to date according to activity in cell lines and animal models and represents the most common types of breast cancer. In addition to those listed within Table 4, soy-based extracts, curcumin and piperine have been studied and we will discuss these in detail as the most promising plant based targets in the prevention, treatment and progression of breast cancer.

## 9. Promising plant based targets in the prevention, treatment and progression of breast cancer

### 9.1. Summarizing the individual and combined effects of the soy isoflavones, genistein and diadzein, on mammary tumor development, metastases and invasive breast cancer cells *in vivo* and *in vitro*

Genistein, daidzein and glycitein are the main isoflavones present in soybean and soy-based foods [341] [342]. Out of these, genistein is the mostly studied and dominant isoflavone of soy against breast cancer and has progressed to phase II clinical trials [343]. Soy isoflavones acting upon breast cancer cells *in vitro* and *in vivo* have been studied extensively with varying results and the clinical implications specific to breast cancer have been discussed. Soy isoflavones are structurally similar to female androgen estrogen, and thus they are also known as phytoestrogens [344] and may possibly be competing with the physiological estrogens. Genistein and daidazine (but not glycietein) possesses the ability to transactivate the estrogen receptors.

Utilizing cell based assays on MCF-7 human breast cancer cells, estrogenic agonist actions of soy isoflavones have been studied by Matsumura and co-workers whereby genistein and daidzein exert estrogen response in MCF-7 cells [345] via ER with higher affinity to ERβ1. Similarly, in hepatoma cells transfected with ER, genistein and daidazine bind to both ERα and ERβ but with more affinity to ERβ. Genistein is more potent compared to daidazine [346]. However, these phytoestrogens are 400-600 times less potent compared to 17-β estradiol [347].

*In vitro*, genestein is capable of identifying cells that specifically carry BRCA1 mutation and strongly inhibits the growth of BRCA1 mutant cells compared to cells expressing the wild-type BRCA1 protein [348]. The resistance shown by cells expressing wild type BRCA1 protein has been attributed to increased AKT and decreased p21 (WFA1/CIP1) protein levels [349].

| Natural Product; Active Ingredient | Effect | Ref. |
|---|---|---|
| White button mushrooms (*Agaricus bisporous*); Conjugated linoleic acid and its derivatives | Decreased both tumor cell proliferation and tumor weight with no effect on rate of apoptosis in MCF-7aro cells and nude mice injected with MCF-7aro cells. | [324] |
| *Taxus brevifolia* (Pacific Yew) and other Taxus derivatives; Paclitaxel | Promotes tubulin polymerization and stabilization of microtubules against depolymerization. | [328] [329] |
| Dysoxylum binectariferum; Flavopiridol | Inhibition of MMP-2 and MMP-9 secretion of in MDA-MB-435 (parental) and 435.eB (stable transfectants) breast cancer cells. | [330] |
| Green Tea; *Epigallocatechin gallate* | Suppresses receptor (ERα) MBA-MB-231 breast cancer cell growth *in vitro* and *in vivo* in combination with curcumin. | [331] |
| Bloodroot *(Sanguinaria Canadensis);* Sanguinarine | Induced apoptosis through mediation of ROS production in MDA-MB-231 breast carcinoma cells, decrease in mitochondrial membrane potential, release of cytochrome c, activation of casp-3, and casp-9 and down regulation of Bcl-2. | [332] |
| *Garcinia hanburyi.* (Gamboge tree); Gambogic acid | Upregulation of p53 and down regulation of Bcl-2 resulting in apoptosis in MCF-7 cancer cells. | [333] |
| Ganoderma lucidum; Ganoderic acids | Inhibits AP-1 and NF-κB activity; inhibition of u-PA secretion from MDA-MB-231 cells. | [334] |
| Ginger; Acetoxychavicolacetate | Decreased cell viability in MCF-7 and MDA-MB-231 cells via casp-3-dependent increase in apoptosis. | [335] |
| Grapes, fruits, and root extracts of the weed Polygonum cuspidatum); Resveratrol | Suppresses NF-κB activation and cell proliferation in MCF-7 cells, reduced expression of Cox-2 and MMP-9 (with a reduced NF-κB activation). | [336] |
| *Garcinia indica* (kokum); Garcinol | Induced apoptosis in MCF-7 and MDA-MB-231 cells via caspase activation and down-regulation of NF-κB regulated genes. | [337] |
| Plumbago europaea (Plumbago); Plumbagin | Induced apoptosis with concomitant inactivation of Bcl-2 and the DNA binding activity of NF-κB in MCF-7aro breast cancer cells. | [338] |
| Rotenone; Deguelin | Arrests cells at the S phase resulting in anti-proliferative effect in MDA-MB-231 cancer cells. | [339] |
| Silymarin; Silibinin | Reduced PMA-induced invasion of MCF-7 cells through specific inhibition of AP-1-dependent MMP-9 expression. | [340] |

**Table 4.** Plant Based Natural Products indicated in Breast Cancer

Cyclooxygenase-2 (COX-2) expression, which is associated breast cancer risk [350], can also be inhibited by soy isoflavones [351]. Thus, it seems that soy isoflavones are capable of curtailing breast cancer risk factors.

The influences of soy isoflavones on cell growth, cell cycle and apoptosis are all relevant to their effectiveness as chemopreventive agents for breast cancer. A number of studies have indicated the potential of genistein to inhibit proliferation of breast cancer cells in culture by causing cell cycle arrest and/or apoptosis. Genistein induces G2/M cell cycle arrest [352-354]. This effect was seen both in hormone sensitive and hormone independent cells [352]. According to Li *et al*. [2008] G2/M cell cycle arrest occurs, via stable activation of ERK1/2 pathway [354].

Demarcation on the relative importance of cell adhesion, invasion and migration for primary tumor growth verses metastatic tumour growth is not clear. However, motility, migration and adhesion are more connected to metastasis which is undoubtedly the most life-threatening aspect of breast cancer. Thus, it is crucial to identify the effects of soy isoflavones on disease metastasis.

Microarray analysis of genistein treated HCC1395 cells, a cell line derived from an early stage primary breast cancer, has indicated up-regulation of genes that inhibit invasion and down-regulation of genes that promote invasion [355]. Genistein enhances the adhesion of breast cancer cells [356, 357]. This may possibly be one method utilized by genistein to reduce metastasis.

A study by Vantyghem *et al* (2005) describes the ability of dietary genistein to affect metastasis in a post–surgical model in mice [358]. This test model mimics the clinical situation where primary tumors are surgically removed and therapeutic strategies are applied to prevent the growth of any cancer cells seeded to other locations prior to surgery. In this study, primary tumours were established by injecting human breast carcinoma cells, MDA-MB-435/HAL, into the mammary fat pad of nude mice. After 5 weeks, tumours were surgically removed and mice were maintained with a soy free diet or genistein supplemented diet. At the end of 5 weeks, a 10 fold reduction in percent lung metastasis in mice fed on a genistein supplemented diet was seen. In another study, as described by Zhang *et al*., genistein has shown its ability to reduce the number and volume of osteolytic bone metastases in Balb/c(nu/nu) mice injected with MDA-MB-231 human breast cancer cells [359]. As there are clear indications of the ability of this compound to inhibit breast cancer metastasis, as described in the various animal studies, it would be useful to identify the associated molecular mechanisms. Although there are no conclusive findings, a number of different mechanisms have been suggested.

In studies focused on determining related mechanisms, scientists have given more attention to molecules that are overtly expressed in malignant breast tumours. For example, much attention has been invested into the actions of focal adhesion kinase (FAK), a tyrosine protein kinase. As described previously, cell motility is an integral part of metastasis and it is justifiable to investigate the components directly involve in cell motility. FAK has been designated as a regulator of cell migration and invasion [360]. Since over expression of FAK in human tumors occurs, it has been proposed as a potential therapeutic target [361]. Increased expression of FAK expression in invasive breast carcinomas is associated with an aggressive phenotype [362]. In a transgenic model of breast cancer, mammary epithelial specific disruption of FAK blocks transition of premalignant hyperplasias to carcinomas (and their subsequent metasta-

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sis) indicating direct involvement in mammary tumor progression [363]. Further, attenuation of FAK function dramatically increased apoptosis in breast cancer cells [364]. Disruption of FAK signaling by expressing the N-terminal domain FAK in human breast carcinoma cells has led to rounding, detachment and apoptosis [365]. To gain insight into the influence of genistein and daidazine in this important pathway in breast cancer, *in vitro* and *in vivo* studies have been conducted. According to an *in vitro* study, the soy isoflavones genistein, daidzein and 17β estradiol increased the number of focal adhesions and FAK activity in ERα +ve (T47D cells) as well as in ERα -ve (MDA-MB-231) breast cancer cells indicating possible involvement of novel signaling pathways and independent of estrogen receptors. Authors of this study suggested a progressive role (to metastasis) for soy isoflavones in the activation of multiple FAK regulated signaling pathways relevant to breast cancer [366], however the mechanism was not investigated. The studies of Mitra and co-workers may possibly explain the mechanism of FAK in breast cancer metastasis [367]. According to this study, reduced FAK activity or expression blocked 4T1 breast cancer cell invasion through matrigel and the blocking was associated with a 2-3 fold reduction in the expression of urokinase plasminogen activator (uPA) [367]. uPA is a serine protease that cleaves extracellulaer matrix and stimulate plasminogen to plasmin. Cancer cells are known to digest the ECM via substances like uPA and matrix metalloproteases (MMPs) as a means of invading surrounding tissue. This idea is supported by the fact that breast cancer patients with higher level of MMP-9 in tissue is associated with lymph node metastasis; thus, MMP-9 levels in serum, tumour tissue and urine are used as prognostic markers [368]. Furthermore, a study on the role of membrane-type 1 matrix metalloproteinase (MT1-MMP) *in vitro* and in SCID mice reports that the down regulation of mammary cancer cell MT1-MMP has no effect on primary tumour growth and lymph node metastasis, but reduces the occurrence of lung metastasis [369]. Interestingly, uPA secretion from mammary carcinoma cells can be influenced by genistein. This property of genistein has been shown *in vitro* and *in vivo* and the implication on tumour angiogenesis has been studied using F3II mammary carcinoma cells in culture as well as in a syngeneic mouse model. Accordingly, non-cytotoxic concentrations of genistein (0.1-50μM) significantly reduced motility in F3II mammary carcinoma cells and inhibited the secretion of uPA from cell monolayers. Once F3II cells were implanted in syngeneic mice receiving a treatment of genistein (10mg/kg/day), anti antiangiogenic effects were evident [357].

These studies indicate the effectiveness of genistein to inhibit angiogenesis and metastasis by inhibiting proteolytic substances such as uPA. In this respect, our attempt to further explore the value of soy isoflavones in modulating metastasis enabled us to review some important findings. Studies by Shao *et al*, [1998] reported that genistein inhibited the invasion of MCF-7 and MDA-MB-231 cells *in vitro* and the inhibition was characterized by down regulation of MMP-9 (matrix mettaloproteinase-9) and up regulation of TIMP-1 (tissue inhibitor of mettaloproteinase-1) [370]. The same effects were seen in nude mouse xenografts of MCF-7 and MDA-MB-231 cells [371]. Furthermore, in MDA-MB-231 xenografts, genistein inhibited tumour growth, stimulated apoptosis, regulated p21 WAF1/CIP1 expression, inhibited angiogenesis with reduced vessel density and decreased the levels of vascular endothelial growth factor and transforming growth factor β1 [370]. Further studies by the same authors reported that genistein inhibits both constitutive and epidermal growth factor stimulated

invasion in ER- human breast carcinoma cells as characterized by up regulation of TIMP-1 as well as other trypsin inhibitors like protease nexin-II (PN-II) and alpha 1-antitrypsin (alpha 1-AT) [371]. Kousidou *et al* [2005], examining normal mammary cells (MCF-12A), low invasive (ER+ve) MCF-7 cells and high invasive MDA-MB-231 (ER-ve) cells (in parallel) showed differences in the effect of genistein on highly invasive and low invasive cells. Accordingly, all cell types expressed genes of MMP-2, MMP-9, membrane-type matrix metalloproteinase (MT-1, MT-2, MT-3), MMP and TIMP-1, -2 and -3. However, once genistein was added, down regulation of all MMP genes in highly invasive cells and down regulation of many genes in low invasive MCF-7 cells was observed [372].

Based on the above findings, genistein has a role in reducing metastasis and this appears to arise from its ability to suppress uPA and MMPs thereby invading barriers with no direct effect on the capacity of cell mobility. According to the literature, expression of uPA and MMPs is regulated by NFk-B [373] [307]. Therefore it would be worthwhile to review any association between genistein and the NF-kB pathway.

### *9.1.1. Genistein may act via inhibition of the NF-κB pathway*

The possible connection between NF-κB and breast cancer has been extensively studied [374]. Using a doxycycline-inducible new mouse model to inhibit NF-κB activity, specifically within the mammary epithelium at the time of tumor development, Connelly *et al* (2011) indicated the active contribution of NF-κB in mammary tumor progression [301]. In this model, inhibition of NF-κB activity showed an increase in tumor latency and a decrease in tumor burden [301]. Specifically, soy isoflavones inhibited the tumors by suppressing the NF-κB pathway. Furthermore genistein potentiates the activity of a number of NF-κB mediated chemotherapeutic agents by increasing apoptosis in various cancer cells, including MDA-MB-231 breast cancer cells [375]. In the same cell line, genistein induces G2/M cell cycle arrest via stable activation of ERK1/2 pathway [354],[376]. Furthermore, the MDA-MB-231 cell line has the ability to selectively block NF-κB transactivation of IL-6, a cytokine that is known for estrogen independent tumorigenesis activity [377]. Inhibition of proteosome activity by genistein in MCF-7 breast cancer cells has also been associated with NF-κB inhibition [378]. This property of genistein is particularly important for ER deficient breast cancer as constitutive NF-κB and Mitogen- and Stress- Activated Protein Kinase-1 (MAPK) /MSK activity are linked with aggressiveness and the metastasis.

It is clear that almost all of the studies that show beneficial effects of soy isoflavones utilized genistein. However, within the natural products of soy and soy food not only genistein, but daidzein and glycitein are present. A recent study testing genistein, daidazine and glycitein separately has indicated interesting results. According to this study, only genistein induced apoptosis in MCF-7 breast carcinoma cells whereas daidzein caused a slight cell-stimulating effect in the absence of E2; thus, the authors pointed toward the possible risk of breast cancer in postmenopausal women who take soy supplements [379]. This statement is important as a number of soy supplements available in the market contain high levels of daidzein [380, 381].

An animal study using nude mice and MDA-MB-435 breast cancer cells reported the individual and combined soy isoflavones exerting differential effects on metastatic cancer progression

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[382]. As described in this study, daidazine increased mammary tumour growth by 38% while genistein decrease tumor growth by 33%. Moreover, the combined isoflavones increased metastasis to all the organs examined, although no effect on primary tumour growth was noted. These results have led authors to include the consumption of soy foods as a cause of increased breast cancer metastasis. Also, a number of studies by Ju *et al* [383-385] have cited enhanced growth effects of soy components in ER+ve breast cancers. However, with these studies conducted in immune compromised mice, the relevance of these findings have been criticized [386], especially as pre-treatment with genistein has shown to been protective against mammary tumors [387]. More recently, the antiproliferative activity of both genistein and quercetin has been indicated in the prevention and treatment of HER2-overexpressing breast cancer via inhibition of NFκB signaling [388]. In this study, these specific phytoestrogens inhibited proliferation in MCF-7 cell lines accompanied by an increase in intrinsic apoptotic indicators, induction of the extrinsic apoptosis pathway (up-regulating p53), a reduction in the phosphorylation level of IκBα, and negated the nuclear translocation and subsequent phosphorylation of nuclear p65 [388].

### *9.1.2. Sources of soy*

Soybeans can be considered as the richest source of isoflavones in the human diet [389, 390], and are available in fermented and non-fermented forms. Fresh green soy beans, whole dry soybeans, whole-fat soy flour, soy milk and soymilk products such as tofu, okara and yuba are non-fermented while soy sauce, temphe, miso and natto are fermented products [391]. Additionally, products such as soy dairy substitutes, soy cheese, soy yogurt, and soy burgers seem to be popular in Western countries. The isoflavone content in various soy-based food products greatly differ. Other than soy food products, soy supplemented (categorized as a class of complementary medicine) nutraceuticals are widely consumed by Western communities. The quality and standard of these supplements are questionable. For instance, a survey carried out in the Eastern Washington Region of U.S.A. tested 13 products (7 tablet and 6 capsule formulations) by HPLC and showed that only 4 of the 13 products contained the minimum of 90% isoflavone content claimed on the label and variations in composition over time were noted [392]. Interestingly, a recent review shows that overall, most commercially available nutraceuticals are poor in quality [393].

### *9.1.3. Clinical studies*

Although there have been a large number of studies carried out to evaluate a possible soy-breast cancer link, evidence is inconclusive. The two main theories tested involve the effect of soy isoflavone consumption in risk of breast cancer incidence and its effect on recurrence. The largest population based cohort study, including 5,042 female breast cancer survivors, shows that soy food consumption is significantly associated with decreased risk of death and recurrence [394]. Another cohort of 1,954 female breast cancer survivors, who consumed soy isoflavones at the levels comparable to the Asian population, while undergoing tamoxifen therapy showed a reduction in the risk of cancer recurrence and no interference in the efficacy of tamoxifen [395]. Further studies have indicated that soy intake prior to cancer diagnosis is

unrelated to disease-free breast cancer survival and that the association between soy protein intake and breast cancer survival does not differ according to the presence of other risk factors such as ER/PR status, tumor stage, age at diagnosis, body mass index (BMI), waist to hip ratio (WHR), or stage of menopause [396]. No variations were noted in the soy-survival association of indicated polymorphisms in ER$\alpha$ and ER$\beta$ indicating that soyfoods do not have an adverse effect on breast cancer survival. A recent meta-analysis by Dong and Qin (4 studies of breast cancer recurrence and 14 studies of breast cancer incidence) revealed that the consumption of soy isoflavone is inversely associated with risk of breast cancer incidence [397]. However, the protective effect is only observed among studies conducted in Asian populations, unlike those reported in Western populations [397]. One of the previous meta-analysis studies by Wu *et al* [2008] show a similar trend. Accordingly, in Asian populations a higher intake of soy isoflavones, as compared with lower intake, is associated with 29% reduction in the risk of developing breast cancer [398]. Hence, the consumption of soy food at levels similar to those consumed by Asian populations may have protective effects. However, there is evidence in the literature to show possible adverse effects of soy due to its known stimulatory effect on the premenopausal female breast as indicated by increased secretion of breast fluid, the appearance of hyperplastic epithelial cells and elevated levels of plasma estradiol [399]. Some animal studies support the idea of related disadvantages of consuming soy isoflavones. In overiectomized athymic nude mice, physiological concentrations of dietary genistein stimulates the growth of estrogen dependent MCF-7 tumors in a dose dependent manner [383, 400]. Furthermore, the same test model showed that dietary genistein reverses the inhibitory effect of tamoxifen on the growth of MCF-7 tumors [384].

### 9.2. The role of curcumin and piperine in breast cancer prevention and its effects on normal human breast stem cell renewal and signaling

Curcumin is a plant derived polyphenol which gives rise to the yellow colour in the spice, tumeric. This pigment is obtained from the plant *Curcuma longa* and has been noted to have power against cancer. In their review of the mechanisms of cell cycle regulation by curcumin, Gaurisankar and Das have named it as a multiple edged sword [401] because of its ability to regulate the cell cycle as well as apoptosis. Distorted cell cycle regulation and programmed cell death/apoptosis are characteristic features of cancer and curcumin has been shown to target both mechanisms. The ability of curcumin to inhibit telomerase activity [402] and to disrupt mitotic spindle structure causing [403] micronucleation in MCF-7 breast carcinoma cells has been reported. Also, curcumin is known to induce anti-proliferative activity via the decreased expression of cyclin D1 and CDK-4 in MCF-7 breast carcinoma cells [404] and can induce apoptosis through p53 dependent Bax induction [405]. Curcumin is able to disrupt breast tumor growth, but also to inhibit metastasis.

As with genestein, curcumin has been shown to mediate its anti-cancer effects via regulation of the NF-κB signaling pathway. In the nude mouse model, curcumin suppresses the paclitaxel-induced NF-κB pathway resulting in the inhibition of lung metastasis of human breast cancer [406]. The modification of NF-kB signaling eventually leads to pro-apoptotic events and perhaps inhibition of ECM breakdown. Curcumin induced apoptosis in MDA-MB-231 cells

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*in vitro* is associated with IkB and p65 phosphorylation and hence reduced activation of NFkB [407]. This leads to reduced expression on MMPs, diminished invasion through a reconstituted basement membrane and a lower number of metastases in immunodeficient mice injected with tumor cells via intra cardiac route [407]. The high level of of MMP-3 expression noted in MDA-MB-231 invasive breast carcinoma cells is not evident on MCF-7 non-invasive breast cancer cells, implicating its importance in invasion and metastasis. The possibility of using the major forms of curcuminoids, curcumin, demethoxycurcumin, and bisdemethoxycurcumin (all of which are found in turmeric powder) as MMP-3 inhibitors to modulate MMP-3 expression has been suggested [408]. According to Chiu and Su (2009), curcumin inhibits proliferation by increasing the Bax to Bcl-2 ratio while inhibiting the migration via decreasing NF-kB p65 expression in breast cancer MDA-MB-231 cells [409]. Utilizing microarray gene expression analysis on MDA-MB-231 breast cancer cells, Bachmeier *et al.* demonstrated the ability of curcumin to downregulate inflammatory cytokines CXCL-1 and -2 via suppression of NF-κB translocation. Moreover, silencing CXCL-1 and -2 resulted in a downregulation of several metastasis promoting genes [410].

Interestingly, curcumin can interfere with estrogen-mimicking pesticides such as endosulfane, DDT and chlordane [411].

### *9.2.1. Sources of curcumin*

Curcumin is generally considered to be the most active component and the principal curcuminoid found in tumeric [411]. The spice tumeric is commonly used in curries and contains 2-8% of this active ingredient [412], however a supplement form is also available.

### *9.2.2. Clinical trials*

Little data is available on the pharmacokinetics and metabolism of curcumin in humans. Dose-limiting toxicity is not reported and high oral doses of curcumin (up to 12g/day) have been tested [220, 413, 414]. In a phase one clinical trial involving individuals with non-invasive cancer and pre-cancerous conditions, oral dosing of 4g, 6g and 8g of curcumin yielded peak serum concentrations of 0.51 +/- 0.11microM, 0.63 +/- 0.06 microM, and 1.77 +/- 1.87 microM, respectively. Peak serum concentrations of curcumin are seen 1-2 hours after oral intake and this gradually declines within 12 hours [220]. In another phase I clinical trial involving 15 patients with advanced colorectal cancer, 3.6g of curcumin daily for up to 4 months was well-tolerated [413]. Another study examined the pharmacokinetics of 450mg-3600mg curcumin (daily for 1 week) in twelve patients with hepatic metastatic disease from primary colorectal adenocarcinomas. Using a high-performance liquid chromatography assay, low nanomolar levels of the parent compound and its glucuronide and sulphate conjugates were found in the peripheral or portal circulation; despite its absence in liver tissue, trace levels of products of its metabolites were detected [415]

Due to the poor bioavailability of curcumin systemically, high priority has been given to study its potential against colorectal cancers. A very recent publication on a phase IIa clinical trial involving men and women 40 years of age or over and smokers that carry 8 or more colorectal

aberrant crypt foci (ACF) indicates that oral dosing of curcumin (4g per day for 30 days) significantly reduces colorectal ACF, a biomarker of colon carcinogenesis. [416] The reported anti-carcinogenic effect of curcumin is not associated with increased levels of curcumin in local tissue but increased levels of conjugate concentrations in suggesting that curcumin may mediate its effects by cuccumin conjugates delivered systemically. The same study showed that the presence of curcumin conjugates in plasma and tissue prior to treatment (believed to be originated from the normal diet of the studied population) were accompanied by a steady increase of curcumin conjugates following the month-long daily dosing [416]. A study examining the pharmacokinetics of curcumin at the concentrations of 10g and 12g in twelve healthy volunteers indicates comparable results. Accordingly, a single dose of orally administered curcumin resulted in the detection of conjugates, glucuronides and sulfates in plasma in all subjects while free curcumin was evident in only one subject [414]. Even though curcumin conjugates and other breakdown products have not been assessed for their anticarcinogenic properties [416], these findings shed some light on the potential of curcumin as a treatment of all cancers, including those of colorectal origin. This may offer a likely explanation of how continuous exposure to small quantities of curcumin via normal diets protects Asian women from breast cancer.

A phase I dose escalation trial of combined effects of docetaxel and curcumin in patients with advanced and metastatic breast cancer was published very recently. This study involved 14 patients and demonstrated the feasibility, safety and tolerability of a combination of curcumin with a standard dose of docetaxel which warrants further investigation and progression to a Phase II clinical trial [417]. Similarly, the curcumin inhibiting effects of chemotherapy induced apoptosis in models of human breast cancer have been identified [418].

*9.2.3. Curcumins' ability to destroy cancer stem cells*

The properties of CSC's are connected with major signaling pathways. The signaling pathways active in mammary stem cells are shown to be Wnt/β catenin, Hh and Notch [60],[51],[185], [206],[269].

A recent study by Karkarala *et al.* has demonstrated the potent inhibitory effect of curcumin and piperine on Wnt/β-catenin signaling in primary human breast epithelial cells [419]. In this study, inhibition of Wnt signaling pathway was shown to affect breast stem cell renewal by inhibiting the mammosphere formation. According to the authors, curcumin and piperine (separately and in combination) inhibited breast stem cell self-renewal; however toxicity to differentiated cells was not reported. The plasma concentration of curcumin in people taking high oral doses has been shown to be very low due to many reasons such as metabolism of the compound in the intestine and the liver, as reviewed by Burgos-Moron *et al.* [420].

The lack of bioavailability of curcumin was known as a potential disadvantage for years and various strategies have been investigated to overcome the problem. One such strategy has been the use of piperine in combination of curcumin. Accordingly, concomitant administration of piperine and curcumin tends to increase the bioavailability (up to 2000%) compared to

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administration of curcumin alone in an experimental group of people [421]. This finding could well be a possibility as piperine has been shown to inhibit P-glycoproteins and CYP3A4 expressed in enterocytes where the bioavailability of many orally ingested compounds are determined [422]. Alternatively, increasing the solubility of curcumin by heat as means of increasing the bio availability has been suggested [423]. This method is easily achievable and a well-cooked curry with tumeric and piperine could be a tasty way of obtaining the goodness of these natural compounds.

Based on the ability of curcumin and piperine to inhibit CSC's as described above, curcumin has great potential as a possible therapeutic agent against breast cancer. The majority of the breast cancer patients have tumors that respond to the naturally occurring hormone, estrogen. Therefore, most of the currently available drugs known to be effective against breast cancer can prevent the action of estrogen and are thus referred to as selective estrogen receptor modulators (SERM). Unfortunately, there is a cohort of patients whose tumours do not express estrogen receptor. SERMs are of no use for this group with ER -ve breast cancers. The potential of curcumin and piperine to suppress the self-renewal of stem cells could prove beneficial in ER +ve as well as ER -ve breast cancer patients.

## 10. Summary

Considering the existence of CSC's in breast cancer, and the inability of current therapeutic approaches to destroy such, we propose targeting CSC's as a tool to investigate the effect of natural dietary compounds. Specifically, we suggest isolates of soy and turmeric, and their effects on breast cancer tumors and metastasis reoccurrence in breast cancer. Similar to other developmental agents aimed at cancer signaling pathways, the optimal dosing, dosing regimens and adverse effects will have to be refined. However, these prospects are notably different than conventional therapeutics in several ways and such should be contemplated upon exploring such molecules. For example, consider the complex cross-talk of both inter- and intra-cell signaling pathways and how feedback mechanism effects may eliminate inhibitors via the actions of a single pathway. Figure 2 summarizes targeted areas of interruption via the major pathways involved in stem cell renewal as well as those pathways that may be responsible for downstream signaling to these major pathways. Additionally, if CSC's are primary targets, then metastasis incidence and/or cancer free survival may be a more appropriate efficacy endpoint in clinical trials than tumor volume. Perhaps then it would be more strategic in breast cancer to design preclinical trials based on incorporating combination regimens at the early stages of drug development while targeting multiple pathways and focusing on appropriate endpoints; all to avoid missing potentially important therapeutic benefits. While plant based derivatives, such as those considered in this chapter hold promise in breast cancer treatment and management, their development should be pursued systematically, guided by sound scientific principles.

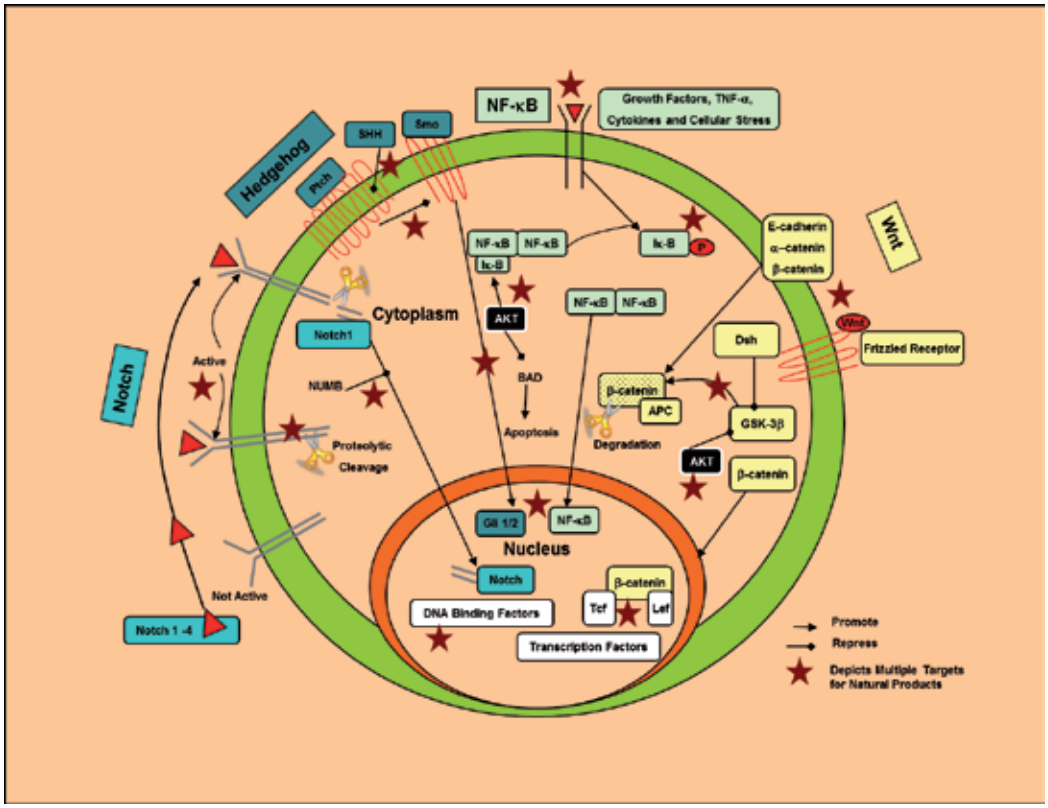


**Figure 2.** (Tobin, GA, 2012) An Overview of the Major Signaling Networks Involved in Breast Cancer and CSC Self-renewal, including Notch, NFκB, Wnt and Hh. Symbols and acronyms have been discussed previously, with the exception of P = phosphorylation, BAD = Bcl-2-associated death promoter, and SHH = Sonic Hh. Also, NUMB references the Protein numb homolog that in humans is encoded by the NUMB gene.

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The medicinal use of plants, animals and microorganisms has been a part of human evolution and likely began before recorded history. Is it possible that this knowledge can be used to create powerful new drugs and solve some of the human health problems facing us today? This book is a collection of an expert team of agronomists, chemists, biologists and policy makers who discuss some of the processes involved in developing a naturally-sourced bioactive compound into a drug therapy. These experts define a natural compound and elucidate the processes required to find, extract and define a naturally-derived bioactive molecule. Finally, they describe the necessity for understanding the fundamental mechanisms of disease before applying bioactive molecules in bioassay-guided drug discovery platforms.

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