

REVIEW ARTICLES

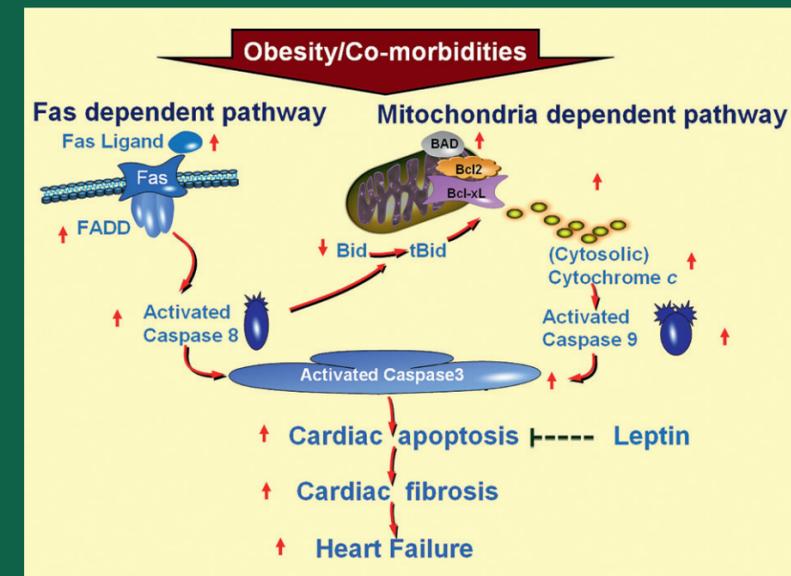
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Review article

Anti-glycative potential of triterpenes: A mini-review

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ABSTRACT

Triterpene compounds occur naturally in many herbs and plant foods. Triterpenes such as ursolic, oleanolic, and betulinic acid definitely possess antioxidative and anti-inflammatory activities as well as an inhibitory effect on advanced glycation end-product (AGE) formation. Furthermore, the effects of triterpenes upon the activity and expression of aldose reductase, sorbitol dehydrogenase, and glyoxalase I, enzymes involved in the polyol pathway, have been examined, with positive results reported. These studies indicate triterpenes as potent antiglycative agents, suggesting that they can benefit the prevention of and/or therapy for glycation-related diseases such as diabetes mellitus and Alzheimer's disease. Further studies should examine their impact on receptors of AGE (RAGEs) and AGE–RAGE interaction in order to bolster the antiglycative application of these natural compounds.

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1. Introduction

Glycative stress from excessive production of advanced glycation end-products (AGEs) is an important contributor for the pathogenesis of several diseases such as diabetes. Several enzymes are involved in the process of AGEs formation. Thus, any agent(s) with the ability to inhibit AGE generation, and decline the activity of responsible enzyme(s) may potentially prevent or attenuate glycative stress and retard the development of associated diseases.

2. Triterpenes

Triterpenes are originally synthesized by plants as metabolites, and are abundant in the plant kingdom in the form of free acids or aglycones [1,2]. So far, both the structure and the

chemical characteristics of at least 80 distinct types have been identified, many triterpenes having been long used as flavors, pigments, polymers, fibers, glues, and waxes. In many Asian countries, some serve formal medical purposes (including folk medicine) to prevent or treat a variety of diseases [3,4]. Recent decades have seen more attention paid to the bioactivities of triterpenoids, earning them consideration as important sources of medications and/or complementary medicines.

According to their structural traits, triterpenes are grouped into euphanes, taraxanes, oleananes, lupaneps, ursanes and baccharanes, with ursanes and oleananes as the major triterpene skeletons in higher plants, including many herbs or plant foods we regularly consume [5,6]. In plant foods, common ursane- and oleanane-type triterpenoids are pentacyclic triterpenes: oleanolic acid, ursolic acid, maslinic acid, uvaol, and erythrodiol. Other groups of triterpenes occur widely in edible or inedible plants.

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Although the content of these pentacyclic triterpenes in edible plants depends on the species, season, and conditions of cultivation, these triterpenes has been reported to be present in herbs such ground ivy (*Glechoma hederacea*), plantain (*Plantago major* L.), thyme (*Thymus vulgaris*), glossy privet (*Ligustrum lucidum* Fructus), and hawthorn fruit (*Crataegi Pinnatifidae* Fructus); fresh fruits such as apple (*Malus domestica* Borkh), blueberry (*Vaccinum dunalianum*), guava (*Psidium guajava*), persimmon (*Diospyros kaki* L.), and loquat (*Eriobotrya japonica*); and vegetables such as olive (*Olea europaea* L.), daylily (*Hemerocallis fulva* L.), spinach (*Spinacia oleracea* L.), and leaf mustard (*Brassica juncea*) [7–9].

Since the consumption of vegetables and fruit is always encouraged in order to improve their health, ever more interest has been raised in understanding the contribution of these special plant food component(s) to health. Therefore, exploring and elucidating the bioactivity and mode of action of triterpenes merits our attention.

3. Bioactivities of triterpenes

Some studies report such compounds as possessing *in vitro* and/or *in vivo* vasodilatory effects [10,11] and anti-inflammatory, antioxidative, and anticancer activities, suggesting that they are potent agents for preventing and/or attenuating disease.

3.1. Anti-oxidative effects

The overproduction of reactive oxygen species (ROSs) and reactive nitrogen species such as the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide, the hydroxyl radical, or nitric oxide has been considered to be a crucial contributor to the development and progression of chronic diseases associated with oxidative stress, e.g., aging, diabetes mellitus, cancer, atherosclerosis, infection, cirrhosis, and Parkinsonism [12–14].

It is widely known that these ROSs and reactive nitrogen species can, via their free radical property, directly attach to the cell apparatus, or can, by acting as signal transduction mediators, regulate the expression of genes involved in cell differentiation and/or apoptosis. This, in turn, evokes oxidative injury, impairs the antioxidative defense system, and causes organ malfunction [15–17]. These free radicals can mediate the gene expression associated with the local or systemic immune system, which subsequently promotes inflammatory reactions and causes inflammatory damage [18,19]. Many *in vitro* and *in vivo* studies highlight the fact that triterpenes possess antioxidative activity via scavenging free radicals, enhancing the activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferase, while sparing nonenzymatic antioxidants such as reduced glutathione, ascorbic acid, and alpha-tocopherol [20–22].

Taken together, the above pieces of evidence supports the fact that triterpenes, through attenuating oxidative stress, retard the development and/or delay the progression of chronic diseases. However, human and even clinical studies are required to further confirm the antioxidative protection bestowed by these triterpenes.

3.2. Anti-inflammatory effect

Inflammation is a normal process whereby the body wards off invaders and repairs tissue damage. Many T-helper cell type 1 and 2 cytokines and chemokines such as interleukin 1-beta (IL-1 β), IL-6, IL-10, tumor necrosis factor alpha (TNF- α), monocyte chemoattractant protein 1 (MCP-1), and prostaglandin E₂ (PGE₂), are essential for the host immune/defense system to protect against stimuli such as pathogens and chemical agents [23–25].

However, as seen in pathological situations such as diabetes mellitus, cancer, and cardiovascular disease, the overproduction of certain inflammatory molecules leads to cytokine imbalance, evokes inflammatory injury, or even causes tissue destruction. In addition, these cytokines and chemokines can activate macrophages and/or mediate factors involved in pathological processes, which in turn favors the development of acute and chronic disease. For example, IL-8 promotes angiogenesis and transforming growth factor beta-1 (TGF- β 1) and enhances fibrosis in cancer [26,27]. The use of appropriate agent(s) with an anti-inflammatory effect could the diminish overproduction of inflammatory stimuli, thus delaying disease progression.

The anti-inflammatory effects and possible modes of action of triterpenes in cell lines, animals, and even humans have been reported [28–30]. These studies indicate that triterpenes regulate both upstream and downstream inflammatory factors, and inhibit the expression, activity, and production of cytokines and/or chemokines, which may consequently alleviate inflammatory stress and mitigate the progression of chronic disease.

3.3. Other bioactivities

Antitumor. Betulinic acid directly triggers mitochondrial membrane permeabilization and causes apoptosis in cancer cells [31]. Ursolic and oleanolic acid cause apoptosis in hepatoma cells, reducing mitochondrial membrane potential and Na⁺-K⁺-ATPase activity [32].

Antiviral. Glycyrrhizin and its derivatives may protect liver cells against damage induced by chronic hepatitis B or C [33].

Antiobesity. Ursolic acid may stimulate lipolysis by translocating hormone-sensitive lipase and decreasing perilipin A expression, as well as upregulating adipose triglyceride lipase in primary culture adipocytes [34].

Based on the above *in vitro* and *in vivo* protective effects and actions, these triterpenes are considered to be potent medicinal compounds, even candidates for new drug development. Information is also available regarding their effect against glycation, another important pathological process involved in many chronic diseases.

4. Glycation and chronic diseases

Non-enzymatic glycation with the formation of Maillard reaction products, also known as advanced glycation end-products (AGEs), is implicated in the pathogenesis of many chronic diseases, e.g., diabetes mellitus, Alzheimer's disease, atherosclerosis, osteoarthritis, inflammatory arthritis, and

cataracts [35–37]. Any agent capable of inhibiting the formation of AGEs may potentially decrease glycative stress and prevent or delay the progression of glycation-related disease.

It is well known that hyperglycemia (as in diabetes) enhances glucose metabolism through the polyol pathway [38]. Aldose reductase, the first and rate-limiting enzyme in this polyol pathway, reduces glucose to sorbitol, which is further metabolized to fructose by sorbitol dehydrogenase (SDH), the second enzyme in this pathway [39,40]. This flux through SDH and elevated fructose level promotes AGE formation and contributes to microvascular abnormalities [41,42]. On the other hand, glyoxalase I (GLI), part of the glyoxalase system present in the cytosol of cells, metabolizes physiologically reactive alpha-carbonyl compounds such as glyoxal and methylglyoxal, and consequently decreases the available precursors for AGEs formation [43].

Because aldose reductase, SDH, and GLI are key factors involved in endogenous glycation reactions, and responsible for the formation and degradation of AGEs, the development of new drugs to mediate this pathway and lower glycative stress should pay more attention to these enzymes. That is, any agent with the ability to suppress the activity and/or expression of aldose reductase and SDH, as well as enhance GLI activity and expression, may curb these glycation reactions and AGE production.

5. AGEs and receptors for AGEs

AGEs are a complex product mixture, formed mainly by reactions between reducing sugars such as glucose, ribose and ascorbate, and the amino groups of lysine or arginine residues from proteins or other moieties (lipids or nucleic acids), followed by Amadori rearrangement.

Next to glucose, reactive dicarbonyl compounds such as methylglyoxal are also precursors for the formation of (extra) cellular AGEs [44]. Methylglyoxal can react with arginine and lysine residues to form imidazolone adducts and N^ε-(carboxyethyl)lysine (CEL), respectively [45]. That is, AGEs are mixtures of protein-bound nitrogen- and oxygen-containing heterocyclic compounds, formed via a complex cascade of dehydration, condensation, fragmentation, oxidation, and cyclization reactions. Except for pentosidine and N^ε-(carboxymethyl)lysine (CML), the structures of many AGEs have not yet been characterized. Although reducing AGEs present in circulation and/or in tissues improve the attenuation of glycative stress, both decreasing AGE formation and increasing AGE degradation and excretion pose a big challenge when developing an anti-AGE drug based on the properties of irreversibly cross-linked, heterogeneous, insoluble protein aggregates.

Glycated hemoglobin, CML, glycated albumin, and pentosidine are common AGEs present in the circulation of patients with diabetes mellitus or Alzheimer's disease, where they may serve as markers of disease progression [46,47]. Circulating AGEs could be from two sources: exogenous and endogenous. The former is from the diet (food); the latter are synthesized in the tissues under normal and pathological conditions. The intake of food rich in glycative products contributes to enriching the circulating AGE pool and elevates

glycative stress [48]. Individuals with glycation-related diseases should thus limit their dietary intake of AGE-containing foods.

Endogenous AGEs may be formed during natural aging, while the progression of diabetes, renal failure, and/or neurodegenerative disease raises endogenous AGE production. The tissue content of AGEs depends on the rates and levels of AGE formation and degradation. Accumulation of AGEs during natural aging is ascribed to the time-dependent nature of advanced glycation coupled with greater oxidative stress, in addition to the progressive reduction in the capacity to neutralize oxidative stress [49]. It is well known that hyperglycemia and oxidative stress accelerate the accumulation of AGEs especially; oxidative stress boosts AGE production via glycooxidation and lipid peroxidation [50,51]. On the other hand, AGE degradation is determined by ligation to macrophage scavenger receptors, protein turnover rate, and renal capability for clearance [52].

5.1. AGEs in diabetes mellitus

AGEs play an important role in the pathogenesis of diabetes-related macro- and microvascular complications: the circulating level of AGEs indicates the clinical stage of patients with diabetic complications such as nephropathy [53,54].

Renal tubular and interstitial cells are direct targets for increased glycative damage. An elevated glucose level stimulates the tubular cells to secrete vasoactive hormones such as angiotensin II, TGF- β , and matrix proteins [55]. These not only lead to AGE formation in the target cells, but also activate intracellular signal transduction systems, generate free oxygen radicals such ROS, and induce redox-sensitive transcription factor and nuclear factor kappa B (NF- κ B), while promoting the expression of genes associated with NF- κ B-mediated inflammation, such as IL-6 [56,57]. On the other hand, circulating AGEs are reabsorbed and metabolized by the proximal tubular epithelial cells.

Massive AGEs, as seen in diabetic nephropathy, spawn renal cellular hypertrophy via decreased protein breakdown [58]. The kidneys are thus vulnerable to AGEs, which can activate various intracellular second messengers such as mitogen-activated protein kinase and nitric oxide synthase, subsequently inducing the expression of adhesion molecules and the production of inflammatory cytokines, as seen in diabetic cardiomyopathy and retinopathy [59,60]. These studies link AGEs to oxidative and inflammatory injury in diabetes. Lowering the level of AGEs in the circulation and the organs is necessary in order to alleviate or delay these complications.

5.2. AGEs in Alzheimer's disease

Two major neuropathological hallmarks are present in the brains of patients with Alzheimer's disease: extracellular senile plaques and intracellular neurofibrillary tangles. Senile plaques contain a core of β -amyloid ($A\beta$) peptide, and neurofibrillary tangles contain hyperphosphorylated microtubule-associated protein tau [61]. AGEs can be detected in both neurofibrillary tangles and senile plaques, in which CML has been found to be localized in the cytoplasm of neurons,

astrocytes, and microglia in both aged brains and the brains of those with Alzheimer's disease [62,63].

Although it remains unclear whether the AGEs present in brain tissue are endogenously synthesized and/or derived from an exogenous dietary intake, several studies have illustrated the accumulation of CML and pentosidine, two major AGE molecules, as being highly associated with the progression of Alzheimer's disease [64,65]; this strongly suggests a crucial role for AGEs in the pathogenesis of this condition. AGEs could directly cause neuronal cell apoptosis by acting as neurotoxins or promoting inflammatory injury and/or as proinflammatory molecule stimulators [66,67]. Deposition of AGEs on the neurovascular wall not only impairs brain function, but also expedites the deterioration of dementia.

CML, along with its glycation-specific precursor hexitol-lysine, is markedly increased in neurons from patients with Alzheimer's disease, especially those with intracellular neurofibrillary pathology [68]. Oxidative stress affects all classes of macromolecule (sugar, lipids, proteins, and DNA), and contributes to an early response in many chronic neurodegenerative diseases, including the normal aging process and Alzheimer's disease. The reported increase of hexitol-lysine or CML is partially due to lipid peroxidation [69], inevitably producing neuronal dysfunction. The oxidative stress hypothesis of Alzheimer's disease suggests that the generation of AGEs in brain tissue is accelerated by an overproduction of oxygen-derived free radicals in cerebrovascular disorders [70].

5.3. AGEs in cancer

The impact of AGEs on the development and progression of cancer has recently attracted more attention. Hypoxia, a microenvironment for tumor cell adaptation, also favors the generation of AGEs by way of oxidative stress and/or damage to cell membranes [71]. AGEs diminish the vascular barrier function and enhance the expression of vascular endothelial growth factor and vascular cell adhesion molecule 1, as well as disturbing the balance of cellular coagulant–anticoagulant properties, thus promoting angiogenesis and tumor growth [72,73]. Methylglyoxal derived from polyol pathways forms adducts with DNA nucleic acid bases and results in the production of the AGE molecule N²-(1-carboxyethyl)-2'-deoxyguanosine, an indicator of DNA damage or mutation [74]. These findings indicate that elevated AGEs level in the circulation could facilitate the growth of an already established malignant tumor.

6. Receptors for AGEs

The receptor for AGE, also called as RAGE, is expressed by a variety of cells, including endothelial and tubular epithelial cells. RAGEs can engage diverse ligands associated with distinct pathological processes. One class of RAGE ligands reacting with AGEs occurs in diabetes, renal failure, or amyloidoses, and is responsible for deterioration in diabetes mellitus and Alzheimer's disease. Elevated RAGE expression has been noted in various cells under diabetic conditions [75], since hyperglycemia is a major stimulator of the production of

RAGE ligands with a capacity to interact with AGEs in the circulation and tissues. AGEs derived from the diet or endogenous sources also enhance RAGE expression [76].

In the development of Alzheimer's disease, RAGE acts as a signal transduction receptor for A β peptide that has accumulated in the affected brain parenchyma and cerebral vasculature. This aggravates neuronal stress and neuroinflammation, and finally impairs memory and learning [77]. RAGE also acts as ligand for S100/calgranulins, high-mobility group box 1 and beta-sheet fibrils, which generates proinflammatory and prothrombotic molecules and ROSs; this eventually leads to augmented damage in the target tissues, such as atherosclerotic plaques and cardiac infarction [78,79]. The targets of RAGE ligands include tumor cells, neurons, endothelial cells, Caco-2 epithelial cells, podocytes, smooth muscle cells, and microglial cells, and RAGE ligands may trigger diverse signaling cascades (MAPKs, p21ras, ERK1/2, p38, JNK, and Jak/STAT) in these targets [80,81].

These RAGEs and ligands play independent roles in the pathology of several diseases, but the AGE–RAGE interaction merits attention as it directly activates many crucial signaling mechanisms. It has been indicated that AGE–RAGE interaction stimulates O₂^{•-} production, raises oxidative stress [82], evokes vascular inflammation and thrombosis via activating NF- κ B [83,84], and enhances the expression of adhesion molecules, chemokines, proinflammatory cytokines, matrix metalloproteinases and the upregulation of RAGE itself [85,86]. Consequently, NF- κ B induces the expression of downstream genes encoding TNF- α , IL-6, and MCP-1, which in turn promote inflammatory reactions and cause impairment in the target tissues [87,88].

Although it is well known that glycative stress from AGEs, RAGEs, and their interaction play a key role in the progression of diabetes mellitus and Alzheimer's disease, the influence of the AGE–RAGE axis on cancer pathology cannot be ignored, since ROSs and cytokines generated from this axis contribute to oxidative and inflammatory DNA damage and initialize carcinogenesis. Besides AGEs and A β , RAGE can bind to other ligands, such as low-density lipoprotein and calgranulins.

The impact of AGEs, RAGEs, RAGE ligands, and their interaction on human health is not limited to glycative stress. Thus, any strategy against glycation-associated chronic diseases must consider: (1) reducing the level of AGEs in the circulation; (2) lowering the level of other RAGE ligands in the circulation; and (3) impeding the interaction of RAGE and its ligands. Any inhibitor(s) blocking AGE formation, suppressing RAGE expression, or interrupting the AGE–RAGE interaction could be a potential candidate for treating diabetes mellitus, Alzheimer's disease, and other glycation-related diseases.

7. Antiglycative potential of triterpenes

Circulating AGEs could be derived from the diet or from endogenous generation. Pentosidine and furosine can be detected in many foods [89,90]. Foods cooked by baking or deep frying also contain high amounts of AGEs [90,91]. In order to reduce circulating levels of AGEs from exogenous sources, the consumption of such foods rich in glycative products should be avoided.

Endogenous AGEs could be formed between reducing the sugars and amino acids present in the blood and/or tissues. Unfortunately, there is a large pool of reducing sugars and amino acids in the human body, and decreasing the intake of reducing sugars and amino acids may not be a good idea because the limitation might impair nutritional status. The alternative is to ingest other natural compound(s) with anti-glycative activities to interfere with AGE formation and/or mediate AGE metabolism.

An *in vitro* inhibitory effect of triterpenes such as astragalosides upon the formation of AGEs such as CML has been reported [92–94]. The results of these studies suggest that triterpenes can halt the interactions between reducing sugars and amino acids, reducing AGE formation and alleviating glycative stress via a nonenzymatic action. Both oxidative and inflammatory stress favor the glycation process; the anti-oxidative and anti-inflammatory effects of several triterpenes, such as ursolic acid and erythrodiol, have already been demonstrated [95–97]. It is quite possible that these triterpenes indirectly attenuate glycation via mitigating oxidative and inflammatory stress.

On the other hand, it is reported that oleanolic and ursolic acid can mediate the activity and/or expression of aldose reductase, SDH, and GLI [98,99]. These authors have indicated that suppression caused by aldose reductase and SDH decreases endogenous AGE generation as well as glycative stress. By upregulating GLI, the metabolism of AGEs is facilitated, and the accumulation of AGEs in the circulation and tissues diminished.

Obviously, the clinical application of any agent with an ability to mediate the enzymes involved in the polyol pathway will be highly beneficial, making it worthwhile to investigate the effect of other triterpenes on the activity and expression of these enzymes. So far, less information is available regarding the impact of triterpenes on RAGE expression and AGE–RAGE interaction. It is clear that suppressing RAGE or interrupting the AGE–RAGE interaction can more effectively mitigate glycative damage and/or retard pathological progression. The exploration of new natural agents and their modes of action is warranted in order to fight glycation-associated disease.

7.1. Blood–brain barrier

One challenge of research focused on triterpenes is their bioavailability. The development of any pharmacological substance acting against Alzheimer's disease has to consider whether this agent can pass through the blood–brain barrier, a tightly packed layer of endothelial cells that surrounds the brain to block high-molecular weight molecules from entering it. The blood–brain barrier not only impedes the influx of intravascular substances from the blood to the brain, but also regulates the transport of substances from blood to brain or from brain to blood via several transport systems such as carrier-mediated transport, active efflux transport, and receptor-mediated transport [100,101]. The blood–brain barrier is vital to brain A β homeostasis and regulates A β transport [102]. Triterpenes with an ability to pass through the blood–brain barrier can provide greater antiglycative potential in the prevention and/or therapy of Alzheimer's disease.

8. Conclusion

Triterpenes are compounds that are naturally present in many plant foods. Based on their marked action against AGE formation, their antioxidative and anti-inflammatory activities, and their regulatory effects on aldose reductase and GLI, these agents may be of benefit in the prevention of and therapy for glycation-associated diseases such as diabetes mellitus and Alzheimer's disease. Future study must probe the effects of triterpenes upon RAGEs, AGE–RAGE interaction, and penetration of the blood–brain barrier.

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Review article

DNA microarray analysis as a tool to investigate the therapeutic mechanisms and drug development of Chinese medicinal herbs

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ABSTRACT

Chinese herbal medicines have been used for the treatment of various diseases for centuries. Although several herbal formulas and herbal components have shown therapeutic potential, the active components and the molecular mechanisms mediating the effects of said formulas remain to be discovered. Microarray analysis has become a widely used tool for the generation of gene expression data on a genome-wide scale. This paper discusses the application of whole genome expression profiling as a tool to investigate the molecular mechanisms governing the therapeutic effects of traditional Chinese medicine. This review also highlights how data derived from DNA microarray analysis can be used to screen for drug targets of various herbal drugs, to predict the therapeutic potential of herbal drugs, to analyze the safety of drugs in the preclinical stage of drug development, and to establish a modern definition of traditional Chinese medicine.

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1. Introduction

Systems biology serves as a translational platform between traditional Chinese medicine and modern science. In this study, we review the technology behind whole genome expression profiling and discuss the biomedical application of the technique to the study of Chinese medicinal herbs.

2. Technology behind genome expression profiling

2.1. Development of whole genome expression profiling

In 1995, Schena and colleagues[1] at Stanford University in Palo Alto, CA, USA, published the first paper on the use of

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complementary DNA (cDNA) microarray probes printed in a two-dimensional grid onto glass slides. They showed that their high-capacity system could simultaneously monitor the expression of many genes. Microarrays prepared by high-speed robotic printing of complementary DNAs on glass are used for measurements of quantitative expression of corresponding genes. Because of the small format and the high density of arrays, hybridization volumes of less than two microliters can be used, enabling the detection of rare transcripts in probe mixtures derived from two micrograms of total cellular messenger RNA (mRNA). Two-color fluorescence hybridization is then used to simultaneously visualize differentially expressed genes. In 1996, Affymetrix began to market commercially available DNA chips. Various microarray experimental platforms have been developed since then.

2.2. Commonly used microarray platforms

Three different types of microarray platforms are commonly used: spotted cDNAs, spotted oligonucleotides, and Affymetrix arrays (Affymetrix, Santa Clara, CA, USA) [2].

Spotted cDNA arrays typically use sets of specific cDNA plasmids in gridded liquid. The inserts of each clone are typically amplified by polymerase chain reaction, and a few pico liters are physically spotted onto glass slides by liquid-handling robots. Spotted cDNA arrays are only used in academic centers because of their flexibility and relatively low cost.

Spotted oligonucleotide arrays are also built on glass slides by liquid-handling robots; however, the input solution comprises synthetic oligonucleotide (often 60–70 mer) rather than plasmids. Most of the process is automated, leading to less sample mix-up and less sample dropout. Disadvantages of spotted oligonucleotides include the relatively high cost of synthesizing large numbers of large oligonucleotides and the nonrenewable nature of the resource. Nonetheless, spotted oligonucleotide arrays are still widely used.

Affymetrix GeneChips (Affymetrix, Santa Clara, CA, USA) are factory designed and synthesized. Design is done using software to choose a series of 11- to 25-mer probes from the 3-foot end of each transcript or predicted transcript in the genome. Synthesis of arrays is done using light-activated chemistry and photolithography methods. Spotted oligonucleotides and Affymetrix arrays have superseded the use of

spotted cDNAs. The manufacturers of commonly used DNA microarray platforms are listed in Table 1.

2.3. Limitations and standardization of microarray platforms

DNA microarrays enable researchers to simultaneously monitor the expression of thousands of genes. However, the current technology has several limitations. The major problems are sensitivity, accuracy, specificity, and reproducibility of microarray results. Studies have shown that, for relatively abundant transcripts, the existence and direction, but not the magnitude, of expression changes can be reliably detected [3]. However, accurate measurements of absolute expression levels and the reliable detection of low abundance genes are difficult to achieve. The main problems seem to be the suboptimal design or choice of probes and some incorrect probe annotations. Marshall [4] compared the reliability of numerous array platforms, including the Affymetrix GeneChip, the Agilent array (Agilent Technologies, Santa Clara, CA, USA) and the Amersham array systems (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and found that more than one-half of the variability observed in the results was attributable to differences in the microarray platforms themselves. Efforts to standardize microarray data have been underway for some time and include the standardization of sample preparation, RNA isolation, cDNA synthesis, hybridization analysis, and quality control checkpoints to ensure reproducibility of data. For example, quality control criteria for RNA isolation include yield, purity, and integrity. An RNA integrity number greater than eight indicates that the RNA sample is suitable for cDNA synthesis. The criteria for cDNA labeling include concentration and incorporation efficiency. An incorporation efficiency of 15 labeled nucleotides per 1000 cDNA nucleotides indicates that cDNA labeling is suitable for hybridization. The gene expression profile obtained using standardized protocols can yield data that are consistent between laboratories and are intrinsically comparable [5].

Use of identical microarray chips and identical protocols would minimize the efforts made by researchers to integrate expression data, thereby allowing for the information embedded in these data to be maximally explored. In 2004, the Microarray and Gene Expression Data (MGED) society wrote an open letter to scientific journals proposing standards for

Table 1 – Manufacturers of DNA microarray platforms.

Manufacturer	Location	Website
Affymetrix	Santa Clara, CA, USA	www.affymetrix.com
Agilent Technologies	Santa Clara, CA, USA	www.agilent.com
Expression Analysis	Durham, NC, USA	www.expressionanalysis.com
Jivan Biologics	Larkspur, CA, USA	www.jivanbio.com
Marligen Biosciences	Ijamsville, MD, USA	www.marligen.com
NanoString Technologies	Seattle, WA, USA	www.nanosttring.com
NimbleGen	Madison, WI, USA	www.nimblegen.com
Oxford Gene Technology	Oxford, UK	www.ogt.uk
PerkinElmer	Waltham, MA, USA	www.perkinelmer.com
Phalanx Biotech Group	Hsinchu, Taiwan	www.phalanxbiotech.com

publication. The MGED society suggested that journals require submission of microarray data to one of two public repositories: Gene Expression Omnibus (GEO) or ArrayExpress. Moreover, they stated that authors should provide a checklist of variables and supply the checklist as supplementary information at the time of submission. Other members of the microarray community welcomed these steps, in particular Brazma and colleagues [6], who proposed the Minimum Information About a Microarray Experiment (MIAME), a guideline that describes the minimum information required to ensure that microarray data can be easily interpreted. The standardization of global gene expression data will make microarray data much more useful and accessible.

In summary, DNA microarray technology has evolved rapidly since its introduction in 1995. Although certain limitations of the current technology exist and have become more apparent during the past couple of years, the ability of microarrays to monitor the expression of thousands of genes simultaneously is unsurpassed [3].

3. Application of whole genome expression profiling to traditional Chinese medicine studies

Whole genome expression profiling can be applied to study the biomedical effects of Chinese medicinal herbs. Extracts prepared from medicinal plants and other natural sources contain a variety of molecules with potent biological activities. Unfortunately, it is often difficult to analyze the biologic activities of these extracts because of their complex nature and the possible interaction of their components. Genome-wide expression monitoring with high-density microarrays provides a simple way to test the biochemical effects of herbs, thereby gaining insight into their potential beneficial effects and negative side effects. DNA microarray has been used to evaluate the toxicity of novel drug candidates and to identify disease targets for drug development. Additionally, the therapeutic efficacy of a given drug can be predicted on the basis of gene expression patterns *in vitro*.

3.1. Evaluation of biologic activity and mechanisms of Chinese herbs

Microarray data have been used to characterize the biologic activities and mechanisms of action of herbal formulae or herbal compounds. For example, PC-SPES is a dietary supplement comprised of extracts from eight different herbs: *Scutellaria baicalensis*, *Glycyrrhiza glabra*, *Ganoderma lucidum*, *Isatis indigotica*, *Panax pseudo-ginseng*, *Dendranthema morifolium*, *Rabdosia rebescens*, and *Serenoa repens*. PC-SPES is also used as an alternative therapy by patients with prostate carcinoma [7–9]. The gene expression profile in cultured cells that have been exposed to PC-SPES shows differential expression of genes involved in modulating cell cycle, cell structure, and androgen response, indicating that alteration of some of those genes may be responsible for PC-SPES-mediated cytotoxicity [10]. Yukmijihwang-tang (YMJ), also known as LiuWei Dihuang Wang, is composed of six different medicinal herbs, including *Rehmannis radix*, *Radix dioscoreae*, *Fructus corni*, *Poria*, *Cortex moutan*, and *Radix alismatis*. YMJ has

been widely used for centuries as an antiaging herbal formula in Asian countries [11]. Microarray data indicate that YMJ enhances memory retention by inducing several genes that are involved in protecting neuronal cells, enhancing cell proliferation, and stimulating neurite growth [12]. *Pinelliae Rhizoma* extract (PRe) is used to treat cough and asthma. However, the mechanism by which PRe exerts its effect on psychological disorders has not been studied. Kim and coworkers [13] used microarray to analyze the effect of PRe in mice exposed to psychological stress. They found that the expression of most genes that are altered in response to psychological stress is restored to normal levels in PRe-treated mice, with recovery rate of 81.5% for up-regulated genes and 85.2% for down-regulated genes. When the interaction network was analyzed, the recovery rate of the core node genes (46 up- and 29 down-regulated genes) in PRe-treated mice was over 95%, indicating that those genes may be the effective targets of PRe. Curcumin, a major chemical component of *Curcuma longa*, is used as a spice to give a specific flavor and yellow color to curry. It is also used as a cosmetic agent and in some medical preparations [14]. Curcumin displays anticarcinogenic properties in animals [15,16]. Microarray-based gene expression patterns indicate that, in addition to anticarcinogenic effects, curcumin may be an effective anti-metastatic agent via the regulation of expression of certain genes [17]. Aristolochic acid (AA), the major constituent of *Aristolochia* species, is associated with nephritis and renal cancer [18–20]. Microarray and network analysis have shown that most AA-altered genes are connected with nuclear factor- κ B (NF- κ B), suggesting that NF- κ B plays a critical role in the pathogenesis of AA-induced renal diseases [21]. Extracts prepared from medicinal plants and other natural sources contain a variety of molecules with potent biological activities; the aforementioned studies suggest that genome-wide expression monitoring with high-density microarrays is an effective method for analyzing the biologic activities of those extracts.

3.2. Establishing a modern definition of traditional Chinese medicine

Chinese herbal formulas consist of several herbal components. However, the mechanisms of action of most Chinese herbal formulas and the relationship between formulae and their components remain to be elucidated. The putative mechanism of San-Huang-Xie-Xin-Tang (SHXXT) and the relationship between SHXXT and its herbal components were analyzed in our laboratory using a microarray technique [22]. Gene-set enrichment analysis indicated that SHXXT and its components displayed a unique anti-proliferation pattern involving p53 and DNA damage signaling pathways in HepG2 cells. Network analysis showed that SHXXT-affected genes were regulated by p53. In addition, clustering analysis showed that *Rhizoma coptis*, the principal herb in SHXXT, shared a similar gene expression profile with SHXXT. These findings indicate that *R coptis* is the principal herb in the herbal combination SHXXT (Fig. 1). To the best of our knowledge, this was the first study to reveal the relationship between a traditional Chinese medicine formula and its

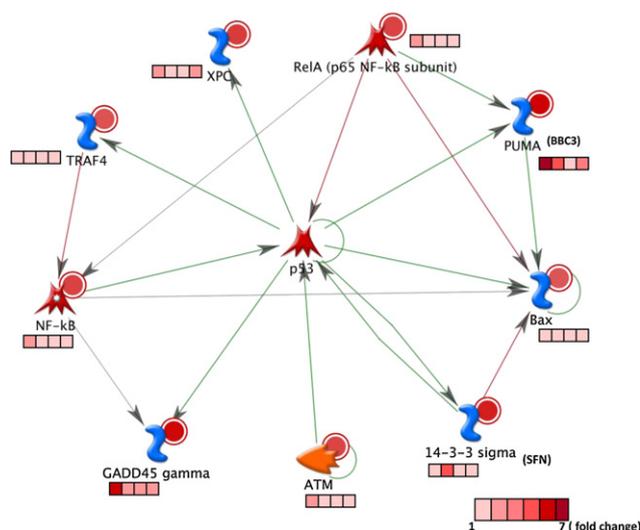


Fig. 1 – Network analysis of SHXXT-regulated genes. We selected the target genes that are regulated by p53 from <http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=searchTFGeneForm> (Cold Spring Harbor Laboratory, NY, USA). To estimate the overall regulatory effect of SHXXT on these target genes, we used the 'geneSetTest' function implemented in the R program (<http://www.r-project.org>) of the Limma package (<http://www.bioconductor.org>) to compare the absolute t-statistic values for these target genes with those for all genes. These target genes were then combined with the differentially expressed genes, which belonged to the Gene Ontology (GO) category 'regulation of biological process,' to investigate their relationship with p53. We used the MetaCore (GeneGo, St. Joseph, MI, USA) Analytical suite to construct the interaction networks between p53-downstream genes and part of the differentially expressed genes. The fold changes in gene expression in SHXXT-, *Rheum officinale*-, *Coptis chinensis*-, and *Radix scutellariae*-treated cells, respectively, are shown at the bottom. SHXXT = San-Huang-Xie-Xin-Tang.

herbal components using microarray and bioinformatics approaches.

3.3. Evaluation of drug safety

Many natural products, including polyphenols, terpenes, alkaloids, flavonoids, and phenolics, are potential therapeutic agents [23]. Previous studies have shown that phytochemicals affect the expression levels of genes involved in drug metabolism [24]. To evaluate whether phytochemicals affect drug metabolism, we analyzed the expression levels of genes encoding phase I and II drug metabolism enzymes in cells exposed to anthraquinone compounds. Phase I drug metabolism genes encode alcohol dehydrogenases, aldehyde dehydrogenases, and cytochrome P450 families, while phase II drug metabolism genes encode glutathione S-transferases, sulfotransferase, and UDP glucuronosyltransferase (UGT) families. We found that genes involved in phase II drug

metabolism were down regulated during anthraquinone compound treatment (Table 2). These data suggest that anthraquinone compounds may slow down the excretion of drugs, thereby increasing the half-life of drugs [25].

3.4. Prediction of the therapeutic potential of medicinal herbs

Vanillin has been shown to inhibit mutagenesis and to suppress the invasion and migration of cancer cells [26]. In our previous studies, microarray data and gene ontology investigation indicated that vanillin affected clusters of genes involved in the cell cycle and apoptosis. Network analysis indicated that Fos might play a central role in the regulation of the gene expression network. Results from reporter assay and Western blot further indicated that vanillin inhibited Fos-related transcription factor activator protein 1 (AP-1) activity via an extracellular signal-regulated kinase pathway. Our data suggest that vanillin exhibits anticancer potential by regulating cell cycle and apoptosis and that its regulation may involve the suppression of AP-1 (Fig. 2) [27,28].

AA belongs to a family of compounds found in the Aristolochiaceae family of plants. *Aristolochia* species in particular have been used for centuries in Asia for medicinal purposes. Although AA is bioactivated in both the kidney and liver, it only induces diseases and tumors in kidney and urinary tract in human and rodents [18]. To elucidate why AA displays such tissue-specific carcinogenicity, Chen and colleagues [29] examined gene expression profiles in kidney and liver of rats treated with carcinogenic doses of AA. They found that the biologic processes related to defense response, apoptosis, and immune responses were significantly altered by AA exposure in kidney but not in liver. These findings may explain why AA induces tumors in the kidney but not in the liver [29].

Ginkgo biloba extract EGb 761 is widely used to treat neurologic disorders [30,31]. In a previous study, we tested the effects of EGb761 on the transcriptional profile of mouse genes.

Table 2 – Analysis of expression levels of genes associated with drug metabolism.^a

Gene symbol	log ₂ ratio	Standard deviation
UGT1A10	-0.23	1.89
UGT2A1	-0.28	1.15
UGT2B11	-1.70	5.42
UGT2B15	-0.85	0.69
UGT2B4	-0.34	0.29
UGT2B7	-0.94	0.79

^a Results were obtained from three independent assays. A total of 219 genes associated with drug metabolism were selected from 'The Pharmacogenetics and Pharmacogenomics Knowledge Base' website (<https://www.pharmgkb.org/index.jsp>). Among these genes, we analyzed the expression levels of phase I drug metabolism genes, including alcohol dehydrogenases, aldehyde dehydrogenases, and cytochrome P450 genes, and phase II drug metabolism genes, including glutathione S-transferases, sulfotransferase, and UGT genes. The log₂ ratio and standard deviation of UGT genes are shown.

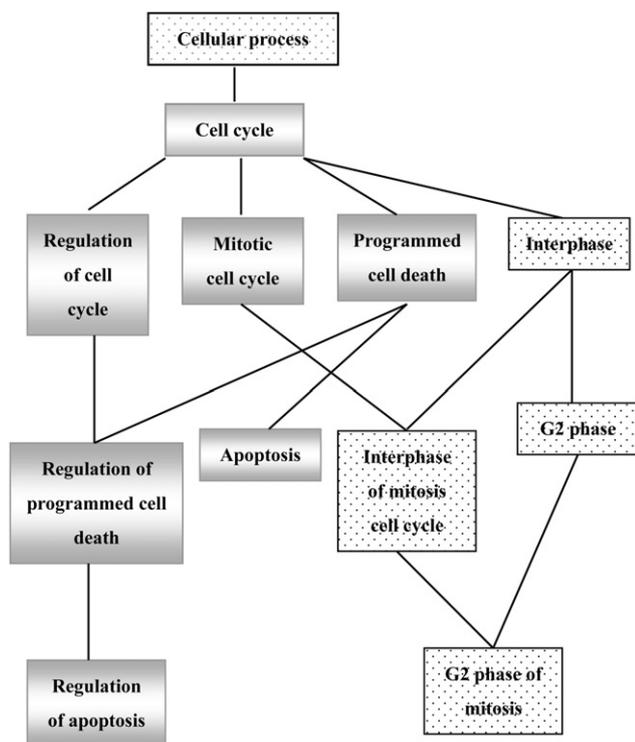


Fig. 2 – Ontology analysis of vanillin-affected genes. Vanillin-affected genes were analyzed by GO on the Gene Ontology Tree Machine website (<http://bioinfo.vanderbilt.edu/gotm/>), a web-based and tree-based data-mining environment for gene sets. We used the WebGestalt tool to test significant GO terms, and the significant GO terms are shown.

A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that EGb761 affected [32] the neuroactive ligand-receptor interaction pathway in brain. A total of 53 genes were significantly affected, and EGb761 up-regulated a subgroup of dopamine receptors, especially dopamine receptor 1a. Immunohistochemical staining confirmed the microarray data. The finding that *G biloba* treatment resulted in increased expression of dopamine receptor 1 in brain may explain why EGb761 is an effective treatment of neurologic disorders such as Parkinson disease (Table 3) [32].

3.5. New drug development

Whole genome expression profiling has also been used for the development of new drug [33–36]. Large-scale gene expression analyses of toxin-treated cells and animals have yielded information on the toxic potential of novel drug candidates [37–41]. In addition, gene expression profiles have been applied to identify the disease targets for drug development [42]. Moreover, the therapeutic efficacy of drugs can be predicted on the basis of gene expression signatures *in vitro* [43,44].

A number of studies have shown that DNA microarray data have potential utility in drug discovery and drug target validation [44,45]. For example, Lamb and others [46] analyzed the expression profiles of 164 small molecules with DNA

Table 3 – Neuroactive ligand-receptor interaction of EGb761-affected genes in the brain and kidney.^a

	Observed (total)	p value
Brain	53 (237)	4.13×10^6
Kidney	0 (237)	0.536

a Fluorescent RNA targets were prepared from 5 µg of total RNA using a MessageAMP aRNA kit (Ambion, Austin, TX, USA) and Cy5 dye (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Fluorescent targets were hybridized to Mouse OneArray Whole Genome DNA microarray (Phalanx Biotech Group, Hsinchu, Taiwan). After an overnight hybridization at 50 °C, nonspecific binding targets were washed away and the slides were scanned with an Axon 4000 scanner (Molecular Devices, Sunnyvale, CA, USA). The Cy5 fluorescent intensity of each spot was analyzed by genePix 4.1 software (Molecular Devices, Sunnyvale, CA, USA). The signal intensity of each spot was corrected by subtracting background signals. Spots with a signal-to-noise ratio of less than 0 as well as those of control probes were filtered. Spots that passed these criteria were normalized by the R program (<http://www.r-project.org>) of the Limma package (<http://www.bioconductor.org>) using quantile normalization [50]. The p value of each gene was calculated by t-statistics using the Differential Expression (T-Rex) tool in the Gene Expression Pattern Analysis Suite (<http://gepas3.bioinfo.cipf.es/>) [51]. These differentially expressed genes ($p < 0.01$) were further analyzed by the KEGG pathway [52]. Pathway enrichment analysis was performed on the WebGestalt website (<http://bioinfo.vanderbilt.edu/webgestalt/login.php>) by the hypergeometric test, which is used to evaluate the p value of the over-represented pathways. The neuroactive ligand-receptor interaction pathway is shown.

microarray. By comparing the genomic signatures of drug candidates or the disease state to this resource, the authors found that it was possible to identify potential mechanisms of action, confirm previous applications of known drugs, and identify additional potential uses for known drugs [46]. Their results demonstrate that the establishment of a huge gene expression database would be useful for finding connections among small molecules that share similar mechanisms of action and that are involved in similar physiologic processes, thereby allowing for the development of disease-fighting drugs.

Several studies have indicated similarities between gene expression profiles and therapeutic activities [46–48]. In addition, genome-wide expression monitoring with high-density microarrays provides a simple way to test biochemical effects of herbs, thereby gaining insights into their potential beneficial effects and negative adverse events [30]. In a recent study, we applied DNA microarray to analyze biologic events, predict the therapeutic potential of drugs, and evaluate the safety of herbal formulas [49]. For seven consecutive days, mice were administered orally with 15 of the most widely used Chinese herbal formulae listed in the Taiwan National Health Insurance Database, and the gene expression profiles in liver or kidney were analyzed by DNA microarray. Our data showed that most formulas altered metabolic pathways, such as the pathways governing glutathione metabolism and oxidative phosphorylation, and regulatory pathways, such as that regulate antigen processing and presentation and insulin-like growth factor signaling. By comparing the gene expression signatures of formulas with those of disease states or drugs, we found that response of mice to formula might be

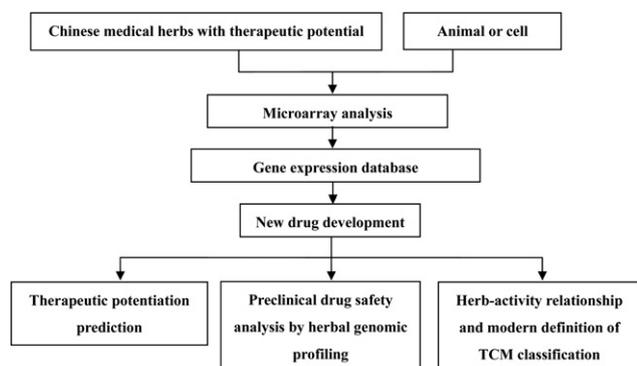


Fig. 3 – Paradigm for the application of whole genome expression profiling as a tool for therapeutic prediction, drug development, and safety evaluation of Chinese herbal medicines.

associated with disease state in said mice, such as metabolic or cardiovascular diseases. Moreover, most formulas altered the expression levels of cytochrome P450, glutathione S-transferase, and UGT genes, suggesting that caution should be paid to possible drug interactions of these formulas. Furthermore, the similarities in gene expression profiles between formulas and toxic chemicals were low in kidney, suggesting that these formulas might not induce nephrotoxicity in mice. This transcriptomic platform will not only help researchers understand the therapeutic mechanisms associated with herbal formulas and gene interactions, but will also help researchers develop novel disease-fighting drugs (Fig. 3).

4. Conclusion

Whole genome expression profiling can provide a basis for investigating the molecular mechanisms governing the therapeutic effects of Chinese herbal medicines and can be used to elucidate the biology of disease progression, identify potential therapeutic targets, and facilitate the development of traditional Chinese medicine-derived biopharmaceutical products.

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Review article

Garlic: Health benefits and actions

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ABSTRACT

Recent years have seen an increasing emphasis on foods and food components in disease prevention. Garlic (*Allium sativum* L.), one of the best-researched herbal remedies, holds a unique position in history, traditionally employed to treat infection, colds, diabetes, heart disease, and a host of other disorders. Clinically, it has been evaluated for lowering blood pressure, cholesterol, and glucose concentration, as well as for the prevention of arteriosclerosis and cancer. Epidemiologically, garlic consumption inversely correlates with the risk of oral, stomach, esophageal, colon, and prostate cancers. In addition, the biological activities of garlic, including antibacterial, antithrombotic, antioxidant, immunomodulatory, and antidiabetic actions and modulation of drug metabolism, have been extensively investigated. Here, we briefly summarize the recent findings on garlic and its sulfur-containing compounds in preventing cardiovascular diseases and cancer, along with its modulation of drug-metabolizing enzymes and membrane transporter activities. Finally, garlic safety and drug interaction are discussed.

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1. Chemicals and bioactive components of garlic

The unique flavor and health-promoting functions of garlic are generally attributed to its rich content of sulfur-containing compounds, i.e., alliin, γ -glutamylcysteine, and their derivatives. Processing a fresh and intact garlic bulb by crushing, grinding, or cutting induces the release of the vacuolar enzyme alliinase, which very quickly catalyzes alliin to allicin [1,2]. Allicin is, however, a very unstable compound, soon rearranged and transformed into numerous lipid-soluble sulfur-containing byproducts, mostly diallyl disulfide (DADS) but also diallyl sulfide (DAS), diallyl trisulfide (DATS), allylmethyl trisulfide, and diallyl tetrasulfide [1]. These compounds emit strong odors and are kept in garlic oil. Under

appropriate conditions, allicin can be transformed into other lipid-soluble products such as ajoene and vinylthiophene. Ajoene is identified as a principal product in garlic extract prepared by using ether as a solvent [3].

In contrast to the processes stated above, alternative pathways occur in case of different means of garlic storage. An aging process caused by immersing intact or sliced raw garlic in alcohol or vinegar for several months results in sulfur-containing compounds in this aged product dramatically different from those found in garlic oil. This aging process is supposed to cause considerable loss of allicin. Meanwhile, with the action of γ -glutamyltranspeptidase, the other sulfur-containing precursor γ -glutamylcysteine is transformed into water-soluble S-allylcysteine (SAC) and subsequent metabolites, including S-allylmercaptocysteine (SAMC)

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and S-methylcysteine. Unlike the oily sulfur compounds, these water-soluble compounds are odorless but have a more delicate and less characteristic flavor [4].

In addition to sulfur-containing compounds as stated above, garlic is also rich in trace elements. In raw garlic, the amounts of zinc, manganese, copper, selenium, and iodine in 100 g fresh weight of garlic are 556.1, 446.9, 143.3, 5.5 and 2.5 μg , respectively [5]. The protein content of raw garlic ranges from 2.6% to 3.0%, depending on the variety of garlic. The average content of free amino acids is 2.13%. Concentrations of dietary fiber and total tocopherols in raw garlic are 2310 and 103.1 mg/100 g fresh weight, respectively. Ascorbic and total polyphenols levels are 73.6 and 1.9 mg in 100 g dry weight [6]. Over 70 fatty acids have been determined, with linoleic (46–53%), palmitic (20–23%), oleic (4–13%), and α -linolenic (3–7%) acids being most abundant, accounting for 80% of the total lipids [7].

2. Garlic preparations and supplements

Because of the complex chemistry of garlic, variations in processing methods can yield quite different preparations. Raw garlic homogenate, the major preparation of garlic, is the most common form of garlic consumed, and allicin the main compound present in fresh raw garlic homogenate. There are currently many garlic supplements on the market, garlic oil, powder, and aged extract being the most popular.

Garlic oil is mostly obtained by steam distillation, with a yield around 2.5–3.0 g/kg fresh garlic. In garlic oil, DAS, DADS, and DATS, differing in their number of sulfur atoms, and allylmethyl sulfide are the four most abundant volatile allyl sulfides [8]. Garlic powder is generated from garlic cloves that have been dehydrated and pulverized into powder. Due to deactivation of alliinase by heat during dehydration, the major active constituents of garlic powder are alliin and a small amount of oil-soluble sulfur compounds.

To overcome the strong and irritant odor and the possible side effects of raw garlic and garlic oil, including growth retardation and destruction of gut microflora, an “aging” process has been applied to garlic. Aged garlic is prepared by soaking whole or sliced garlic cloves in alcohol or vinegar solution for 6–20 months, which removes the several irritant sulfur-containing compounds and also stabilizes some unstable compounds such as allicin [4,9]. The water-soluble compounds SAC and SAMC are the most abundant sulfur-containing components, and trace amounts of oil-soluble allyl sulfides exist in aged garlic. In contrast to odoriferous garlic oil and raw garlic, garlic powder and odorless aged garlic product are currently the most popular garlic supplements on the market.

3. Garlic and cardiovascular disorders

Cardiovascular disease is a common human chronic disease, and it is the leading cause of morbidity and mortality in the USA [10]. The etiology of cardiovascular disorders is multifactorial, with, for example, hypercholesterolemia, hypertension, diabetes mellitus, heredity, hyperhomocysteinemia,

increase in oxidative damage, and smoking as well-demonstrated risk factors [11].

Due to the accompanying inflammation in the plaque, cardiovascular disorders are regarded as chronic inflammation-related diseases [11]. The increased production and release of inflammatory mediators, such as reactive oxygen species (ROSs), tumor necrosis factor alpha (TNF- α), interleukin 6 (IL-6), arachidonic acid metabolites, and nitric oxide is noted in the atherosclerotic lesion [12]. This results in a greater expression of adhesion molecules, including P-selectin, E-selectin, vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), and monocyte chemoattractant protein-1 on the cell surfaces of monocytes, leukocytes and vascular endothelial cells, which accelerates the adherence of monocytes and leukocytes to the vascular endothelium and their subsequent transmigration into the subendothelial space.

Within the intima, activated macrophages release ROSs, scavenge oxidized low-density lipoprotein (oxLDL), become foam cells, and lead to the development of the fatty streak in the early stage of atherosclerosis [13,14]. This explains why phytochemicals with anti-chronic inflammation, hypolipidemic, and antioxidative properties are thought to be capable of decreasing the incidence of atherosclerosis.

Garlic has been regarded as a potent antiatherogenic food [15]. Its lowering of blood cholesterol is believed largely due to a reduction in LDL-cholesterol [16,17], which may be due to inhibition of hepatic hydroxymethylglutaryl-CoA reductase activity by alliin and allicin [18]. Over the past decade, several intervention studies and systemic meta-analytic reviews have investigated the effectiveness and properties of garlic in preventing cardiovascular disease (Table 1).

A double-blind placebo-controlled randomized study including 51 patients with coronary conditions indicated that 12 months' treatment with 300 mg/d garlic powder significantly decreased the total cholesterol and LDL-cholesterol levels [19]. A reduction of 32.9 and 27.3 mg/dL in LDL-cholesterol resulting from the garlic was observed in men and women, respectively.

A similar reduction in total cholesterol and LDL-cholesterol, along with an increase in high-density lipoprotein-cholesterol, were also reported in hypercholesterolemic adults who were administered 10 g/d garlic extract for 4 months, 5 g/d raw garlic for 6 weeks, or 600 mg/d garlic powder for 12 weeks [20–22]. Oily macerate of garlic (1620 mg/day for 30 days) was found to significantly lower the levels of total cholesterol, LDL-cholesterol, and triacylglycerides in 70 hypertensive adults [23]. However, Burggraaf and colleagues reported that 12 weeks of 2.1 g/d garlic powder administration did not change the lipid profiles in overweight subjects with normal blood lipid levels [24].

A meta-analysis including 29 trials recently revealed that garlic supplementation markedly reduced blood total cholesterol levels (–0.19 mmol/L; 95% CI –0.33 to –0.06 mmol/L) and triacylglyceride levels (–0.11 mmol/L; 95% CI –0.19 to –0.06 mmol/L), but exhibited no significant effect on LDL- or high-density lipoprotein-cholesterol [25]. A similar reduction in total blood cholesterol and triglycerides has been reported in systemic reviews [26,27]. Inconsistent clinical evidence warrants more study before reaching convincing conclusions.

Table 1 – Effect of garlic supplementation on human cardiovascular disorders.

Preparation	Subjects/dose	Effect	Reference
Garlic powder	51 patients with CVD, 300 mg/d, 12 mo	↓ total cholesterol and LDL-C	[19]
	42 mildly hypercholesterolemic men, 600 mg/d, 12 wk	↓ total cholesterol and LDL-C ↑ HDL-C	[22]
	90 normolipidemic and overweight adults, 2.1 g/d, 12 wk	No changes in blood total cholesterol, LDL-C, and TG	[24]
Raw garlic	30 hypercholesterolemic adults, 5 g/d, 42 d	↓ total cholesterol and TG ↑ HDL-C	[21]
Garlic extract	23 hypercholesterolemic adults (13 with hypertension), approximately 10 g/d, 4 mo	↓ total cholesterol, LDL-C, and TG ↑ HDL ↓ SBP and DBP	[165]
Aged garlic extract	11 healthy adults, methionine- induced hyperhomocysteinemia, 4 mL/d, 6 wk	↑ NO and endothelium-derived hyperpolarizing factor	[166]
	65 patients with intermediate CVD risk, 250 mg/d co-administered with B vitamins (B ₁₂ , B ₆ , and folic acid) and L-arginine, 12 mo	↓ total cholesterol, LDL-C, and homocysteine ↑ HDL	[140]
Garlic oil	20 hypertensive patients, 250 mg/d, 2 mo	↓ SBP, DBP, and oxLDL	[13]
Oil-macerated garlic	70 hypertensive adults, 1620 mg/d, 30 d	↓ total cholesterol, LDL-C, and TG	[23]

CVD = cardiovascular disease; DBP = diastolic blood pressure; HDL-C = high-density lipoprotein-cholesterol; LDL-C = low-density lipoprotein-cholesterol; NO = nitric oxide; oxLDL = oxidized LDL; SBP = systolic blood pressure; TG = triglycerides.

Garlic is reported to prevent cardiovascular disease by multiple effects, one of which is inhibition of platelet aggregation. A single intravenous dose of aqueous extracts of garlic (10–100 mg/kg) dose-dependently inhibited blood thromboxane B₂ concentration in rabbits [28]. Maximum inhibition of thromboxane B₂ occurred 0.5 hours after injection and lasted until 6 hours afterwards. In another study, oral administration of aqueous extract of fresh garlic inhibited cyclooxygenase (COX) activity in rabbit platelets, resulting in a suppression of thromboxane formation and blood aggregation [29]. Similarly, in eight males (aged 40–50 years), the consumption of one crushed clove of garlic daily for 16 weeks resulted in an 80% reduction in serum thromboxane B₂ levels [30].

Alliin and alliin-derived thiosulfonates are recognized as major compounds responsible for the antithrombotic activity of garlic [31]. Besides an inhibition of COX activity, other possible mechanisms for garlic's inhibition of platelet aggregation include suppression of intraplatelet Ca²⁺ mobilization, an increase in cyclic AMP and cyclic GMP levels, an increase in platelet-derived nitric oxide production, and a reduction in platelet binding to fibrinogen [32].

Garlic's protection against cardiovascular diseases has been partly attributed to its potent anti-inflammatory activity [33]. The ethyl acetate-soluble fraction of garlic is proven to be effective in inhibiting nuclear factor κ B (NF- κ B) activation, as well as expression of COX-2 and inducible nitric oxide synthase in IL-3-dependent murine pro-B-cells Ba/F3 through the Toll-like receptor-dependent pathway [34]. Thiocremonon, a novel organosulfur compound of garlic, inhibits 12-O-tetradecanoylphorbol-13-acetate-induced ear edema in ICR mice, and carrageenan- and *Mycobacterium butyricum*-induced inflammatory and arthritic responses in the paws of Sprague-Dawley rats [35]. Garlic oil reportedly suppresses 1-chloro-2, 4-dinitrobenzene-induced contact hypersensitivity as determined by ear swelling [36].

After 30 days of administration of 600 mg/kg garlic powder, an increase in interferon- γ and a decrease in IL-4 in phytohemagglutinin-activated splenocytes were noted,

suggesting that garlic treatment may favor a T-helper type 2 cell or humoral immune response [37]. 1,2-Vinyldithiin was recently reported to significantly suppress IL-6 and monocyte chemoattractant protein-1 secretion by macrophage-secreted factors stimulated human preadipocytes isolated from the subcutaneous adipose tissue of nonobese young women [38]. DAS has been reported to prevent COX-2 upregulation and prostaglandin E₂ (PGE₂) secretion in primary human synovial fibroblasts and articular chondrocytes induced by IL-1 β and monosodium urate crystal, and ameliorates crystal-induced synovitis, potentially through the NF- κ B signaling pathway [39].

The presence of proinflammatory cytokines initiates numerous physiological changes in vessel walls, such as enhanced adhesion of leukocytes to the endothelium. A recent *in vitro* study indicated that the chloroform extract of aged black garlic attenuated TNF- α -induced VCAM-1 expression via an NF- κ B-dependent pathway in human umbilical vein endothelial cells (HUVEC), hence decreasing the adhesiveness of monocytes on endothelial cells [40]. In primary human coronary artery endothelial cells, aqueous extract of garlic (0.25–4.0 mg/mL) dose-dependently curbs ICAM-1 and VCAM-1 expression induced by IL-1 α [41]. When stimulated by oxLDL, DADS and DATS suppress VCAM-1 and E-selectin expression in HUVEC and the subsequent adhesion of HL-60 to endothelial cells [42].

Taken together, although solid clinical evidence that garlic's effect in protecting blood vessels can be attributed to its anti-inflammatory properties is lacking, the potent anti-inflammatory action of garlic and its sulfur-containing compounds obtained from *in vitro* and animal studies supports the potential value of garlic in preventing atherogenesis.

Evidence indicates that garlic also acts to maintain vascular tone and cardiac function. Experiments on laboratory animals and investigations of humans has proved that diets supplemented with garlic can restore endothelial functions. Alliin is believed to be the active component of raw garlic protecting coronary endothelial function and

vasoreactivity in pulmonary hypertensive rats [43]. Enhancement of nitric oxide synthase activity and greater nitric oxide production partly explained this hypotensive action.

Our recent work demonstrated that DADS and DATS protect the activity and protein expression of endothelial nitric oxide synthase in response to an oxLDL insult to the endothelial cells [44]; this is partly attributable to the mediation of phosphatidylinositol 3-kinase/protein kinase B signaling and prevention of eNOS degradation caused by DADS and DATS [44]. SAC supplementation reduces the incidence of stroke in stroke-prone spontaneously hypertensive rats [45], and lowers mortality and infarct size in a rat model of acute myocardial infarction induced by coronary artery ligation [46].

In an animal experiment inducing diabetes by streptozotocin, rats were orally administered 0–100 mg/kg/d garlic oil for consecutive 16 days; streptozotocin-induced cardiac contractile dysfunction and apoptosis were markedly improved by the garlic oil [47]. In a hypercholesterolemic animal experiment, rats were fed a 1.0% garlic- and 0.5% turmeric-supplemented diet for 10 weeks. Enhanced vasorelaxation in the aortic ring response to adenosine, acetylcholine, and isoproterenol, along with attenuation of the contractile response to 5-hydroxytryptamine, was seen in animals given the garlic- and turmeric-supplemented diet, thus lowering their blood pressure [48].

In a randomized, placebo-controlled, cross-over design involving 15 patients with angiographically proven coronary artery disease, brachial artery flow-mediated endothelium-dependent dilation was improved by aged garlic extract [49]. Similarly to aged garlic extract, garlic oil in a dose of 250 mg/d for 2 months demonstrably improved both systolic and diastolic blood pressure in 20 hypertensive patients [13].

4. Garlic and cancer

The past few decades have seen many epidemiological studies on the correlation between garlic consumption and incidence of cancer, from which an inverse relationship has emerged. Setiawan et al observed a negative dose–response relationship between the monthly intake of garlic and the risk of stomach cancer in Shanghai and Qingdao, China [50]. A recent study found an odds ratios among individuals with a high versus a low intake of garlic and onions that correlated with a starkly reduced risk of colorectal adenoma [51]. In persons who consume a high proportion of garlic, a decreased susceptibility to stomach and colon cancers has also been reported [52].

Based on the US Food and Drug Administration’s evidence-based review system for scientifically evaluating the risk of diverse types of cancer, 19 human studies revealed garlic’s antitumorigenic potential in stomach, colon, rectal, breast, lung, and endometrial cancers. Very limited evidence supports a relation between garlic consumption and reduced risk of colon, prostate, esophageal, larynx, oral, ovary, or renal cell cancer [53].

Several human intervention studies have plotted garlic’s anticarcinogenic traits. A preliminary double-blind, randomized clinical trial using high-dose aged garlic extract (2.4 mL/d) as the active treatment and low-dose aged garlic extract

(0.16 mL/d) as the control was performed involving 51 patients with colorectal adenomas/precancerous lesions of the large bowel [54]. After 12 months of treatment, 37 patients (19 in the active and 18 in control group) completed the study, the size and number of colon adenomas in the high-dose group being significantly lower ($p = 0.04$).

An earlier double-blind intervention study of 5033 subjects (2526 in the intervention and 2507 in the control group) was performed in China. A dose of 200 mg/d DATS in combination with 100 µg/d selenium was taken by the intervention group each month for 3 years. The results showed that DATS offered protection against gastric cancer for males [55]. In this study, it is interesting to note that no such protection occurred in females.

Numerous animal model studies found in the literature were carried out using either garlic extract or individual garlic-derived compounds. The development of aflatoxin B1- or diethylnitrosamine-induced liver cancer in rats was limited by fresh garlic [56] and garlic oil [57]; the latter also protected against ferric nitrilotriacetate-induced kidney cancer growth in rats [58]. DADS suppressed 7,12-dimethylbenzo[*a*]anthracene (DMBA)-induced rat mammary tumor [59]. DAS and DATS protected against DMBA-, phorbol ester-, and benzo[*a*]pyrene-induced skin tumorigenesis in mice [60–63]; DATS also inhibited the growth of PC-3 human prostate cancer xenografts in male nude mice [64]. Similarly, ajoene significantly inhibited B16/BL6 melanoma growth and metastasis to the lung in C57BL/L mice [65]. Aside from oil-soluble organosulfur compounds, water-soluble SAC inhibited the growth and malignant progression of highly metastatic human nonsmall-cell lung carcinoma in nude mice [66].

Although the precise mechanism of garlic’s anticancer efficacy is still not clear, molecular action such as regulation of cell proliferation, increase in tumor apoptosis, blocking of the cell cycle, inhibition of carcinogen activation, increase in phase II drug-metabolizing enzymes, enhanced antioxidation capacity, change in proteasome-dependent protein degradation, and modulation of immune response have been proposed and extensively probed in recent years (Table 2).

In many cancer cells, garlic organosulfur compounds display potential for suppressing the growth of cancer cells and producing cell cycle arrest. DAS increases the accumulation of sub-G1 DNA and the concomitant accumulation of cells in the G2/M phase in a dose-dependent manner in human anaplastic thyroid carcinoma cells [67], as well as in human colon cancer cells [68]. DAS, DADS, and DATS further exhibit differential effects in terms of lowering cyclin-dependent kinase-Cdk7 and

Table 2 – Mechanisms underlying the anti-cancer actions of garlic.

1. Induces apoptosis/arrests the cell cycle
2. Blocks invasion/metastasis
3. Suppresses cell proliferation
4. Inhibits activation of carcinogen
5. Enhances antioxidation
6. Decreases histone deacetylase activity
7. Interrupts tubulin polymerization
8. Changes proteasome activity

raising cyclin B1 protein levels in J5 human liver tumor cells, thus arresting the cells in the G2/M phase [69]. Among those lipid-soluble allyl sulfides, which differ in their number of sulfur atoms, DATS revealed a better growth inhibition of human melanoma A375 cells and skin basal cell carcinoma cells than was seen with DADS and DAS [70]. The induction of apoptosis and cell cycle arrest by garlic allyl sulfides have also been reported in different types of cancer cell, e.g., human lung adenocarcinoma [71], glioblastoma [72], prostate cancer [73,74], neuroblastoma [75], gastric cancer [76], bladder cancer [77], colon cancer [78], and mammary cancer [79].

Garlic organosulfur compounds resulting in cell cycle arrest and apoptosis can be linked to the modulation of several key elements in cellular signal transduction. It has been demonstrated that DATS-induced apoptosis of PC3 human prostate cancer cells involves c-Jun N-terminal kinase (JNK) and extracellular-signal-regulated kinase-mediated phosphorylation of Bcl-2 [80]. Inactivation of the Akt signaling pathway also likely plays a role in DATS-induced mitochondrial translocation of Bad and caspase-mediated apoptosis in PC3 and DU145 human prostate cancer cells [81]. Likewise, the DATS arrest of DU145 cells in G2/M phase is effected by hyperphosphorylation of Cdc25C [82] and delayed cdk1 translocation into the nucleus [83], as well as by oxidative modification of beta-tubulin in human colon cancer cells, which impedes the polymerization of tubulin [84].

A similar interruption of tubulin polymerization has been reported by treating SW480 and NIH3T3 fibroblasts with SAMC; this subsequently arrests the cells in mitosis and triggers the JNK1 and caspase-3 signaling pathways, leading to apoptosis [85]. In B16F-10 melanoma cells, DADS-induced apoptosis is attributed to the mitochondrion-dependent pathway by upregulating p53 and caspase-3 while down-regulating NF- κ B-mediated Bcl-2 activation [86]. Recently, both the extrinsic and intrinsic death pathways have been shown to be involved in allicin induction of apoptosis in gastric SGC-7901 cancer cells [87].

Garlic organosulfur compounds may also act epigenetically and exert anticarcinogenic activity. Histone acetylation notably increases in colonocytes isolated from DADS-treated rats and also in erythroleukemia cells from SAMC-treated mice, suggesting that histone deacetylase is the target of garlic allyl compounds [88, 89]. In addition to DADS, other garlic organosulfur compounds have been tested; allyl mercaptan, a metabolite of DADS, has been shown to exert the most potent inhibitory effect on histone acetylase in assays with HeLa nuclear extracts, lysates from human colon cancer cells, or purified human histone deacetylase-8 [78,90]. Allyl mercaptan inhibition of histone deacetylase activity results in increasing histone acetylation and Sp3 transcription factor binding to the p21WAF1 gene promoter region, elevating p21 expression and producing cell cycle arrest in HT29 colon cancer cells [90]. Enzyme kinetics assays further reveal an inhibition of allyl mercaptan on histone deacetylase via a competitive mechanism ($K_i = 24 \mu\text{M}$) [90].

Evidence indicates that tumor invasion and metastasis are suppressed in the presence of garlic and its organosulfur compounds. DATS administration retards the growth of PC-3 human prostate cancer xenograft cells in athymic mice [64], and prevents progression to invasive carcinoma and lung

metastasis in transgenic adenocarcinoma of mouse prostate (TRAMP) cells [91]. In *in vitro* experiments, the DADS-suppressed invasion of human prostate cancer LNCaP cells was attributed to an inhibition of matrix metalloproteinase-2 (MMP-2) and MMP-9 activity and to a tightening of the tight junctions [92].

Garlic's suppression of tumor invasion may also be attributed to its action on E-cadherin expression. SAC and SAMC restoration of E-cadherin expression suppresses the proliferation and invasion of prostate cancer cells [93]. This increase in E-cadherin expression and inhibition of cell proliferation are also noted in oral squamous cancer CAL 27 cells in the presence of SAC [94]. The invasive activities of SW480 and SW620 colorectal cancer cells are inhibited by aged garlic extract, whereas aged garlic extract has no effect on the invasion of HT29 cells, suggesting that the anti-invasive action of aged garlic extract is cancer cell-dependent [95]. In the presence of ajoene, human leukemia HL60 cells were arrested in the G2/M phase; both trypsin- and chymotrypsin-like proteasome catalytic activities were inhibited [96].

Taken together, most animal and cell studies suggest that garlic is a potent chemopreventive agent for several types of cancer, acting by inhibiting cell proliferation, arresting the cell cycle, inducing cell apoptosis, and blocking invasion and metastasis.

5. Garlic and the detoxification system

The cancer-chemopreventive effect of garlic organosulfur compounds is believed to be associated with the modulation of carcinogen metabolism, including effects on phase I and II detoxification enzymes. Phase I enzymes, mainly cytochrome P450 (CYP), detoxify a variety of endogenous and exogenous chemicals and activate many carcinogens [97]. Phase II enzymes catalyze the conjugation of phase I metabolites to various water-soluble molecules, such as glutathione (GSH), glucuronic acid, or sulfate, accelerating the metabolite excretion rate. The efficacy of DAS, DADS, and DATS in the transcriptional regulation of phase I and II detoxification enzyme expression positively correlates with the suppression of aflatoxin B1- and benzo[a]pyrene-induced liver and forestomach neoplastic formation in mice and rats [98,99].

Decreased 7,12-dimethylbenzo[a] anthracene-induced DNA adduct formation in rat mammary tissue by DADS [59], protection against benzo[a]pyrene-induced skin tumorigenesis and micronucleated reticulocyte formation in mice by DAS [63], and the suppression of aflatoxin B1-induced DNA breaks by allicin, DAS, DADS, and SAC in HepG2 cells [100] can also be explained by their effectiveness in modulating metabolism of carcinogens.

Among the CYP isozymes, a decrease in CYP2E1 activity and protein levels has been reported in rats fed a diet containing 5% garlic powder [101]. This downregulation of CYP2E1 by garlic suppresses the formation in rats of hepatic preneoplasia induced by diethylnitrosamine [102]. The formation of lycidamide, an active metabolite of acrylamide, in rat liver tissues falls because of the inhibition of CYP2E1 by DAS [103]. In addition to DAS, a reduction in the activity and expression of CYP2E1 results from garlic oil, DADS, and allyl methyl

sulfide [104,105]. In contrast to downregulation of CYP2E1, the activities of isozymes CYP1A1, CYP1A2, CYP2B1, and CYP3A2, as well as their protein and mRNA levels, are upregulated by garlic organosulfur compounds. Dosing rats with 200 mg/kg DAS and allyl methyl sulfide raises CYP1A1, CYP1A2, and CYP3A2 protein levels in a time-dependent manner, a rise being noted 24 hours after treatment [105]. A dose-dependent increase in rat liver CYP1A1, CYP2B1, and CYP3A1 activities and gene transcription is also caused by garlic oil (30–200 mg/kg body weight), probably from the combined effect of the three major allyl sulfides, DAS, DADS, and DATS, in the garlic oil [104,106,107].

Besides acting at the stage of gene transcription, the constituents of garlic may bind to CYP and change its enzyme activity. Using human liver microsomes, the activity of CYP2C9, CYP2C19, CYP3A4, CYP3A5, and CYP3A7, but not CYP2D6, is inhibited by incubation with garlic oil or extracts of fresh garlic, garlic powder, or aged garlic [108]. In the case of CYP2E1, diallyl sulfone and diallyl sulfoxide, metabolites of DAS, act as suicide substrates [109]; this inhibited CYP2E1 activity explains partly the action of DAS in attenuating acetaminophen-, carbon tetrachloride- and ischemic-reperfusion-induced toxicity in rat livers [110,111].

Phase II detoxification enzymes are known to play a key role in accelerating the excretion rate of numerous xenobiotics. Induction of phase II enzymes such as glutathione S-transferase (GST), epoxide hydrolase (EH), UDP-glucuronyl transferase (UGT), sulfotransferase, and NAD(P)H quinone oxidoreductase 1 (NQO1) is considered to be a crucial mechanism protecting organisms against chemical insults. It is thus reasonable to speculate that the induction potency of phytochemicals on phase II enzymes is associated with their efficacy in chemoprevention [112,113].

GST is among the most important phase II enzymes, its vital role in cancer prevention being supported by the finding that the incidence of 7,12-dimethylbenzanthracene-induced skin cancer was significantly elevated in the pi form of GST (GSTP)-null mice [114]. The increase in GST activity caused by garlic organosulfur compounds, including allyl methyl trisulfide, allyl methyl disulfide, DATS and DAS, strongly correlates with the inhibition of benzo[a]pyrene-induced forestomach neoplasia [115]. The effect of DAS, DADS, and DATS on the transcriptional regulation of GST enzyme expression is also positively correlated with their suppression of aflatoxin B1- and benzo[a]pyrene-induced liver and forestomach neoplastic formation [98,99]. The decrease in 7,12-dimethylbenz[a]anthracene-induced hamster buccal pouch carcinogenesis caused by SAC is also accompanied by enhanced GST activity and an increased GSH level [116].

Upregulation of phase II detoxification enzyme gene transcription involves a series of signaling pathways and transcriptional factors. Among these, the pivotal role of nuclear factor E2-related factor 2 (Nrf2) is well documented [112,117]. Activation and binding of Nrf2 to the promoter antioxidant response element/electrophile response element increases the transcription of GST, NQO1, UGT, and sulfotransferase. After treatment with garlic organosulfur compounds, Nrf2 nuclear translocation is increased and NQO1 expression is upregulated in HepG2 cells and in mice [118,119]. Increased hepatic NQO1 and GST activity helps to attenuate carbon

tetrachloride-induced liver injury in rats orally dosed with 500 $\mu\text{mol/kg}$ DATS for 5 consecutive days [120].

Dozens of organosulfur compounds have been identified in garlic products, and these appear to vary in their biological activity. It is interesting to ask what chemical characteristic of these garlic-derived compounds determines their potency to modulate drug metabolism. Evidence from structure–function relationship studies indicates that the number of both allyl groups and sulfur atoms in each organosulfur compound is a determining factor in the transcription of phase I and II enzymes.

With phase II detoxification enzymes, the number of sulfur atoms and allyl groups correlates positively with their potency to enhance gene transcription. DATS displays the best induction of NQO1, followed by DADS; DAS has only a minor effect [118]. Compared with DATS, DADS at a 10-fold higher dose (100 $\mu\text{mol/kg}$) increased the expression of GST and NQO1 in rat liver, whereas DAS did not [121]. Similar findings (DATS > DADS > DAS) have been reported for the induction of GSTP in rat liver [107,122]. An increase in UGT activity is also noted in HepG2 cells treated with DAS, dipropyl sulfide (DPS), and DADS; the effective concentration of DAS and DPS (50 μM) is much higher than that of DADS (2.5 μM) [123]. Feeding rats a diet containing 5% garlic powders markedly raises hepatic UGT activity in an alliin content-dependent manner [101].

A comprehensive study to examine the effect of the allyl sulfides DAS, DADS, DPS, and dipropyl disulfide (DPDS) on the hepatic, renal, intestinal, and pulmonary phase II enzymes GST, EH, UGT, and NQO, was performed by Guyonnet et al [124]. After orally dosing Wistar rats with 1 mmol/kg of each of the compounds for 4 consecutive days, DADS exerted the greatest inducibility of all phase II detoxification enzymes, with pulmonary EH activity unchanged. In addition, induction of NQO activity was seen only in DADS-treated animals. The increases in GST and EH activity caused by DAS, DPS, and DPDS were only noted in liver. Later, the increase in hepatic GST and NQO1 expression and activity by treatment with allyl-containing compounds was demonstrably greater than that by propyl-containing ones: i.e., DADS > DPDS and DATS > dipropyl trisulfide [118,125,126]. These findings suggest that garlic alk(en)yl sulfides have different potencies for inducing phase II enzymes, and that such induction is tissue-specific.

As for phase I enzymes, garlic components with an allyl side chain are better at inducing most CYP isozyme expressions than are propyl- or methyl-containing ones. However, the effect of sulfur atom number on CYP expression differs from that seen in their action on phase II enzymes: garlic compounds with a higher number of sulfur atoms displayed lower inducibility [105,107,115,122,126]. This discrepancy suggests that the regulatory mechanism of garlic organosulfur compounds on phase II and CYP isozymes is different, and the precise active mechanism warrants further study.

6. Garlic and antioxidation

Oxidative stress is a state wherein the balance between radicals generated and the free radical- or oxidant-scavenging capacity of the endogenous antioxidant system is disrupted. Oxidative stress is documented as being involved in the

pathogenesis of chronic diseases, including cardiovascular disorders and cancer. Hence, compounds with antioxidant properties may be used to prevent oxidative stress-mediated diseases [127].

Numerous studies have demonstrated garlic and its organosulfur compounds to be potent antioxidants by displaying radical-scavenging activity and modulating cellular antioxidant enzyme activity. Aged garlic extract and SAC have been shown to scavenge ROSs, protect endothelial cells from injury by oxLDL [128], and defend PC12 neuron cells from damage by hydrogen peroxide [129]. Garlic extract has been proven to be as effective as N-acetylcysteine in lessening ROS formation and GSH depletion induced by acetaminophen in rat primary hepatocytes [130]. Garlic pretreatment with 1 g/kg for 5 weeks reduces iron-catalyzed lipid peroxidation by lowering malondialdehyde levels in rat liver and colon, along with enhancing the status of the antioxidants [131]. Likewise, garlic reduces iron-induced cell proliferation and autophagy and protects mitochondrial membranes by lowering iron storage in the liver [131]. Garlic oil is effective in reducing tributyltin-induced oxidative damage in mice and human amniotic cells [132], as well as decreasing sodium nitrite-induced neurotoxicity in rats [133].

The aforementioned garlic protection against oxidant-induced damage can be attributed to an increase in the activities of superoxide dismutase, GSH reductase, γ -glutamate cysteine ligase, and GST, and also in GSH production [133–135]. Activated Nrf2 demonstrably plays a key role in garlic enhancement of both antioxidant defense capability and drug metabolism enzymes, as described above [134].

The antioxidant properties of garlic have been ascertained in animal models of disease. In the fructose-induced metabolic syndrome model in rats, aqueous garlic extract attenuates oxidative stress and prevents vascular remodeling by suppressing NAD(P)H-oxidase [136]. In db/db mice with type 2 diabetes, the consumption of 5% freeze-dried aged black garlic for 7 weeks significantly raised superoxide dismutase, catalase, and glutathione peroxidase activity and lessened lipid peroxidation in the liver [137]. In rats with streptozotocin induced-diabetes, garlic oil helps to normalize impaired antioxidant status [138]. Less neuron damage accompanied by increased levels of synaptophysin and presynaptic SNAP25 (synaptosomal-associated protein of 25 kDa, a member of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors, which play a key role in presynaptic vesicle fusion and exocytosis), have been seen in Alzheimer's APP transgenic (Tg) mice treated with a diet containing 2% aged garlic extract and its active component SAC (20 mg/kg diet) [129]. SAC also reduces lipid peroxidation and superoxide radical production, and elevates Cu-Zn-superoxide dismutase activity in 1-methyl-4-phenylpyridinium-induced parkinsonism in mice [139].

In recent years, several human intervention studies have examined the antioxidant potency of garlic in humans. Two months of garlic oil (250 mg/d) supplementation greatly reduced oxLDL and 8-iso-prostaglandin F₂ alpha levels, accompanied by a significant decline in both systolic and diastolic blood pressure, in hypertensive patients [13]. A similar fall in oxLDL production has been reported by dosing 70 hypertensive adults with 1620 mg/d oily macerate of garlic for 30 d [23]. In double-blind placebo-controlled study, plasma

oxLDL levels sharply fell in those administered 200 mg/d aged garlic extract combined with multi-micronutrients (folic acid, vitamins B₆ and B₁₂, and L-arginine) for 1 year, compared with controls [140]. Taken together, these results suggest that garlic has potent antioxidant activity in delaying the onset and development of cardiovascular disease, cancer, diabetes, and neurodegenerative diseases caused by an imbalance between free radical production and antioxidant defense.

7. Garlic and drug interaction

As stated above, garlic definitely modulates drug-metabolizing enzyme activity and membrane transporter levels in the liver, lung, kidney, and intestinal tissues. This raises some possibility that garlic supplementation could cause interactions between food and drugs and change the therapeutic efficacy of any drugs administered. To resolve this question, *in vitro* and *in vivo* experiments have multiplied in recent years. Increased toxicity of the human immunodeficiency virus protease inhibitor ritonavir has been reported in patients with AIDS who were co-administered garlic [141]. This can be explained, at least in part, by its inhibition of the excretion of ritonavir: allicin, for example, has been reported to inhibit the p-glycoprotein-mediated efflux of ritonavir in Caco-2 cells [142].

However, examining the permeability of rat jejunum and the Caco-2 cell monolayer has shown that aged garlic extract raises saquinavir and darunavir efflux [143]. A higher efflux of darunavir after addition of aged garlic extract has recently been noted in rat liver slices and isolated hepatocytes, whereas the efflux of saquinavir decreases [144]. The authors propose the competitive binding at the same binding sites and a positive cooperative effect with distinct binding places is likely to be responsible for garlic's effect in altering the efflux of saquinavir and darunavir, respectively [144]. Greater multidrug resistance-associated protein 2 expression is also reported in kidney brush-border membranes with DADS, but not with SAC [145].

For *in vivo* models, the pharmacokinetics of the diuretic drug hydrochlorothiazide in rats has been calculated following 3 weeks' administration of garlic homogenate. The results show that garlic homogenate increases the bioavailability and half-life of hydrochlorothiazide while decreasing its clearance [146]. The diuretic effect of hydrochlorothiazide is concomitantly increased by garlic homogenate. Enhancement of the antihypertensive and cardioprotective efficacy of captopril in rats by garlic homogenate and SAC was also reported in a later work [147].

In our laboratory, the effect of garlic oil on the pharmacokinetics of atorvastatin has recently been determined. Rats were orally administered 50 mg/kg of garlic oil for 5 consecutive days, and then a single dose of atorvastatin (10 mg/kg) was given. The rise of p-glycoprotein levels in liver and 3A1/2 activity in both intestinal and liver tissue appear to be negatively correlated to the area under the curve (AUC) of plasma concentration of atorvastatin and its metabolite 2-OH-atorvastatin (unpublished data).

It would also be intriguing to learn whether and how garlic supplementation interacted with drugs in humans and changed their therapeutic efficacy. To date, limited research

Table 3 – Garlic and drug interactions.

Preparation	Subjects/dose	Effect	Reference
Garlic extract	12 healthy males, 2 g/d (3.71 mg allicin/tablet), 2 wk	No changes in the pharmacokinetics of warfarin	[150]
Garlic extract	10 women with breast cancer, 600 mg/d (3.6 mg allicin/tablet), 17 d	No changes in the pharmacokinetics of docetaxel	[151]
Garlic extract	10 healthy males, 600 mg/d (12 mg γ -glutamyl- cysteine/4.8 mg alliin), 21 d	\uparrow duodenal p-glycoprotein level No change in CYP 3A4 expression No changes in bioavailability of simvastatin, pravastatin, and saquinavir	[148]

has been carried out (Table 3). In a clinical trial involving 10 healthy subjects, 600 mg garlic extract was given daily for 21 days, the results indicating that garlic extract increased intestinal p-glycoprotein expression and decreased the AUC of plasma concentration of saquinavir [148]. Our study evaluated the pharmacokinetics of two hypocholesterolemic drugs, simvastatin and pravastatin, whose AUCs were not changed.

Due to its antithrombotic activity, garlic ranks among the most widely used herbal medicines, typically ingested by people receiving warfarin [149]. Changes in the pharmacokinetics of warfarin as a result of garlic have been determined in a clinical trial involving 12 healthy male volunteers. The results showed that the plasma concentration–time profile of warfarin and platelet aggregation unaltered when warfarin was co-administered with garlic (2 g/d) for 2 weeks [150].

An influence of garlic on the pharmacokinetics of docetaxel was also rated in 10 women with metastatic breast cancer [151], treated with 30 mg/m² docetaxel given weekly for 3 of 4 weeks. Three days after the initial dose of docetaxel, patients received 600 mg of garlic twice daily for 12 consecutive days. The results indicated that the clearance of docetaxel and additional pharmacokinetic parameters including peak concentration, AUC, and half-life were not affected.

Although garlic had no significant effect on the pharmacokinetics of docetaxel, these authors found that patients with the CYP3A5*3C/*3C genotype had a lower mean AUC ratio than those with the CYP3A5*1A/*1A genotype [151]. This finding suggests that genetic background is a determining factor in the outcome of garlic and drug interaction. Understanding the genotype of each individual tested may help in evaluating whether garlic interacts with the particular drug and changes its therapeutic efficacy.

Although the results remain inconsistent and contradictory, the possibility that garlic will affect the therapeutic efficacy of certain drugs cannot be excluded based on its potency in terms of modulating drug-metabolizing enzymes and the activity and expression of membrane transporters.. More well-designed studies are warranted to clarify whether garlic affects the metabolism of drugs and alters their pharmacokinetics.

8. Safety of garlic

Consumed for hundreds of years, garlic is regarded as a safe food. However, in addition to the possible interaction with drugs cited above, several health risks have been reported to be associated with the excess consumption of garlic, or with

contact with garlic it in the workplace. In particular, gastrointestinal tract injury and allergic reactions caused by garlic attract concern. Increased exfoliation of the gastric surface epithelial cells in healthy subjects has been reported after the intragastric infusion of a single dose of raw garlic of over 0.75 g [152]. By injecting 0.5 mL of raw garlic juice into the ligated duodenum of rats, injury to the duodenal mucosal lining followed 2 hours after exposure, with severe damage including ulcers and bleeding occurring after 24 hours [153].

Damage to the stomach and intestine may account for the decrease in body weight seen after rats were given aqueous extracts of garlic (300 or 600 mg/kg/d for 21 days) and garlic oil (200 mg/kg, three times a week for 6 weeks) [36,154]. In a chronic toxicity test, however, no differences in body weight gain and in urinary, hematological, serological, and histological examinations were observed in Wistar rats given garlic extract at doses of 2 g/kg five times a week for 6 months [155]. These inconsistencies require more careful experimental designs to clarify whether garlic displays an adverse effect on gastrointestinal tract and growth; for instance, differences in garlic species, garlic preparations, and the dosage tested merit consideration.

Over recent decades, the allergenic potential of garlic has become well recognized. Cases of allergic reactions – e.g., contact dermatitis, asthma, urticaria, pemphigus, and anaphylaxis – have been reported in association with garlic use [156]. Allergic contact dermatitis in response to garlic was initially reported in 1950; to date, most cases have appeared in chefs and housewives in frequent contact with garlic [157–161]. Among Fernández-Vozmediano et al.’s 13 curry chefs, four tested DADS-positive, all showing dermatitis of the nondominant hand, with hyperkeratosis and fissuring of the thumb, index, and middle finger [162]. Allergy of the hands in the case of a 58-year-old male taking garlic to treat his hyperlipidemia also related to his use of garlic tablets [163]. Based on such evidence, garlic is classified as a type I allergen [159], the allergens being identified as DADS, allylpropyl disulfide, allylmercaptan, and allicin [164].

9. Conclusions

Past decades have seen myriad studies, especially *in vitro* and in animal models, addressing the protective effect of garlic against cardiovascular disease and cancer. This protection can arise from its diverse biological activities: enhanced antioxidant defense, lowering of blood lipids, inhibition of blood aggregation, enhancement of cancer cell cycle arrest/apoptosis, inhibition of invasion and/or metastasis, and

modulation of drug metabolism and/or the immune response. However, the results observed in human clinical and intervention studies have been inconsistent. The risk of garlic–drug interactions is attracting increasing interest, especially in the elderly and in those with chronic diseases. Further experiments are warranted to understand the actual health benefits and impact of garlic.

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Review article

Angelica sinensis: A Chinese herb for brain cancer therapyPo-Cheng Lin^{a,b,1}, Po-Yen Liu^{c,1}, Shinn-Zong Lin^{b,d,**}, Horng-Jyh Harn^{e,f,*}^a Department of Stem Cell Applied Technology, Gwo Xi Stem Cell Applied Technology, Hsinchu, Taiwan, ROC^b Center for Neuropsychiatry, China Medical University and Hospital, Taichung, Taiwan, ROC^c Graduate Institute of Chinese Medical Science, China Medical University, Taichung, Taiwan, ROC^d Department of Neurosurgery, China Medical University Beigan Hospital, Yunlin, Taiwan, ROC^e Department of Medicine, China Medical University, Taichung, Taiwan, ROC^f Department of Pathology, China Medical University and Hospital, Taichung, Taiwan, ROC

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ABSTRACT

Herbs are an important aspect of traditional Chinese medicine, as well as a rich source of unique chemicals. Among the medicinal herbs, *Angelica sinensis* is the most popular used in Chinese medicine. The main compounds found in the acetone extract of *Angelica sinensis* (AS-AC) are ferulic acid, ligustilide, brefeldin A, butylidenephthalide, and polysaccharides, the latter with potential therapeutic effect on various human cancers. Based on molecular evidence from *in vitro* and *in vivo* studies, we discuss here how butylidenephthalide suppresses tumor cell proliferation and promotes tumor cell apoptosis. The molecular mechanisms involved include butylidenephthalide-stimulated translocation of Nur77 from the nucleus to the cytoplasm, leading to tumor apoptosis. Butylidenephthalide likewise suppresses telomerase activity, resulting in tumor senescence. Finally, a controlled release system can increase localized butylidenephthalide concentration. Importantly, butylidenephthalide can cross the blood–brain barrier. Current evidence suggests its efficacy against brain tumors and therefore potential clinical applications.

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1. Introduction

For centuries, natural plant remedies (many of them obtained from herbs) have been used to treat disease and maintain health, especially in China. More than 3200 herbs and 300 minerals (and even animal extracts) are routinely applied to patients as a mixture or formula. Practitioners of traditional Chinese medicine believe that disease primarily arises from imbalances in the body. The therapeutic purpose of a Chinese

doctor is therefore to bring the human body back into equilibrium.

Herbal drugs are used with the goal of restoring this balance by nourishing the body, including the energy, *qi* (breath circulation), and spirit, to maintain health rather than to treat a particular disease or medical condition. This mindset reflects an emphasis on preventive medicine. Treatments undertaken with this goal are called *Fu Zheng* and are given as complementary therapy intended to reduce the side effects of

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conventional Western medical treatment. Chinese herbal medicine is independent of conventional Western concepts of medical diagnosis and treatment.

One aspect of Chinese medicine is to bolster resistance to disease by strengthening a person's immunity. Chinese herbs attempt to prevent and treat physiological imbalances, such as those caused by cancer and other diseases, with combinations of herbs, minerals, and plant extracts. Diseases that cannot be cured by modern medical treatments, such as diabetes and cancer, may be alleviated by traditional Chinese medicine.

2. *Angelica sinensis* (dong quai) as a traditional Chinese medicine

Dong quai, the root of *Angelica sinensis*, is the most popular herbal medicine in China, present in many formulas that have been used over thousands of years. It mainly treats gynecological conditions, menstrual disorders, anemia, coronary heart disease, carbuncles, and sores. Several extracts or single compounds from *Angelica sinensis* have been investigated for their potential to increase myocardial blood flow, reduce radiation damage, and improve kidney function [1–4]. Polysaccharides, its main components, demonstrably protect against gastrointestinal damage and hepatic injury [5–8]. Diverse components from *Angelica sinensis* may thus have myriad pharmacological activities.

Natural products, such as plants, microorganisms, and marine life, are abundant sources of anticancer drugs [9]. In *Angelica sinensis*, the major compounds are ferulic acid, ligustilide, brefeldin A, butylidenephthalide, and polysaccharides [10]. Ferulic acid and ligustilide serve as chemical markers to assess the quality of *Angelica sinensis* plants [10]. In another report, Z-ligustilide was studied for its potential cytotoxicity against two tumor types, L1210 and K562 [11]. Butylidenephthalide is used as a standard profile for identifying *Ligusticum chuanxiong*. Ferulic acid can inhibit platelet aggregation, whereas ligustilide has antiasthmatic activity [12,13].

Angelica sinensis has been used to relieve the side effects of radiation treatment on lung tissue. Other studies highlight the potential clinical application of *Angelica sinensis* extracts, e.g., to inhibit the progress of radiation-induced pulmonary fibrosis by downregulating tumor necrosis factor alpha (TNF- α) and transforming growth factor beta-1 [14–16].

3. *Angelica sinensis* in anticancer studies

To test the antitumor effects of *Angelica sinensis* extracts, Cheng et al used bio-based assays with different human cancer cell lines [17]. Their study showed that AS-AC had a dose-dependent antiproliferative effect on human cancer cell lines derived from the lung (A549), brain (DBTRG-05MG), liver (J5), and colorectal tissue (HT29). Their results also demonstrated that the AS-C induced the arrest of human cancer cells and activated the mechanism of apoptosis. In human brain tumors, both AS-AC and AS-C significantly inhibited proliferation by 30–50%; both treatments also suppressed cathepsin B and vascular endothelial growth factor

expression. In an animal study, AS-AC or AS-C alone suppressed tumor growth by 30% and 60%, respectively [18]. Further, AS-AC and AS-C were able to inhibit microvessel formation in tumors in nude mice [18].

In 1994, Choy and colleagues identified a low-molecular-weight polysaccharide from the *Angelica sinensis* rhizome and proved its strong antitumor and immunostimulatory activities [19]. Additional polysaccharides have since been identified from *Angelica sinensis*: APS-1, APS-3a, APS-3b, and APS-3c [20,21]. These display diverse structural features and antitumor activities: e.g., the backbone of APS-1 consists of (1,4)- α -D-glucopyranosyl residues, its branches being (1,6)- α -D-glucopyranosyl residues with a terminal β -L-arabinofuranose residue. Notably, APS-1 had antitumor effects *in vitro*, especially against human cervical cancer HeLa cells [20]. This antitumor activity coincided with a greater expression of mRNAs encoding interferon-gamma, interleukin-2, and interleukin-6 in splenocytes, as well as greater nitric oxide and TNF-gamma production in macrophages [21].

Butylidenephthalide was recently identified as the active component of AS-AC [22]. Natural phthalide compounds such as butylidenephthalide are considered to be candidate antitumor agents [23,24]. Indeed, butylidenephthalide has proven anticancer potential against colon cancer. As well as its antitumor effect, butylidenephthalide has demonstrated an ability to prevent benzo[a]pyrene-induced forestomach cancer in mice [25].

Butylidenephthalide and other agents (senkyunolide A and Z-ligustilide) act synergistically to reduce tumor cell proliferation [26]. A synergistic antiproliferative effect is noted when butylidenephthalide is combined with the chemotherapeutic drug 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine or BCNU) [27]. This synergism is mediated by downregulation of the gene MGMT, which encodes the DNA repair enzyme O-6-methylguanine-DNA methyltransferase [27].

4. Antitumor effects of AS-C *in vitro* and *in vivo*

Throughout its long record of use in traditional Chinese medicine, *Angelica sinensis* has exhibited many biological activities, e.g., immune system regulation, relief from menopausal symptoms, and improvement in myocardial blood flow [28–33]. Currently, few drugs treat malignant brain tumors, partly owing to the difficulty of producing drugs that cross the blood–brain barrier.

Tsai et al [34] were the first to confirm that AS-C displayed strong activity against glioblastoma (GBM) *in vitro* and *in vivo*. In *in vitro* cytotoxic assays, AS-C exhibited efficacy against not only brain tumors, but also other tumor cells; normal fibroblast cells were resistant to AS-C in the same experiment. Importantly, there was no evidence of AS-C-induced cytotoxicity in either the liver or kidney after injection of 500 mg/kg AS-C (either intraperitoneally or subcutaneously). In that study, AS-C had better anticancer capacity than carmustine and proved less cytotoxic to normal cells than Taxol [34].

In clinical studies, various antitumor drugs, such as temozolomide, only slightly extended survival time for patients with GBM [29,35–42]. In line with the *in vitro* cytotoxic

results, AS-C had a correspondingly greater cytotoxic effect on GBM cells than on other tumor cells. In a subcutaneous tumor model, AS-C inhibited rat GBM tumor growth and extended survival, especially reducing the tumor volume of rat GBM *in situ* [34], suggesting that AS-C crosses the blood–brain barrier and inhibits GBM cell growth. As such, it inhibits GBM tumor growth and induces GBM tumor cell apoptosis, as demonstrated both *in vitro* and *in vivo*.

Further, human GBM cells may have a different metabolism than rat GBM cells, as a result possessing a distinct susceptibility to AS-C treatment. AS-C can also inhibit human GBM tumor growth in a subcutaneous tumor model [34]. These *in vivo* cytotoxicity studies suggest that AS-C should be further developed to yield effective and safe anti-GBM drugs.

5. Butylidenephthalide: *in vitro* and *in vivo* antitumor effect of *Angelica sinensis*

The antitumor activity of AS-C against human GBM [34] made it critical to isolate the antitumor components from AS-C. The major ones were found to be BP (molecular weight 188.22 Dalton) and K2 (molecular weight 190.23 Dalton). Butylidenephthalide emerged as key anticancer component (>30% of crude AS-C) [22]. Our study shows that BP induces cell cycle arrest (at G0/G1) and apoptosis in GBM and hepatocellular carcinoma [22,43,44]. BP promotes apoptosis through both the p53-dependent and p53-independent pathways *in vitro*. In an *in situ* rat model of GBM, BP inhibited tumor growth while decreasing GBM volume, suggesting an anticancer potential [22,43,44].

Studies from the Harn group indicate that *Angelica sinensis* suppresses telomerase activity [22,43,44]. BP may target telomerase to effect its dramatic antitumor activity both *in vitro* and *in vivo*. To test this hypothesis, we monitored its inhibition of telomerase activity in brain cancer cell lines DBTRG-05MG and GBM 8401. BP did indeed inhibit telomerase mRNA transcription and reduced telomerase protein expression in these cells, whereas the expression of the telomerase RNA component was not affected by BP treatment. Reduced telomerase expression emanated from transcriptional inhibition of human telomerase reverse transcriptase, with a consequent reduction in telomerase activity. Results suggest that BP inhibits GBM by downregulating both telomerase expression and activity [45].

6. Antitumor effects of BP in animal models of autologous GBM

This activity has been probed *in vivo* in animal experiments. BP inhibited the growth of RG2, a spontaneous GBM tumor type in rat, whereas tumors continued to grow in the control group treated with the vehicle [22]. There was no significant intergroup variance in body weight [22]. The expression of Ki-67 apparently declined, indicating BP's antiproliferation activity *in vivo*. On the other hand, the level of the apoptotic marker cleaved caspase 3 increased after BP treatment, with a consequent apoptosis of tumor cells *in vivo* [22].

Tsai and co-workers implanted RG2 tumors into rat brain and subsequently treated the animals with BP. Magnetic resonance imaging revealed a significant reduction in tumor size upon subcutaneous administration of BP (300 mg/kg/d) for 5 days [22]. This result is similar to those obtained with a subcutaneous RG2 tumor model, suggesting that BP exhibited equivalent potency across the blood–brain barrier. Finally, on Day 19, the BP-treated group had a significantly higher survival rate (50%; 3/6) than the control group (16.7%; 1/6).

7. Antitumor effect of BP in a nude mouse model of heterologous human GBM

The antitumor effect of BP has been tested in a human GBM xenograft nude mouse model. After mice were grafted with human GBM, BP was administered subcutaneously in larger doses (70, 150, 300, 500, and 800 mg/kg) from Days 1 to 5. As noted in the RG2 experiments, tumor growth was significantly suppressed in all five dosage groups compared with the control group.

At 200 days post-treatment, survival rates were 16.7% (1/6), 33.3% (2/6), 50% (3/6), 80% (4/5) and 100% (6/6) for the respective BP dosage groups [22]. In the control group (vehicle only), the average tumor size was over 1000 mm³ compared with 171.7 mm³ in the 800 mg/kg BP treatment group. In this nude mouse model, survival at Day 200 was significantly prolonged by BP treatment. The survival rate in the various groups was as follows: untreated, 0% (0/6); 70 mg BP, 20% (1/5); 300 mg BP, 50% (3/6); 800 mg BP, 83.3% (5/6). Immunohistochemical staining for cleaved caspase 3 indicated an increased expression of this protein, while that of Ki-67 decreased, hinting that BP can inhibit tumor proliferation and induce tumor cell apoptosis. Collectively, this evidence indicates that BP can decrease tumor size and increase survival rate in mice.

Finally, after 200 days, there were no changes in the specific histology of the hollow organs or in body weight, even in the highest dosage group (800 mg/kg BP for 5 consecutive days). This suggests that BP has no acute or subacute toxicity.

8. Assessing target genes for BP using oligodeoxynucleotide-based microarrays

To investigate the gene(s) targeted by BP in malignant brain tumors, a BP-treated GBM cell line was subjected to RNA microarray analysis after 3 hours or 24 hours of treatment. Subsequently, western blotting and real-time polymerase chain reaction confirmed the microarray results compared with the vehicle group (the tumor cell line treated with only dimethyl sulfoxide). Interesting, among the 30,000 genes assessed, 422 were upregulated in response to BP (49 in 3 hours, and 373 in 24 hours).

Subsequent clustering analysis highlighted a particular family of receptor-encoding genes that included *NOR-1*, *Nurr-1*, and *Nur77*. The proteins encoded by these genes have a similar structure; because they are orphan receptors, however, their ligand(s) are unknown [46]. *NOR-1*, *Nurr-1*, and *Nur77* are early response genes, and their encoded receptors can be induced by apoptotic stimuli, serum, growth factors,

and ligand-binding [47–49]. Nur77, NOR-1, and Nurr-1 are reportedly related to tumor apoptosis and cell growth [50].

Moreover, the mechanism of Nur77-mediated apoptosis in T-cells and several cancers has been extensively studied [46,47,51–53]. There are two proposed mechanisms for Nur77-mediated tumor cell apoptosis. First, upon translocation of Nur77 from the nucleus to the cytoplasm and mitochondria, as well as the conversion of Bcl-2 into its phosphorylated form, cytochrome c is released into the cytoplasm, which leads to tumor cell apoptosis. This mechanism has been reported in prostate cancer and other cancers [46,51].

Second, Nur77 can act as a transcription factor that binds the promoters of the apoptosis genes, e.g., those encoding Fas-ligand, the Nur77 downstream gene 1, and the Nur77 downstream gene 2; Nur77 also participates in TNF-related apoptosis [54–56]. Many chemicals induce apoptosis mediated by Nur77 overexpression, e.g., retinoid-related 6-[3'-(1-adamantyl)-4'-hydroxyphenyl]-2-naphthalenecarboxylic acid (also known as CD437), tetradecanoylphorbol-1, 3-acetate, etoposide (VP-16), cisplatin, and insulin-like growth factor binding protein-3 [36,46,52,57].

9. BP-induced Nur77 nucleo-cytoplasmic translocation leads to tumor cell apoptosis

To determine the role of Nur77 in BP-induced cancer cell apoptosis, Lin and co-workers used a fluorescently labeled Nur77-specific antibody to trace the location of Nur77, reporting that Nur77 was translocated from nucleus to cytoplasm for 24 hours after BP treatment [44]. Nur77 translocation to cytoplasm was recently shown to be induced by activation of c-Jun N-terminal kinase or inhibition of AKT [16].

In addition, the role of T-cells in thymocyte apoptosis and the protein kinase C activation pathway that results in Nur77 expression have also been confirmed [58]. Lin et al separated cytosolic and nuclear proteins and confirmed Nur77 translocation by western blotting. In addition, Nur77 translocation was able to trigger cytochrome C release into the cytoplasm, accompanied by activation of caspase 9 and then caspase 3, resulting in tumor cell apoptosis [44]. Importantly, when small interfering RNA (siRNA) targeting Nur77 mRNA was used to downregulate the Nur77 level in the presence of BP, BP-induced tumor apoptosis was reversed. In sum, BP-induced GBM apoptosis is mediated by a targeted overexpression of Nur77 and its subsequent translocation from nucleus to cytoplasm.

10. BP suppresses telomerase activity, resulting in tumor senescence

The expression of the Nur77 siRNA in BP-treated cells restored their viability by only 40%, suggesting that BP has yet another target(s) besides Nur77 [44]. Because BP can suppress telomerase activity in a dose-dependent manner, it is reasonable to speculate that BP induces cell senescence by (in)directly inhibiting telomerase activity, resulting in tumor cell apoptosis. Since cell viability is a function of both proliferative capacity and apoptotic effects, BP-stimulated suppression of

telomerase activity can induce cytostatic effects and thereby decrease cell viability. In our studies, transfection of a telomerase-encoding transgene and the subsequent restoration of telomerase activity increased the viability of BP-treated GBM cells by 90% when combined with Nur77 siRNA [44]. At present there exist two potential targets: Nur77, which mediates cytotoxicity, and telomerase, which promotes cytostatic state. These dual effects contribute almost equally to reducing brain tumor cell viability in the presence of BP.

In terms of the mechanism by which a telomerase gene is regulated, several transcription binding sites have been reported, including the Myc/Mad sites (E-boxes) [59]. Wu et al reported c-Myc binding to E-boxes and activating telomerase mRNA transcription in tumor cells [60].

A binding site for transcription factor Sp1 is another vital motif present in the telomerase promoter. Kyo et al reported five binding sites for the transcription factor Sp1 in the telomerase core promoter region, indicating Sp1 as vital regulator of the telomerase gene [61]. Most tumor cells have high telomerase activity due to upregulation of Sp1 level. Kyo et al also report a marked decrease in telomerase activity after Sp1 mutation [61].

Our western blot analysis showed no significant decrease in c-Myc level after BP treatment in GBM cells, whereas telomerase activity markedly decreased [44]. The electrophoretic mobility shift assay indicated, however, that Sp1 protein expression declined after BP treatment, with a concomitant drop in Sp1 binding activity at the telomerase promoter. The Sp1 regulatory element in the telomerase promoter may prove to be critical for telomerase transcription in BP-treated GBM cells [44].

The absence of telomerase activity is associated with cell senescence and cell cycle proliferation. Our study showed BP has an antiproliferative effect and induces tumor cell senescence [45]. Cellular proliferation capacity can be restored, with concomitant resistance to senescence, after transfection and overexpression of telomerase – even in the presence of BP. This implicates BP-mediated inhibition of cell proliferation as a central factor in promoting cell senescence and subsequent apoptosis.

Immunohistochemical staining of X-gal, a senescence marker, indicated that BP not only increases cell age by decreasing telomerase activity, but also affects the expression of three major proteins involved in cell cycle regulation and senescence: p53, p21, and p16 [62]. On the other hand, senescence-associated markers p21 and p16 increased after BP treatment [45]. Future experiments must confirm the mechanisms underlying BP-induced tumor cell senescence.

11. Conclusion and future perspectives

Current information suggests BP as potential treatment against brain tumors, with minimal toxicity. However, brain tumors are hard to treat, since any efficacious drug must cross the brain–blood barrier. As such, the means by which this prospective drug could be delivered (including the possibility of local interstitial delivery) poses a formidable task. BP is oil-like and thus less water soluble owing to its hydrophobic character, another issue warranting resolution. Delivering it via

nanoparticles or as prodrug may constitute a viable drug formulation. Because BP targets genes encoding Nur77 and human telomerase reverse transcriptase, we view it as a potential target drug for use against brain tumors, and therefore worth developing for clinical use.

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Review article

Possible pathophysiology of heart failure in obesity: Cardiac apoptosis

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ABSTRACT

Obesity has traditionally been considered an independent risk factor for heart failure whose pathophysiology is generally believed to be associated with the consequence of myocyte hypertrophy, myocardial fibrosis, and abnormalities of intracellular calcium handling. Obesity-related comorbidities like chronic inflammation, coronary artery disease, diabetes, and hypertension play some causative roles in the development of heart failure. Currently, cardiac apoptosis and cardiac fibrosis are found in obesity and leptin-deficient animal models. Leptin pretreatment exerts antiapoptotic effects in cardiomyocytes. In obese rat hearts, key components of Fas-dependent apoptosis (Fas ligand, tumor necrosis factor- α , Fas death receptors, Fas-associated death domain, activated caspase 8, and activated caspase 3) as well as those of mitochondria-dependent apoptosis (Bad, Bax, Bax-to-Bcl2 ratio, cytosolic cytochrome c, activated caspase 9, and activated caspase 3) manifestly increased compared with lean controls. Obesity will activate cardiac Fas- and mitochondria-dependent apoptotic pathways while increasing cardiac fibrosis, which may provide one of the possible mechanisms for developing heart failure in obesity.

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1. Introduction

Heart failure, in pathophysiologic terms, can be defined as the inability of the heart to deliver blood and, hence, oxygen at a rate necessary for adequate tissue metabolism. Obesity or abdominal obesity is traditionally viewed as an independent

risk factor for heart failure [1,2]. Obesity was considered as a major risk factor for the development of heart failure in relative risk ranging from 1.8 to 5.6, depending on degree of obesity, even with other known risk factors excluded [3,4]. Elevated body mass index was associated with an increased risk of heart failure, even in less obese people [5]. Severe

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obesity in humans has long been recognized as causing various cardiac abnormalities characterized by markedly higher rates of chronic volume overload, development of heart failure, hypertension, and left ventricular hypertrophy [3,4,6,7]. Various types of heart failure-associated abnormalities like biventricular failure and left ventricular dysfunction were found in morbidly obese patients [8,9]. However, the relationship between heart failure and obesity is complex and not completely understood.

2. Possible pathophysiology of heart failure in obesity

The pathophysiology of heart failure with diastolic function abnormality is generally believed to be associated with a consequence of myocyte hypertrophy, myocardial fibrosis, and abnormality of intracellular calcium handling [10]. Hypertension, diabetes, and coronary artery disease play causative roles in developing heart failure in obesity [10]. Past years have seen conventional risk factors like hypertension, type 2 diabetes, and dyslipidemia implicated in heart failure; several recent studies highlight a pivotal role of obesity as an independent risk factor [1,2,11]. Chronic inflammation was also considered as a novel metabolic risk factor [12,13]. Virious and complicated obesity predisposes patients to heart failure due to the presence of many comorbidities and intrinsic pathophysiologic mechanisms. Hence, it is difficult to indentify the pathophysiology of heart failure in obesity independent of obesity-related comorbidities.

3. Cardiomyopathic changes in obesity

Obesity is often associated with hemodynamic overload, ventricular remodeling, and higher cardiac output due to augmented stroke volume and an increase in heart rate [6,14]. Obesity cardiomyopathy typically occurs in severe and long-standing obesity, which may progressively develop cardiac abnormalities like dilated heart, congestive heart failure, and sudden cardiac death [6]. In our prior study, 5- to 6-month-old genetic obese Zucker rats appeared to increase their relative cardiomyopathic changes, such as myocardial disarray and minor cardiac fibrosis. We speculate tha tobese rats progressively develop deleterious cardiomyopathic changes at an age earlier than 5–6 months. In obese Zucker rats, the cardiac hypertrophic effect will be underestimated if we only use the ratio of whole heart weight to whole body weight, traditionally regarded as an index of cardiac hypertrophy. Potential inducers of cardiac hypertrophy and cardiac apoptosis include hypertension [15], volume overload, hypoxia [16,17], and oxidative stress [18,19]. However, it is difficult to identify pathophysiology of cardiomyopathic changes in obesity independent of obesity-related intrinsic abnormalities or comorbidities. Specific factors or comorbidities like hypertension, diabetes, lipotoxic, volume overload, nocturnal hypoxemia, oxidative stress, or other unclear factors may potentially cause these cardiomyopathic changes.

4. Cardiac apoptosis in obesity

Cardiomyocyte apoptosis is a component of cardiac remodeling that contributes to heart failure in obesity [20]. Apoptosis, a physiologic program of cellular death, may contribute to many cardiac disorders [15,18]; its occurrence has been reported to contribute to the loss of cardiomyocytes in cardiomyopathies and is recognized as a predictor of adverse outcomes in patients with cardiac diseases or heart failure [21]. One study showed myocardial DNA laddering in obese rat hearts reaching 20 times the normal lean level, hinting that cardiac dysfunction in obesity is caused by lipoapoptosis and is prevented by reducing cardiac lipids [22]. In our previous study [23], increased body weight, increased whole heart weight, increased left ventricular weight, increased ratio of whole heart weight to tibia length, abnormal myocardial architecture, increased myocardial disarray, increased terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive apoptotic cardiac cells and increased minor cardiac fibrosis were observed in genetic obese rat models. High levels of metabolic products are previously proposed to cause common complications of obesity like insulin resistance and cardiovascular disease, ultimately promoting programmed cell death [24]. Leptin-deficient and -resistant mice exhibit increased apoptosis, DNA damage, and mortality compared with wild type mice, suggesting that obesity or impaired leptin signaling enhances excess age-associated DNA damage and premature mortality [25]. By contrast, leptin pretreatment in hypoxia/reoxygenation H9c2 cells attenuated hypoxia/reoxygenation increased DNA fragmentation, TUNEL staining and caspase-3 activity, suggesting that leptin exerts anti-apoptotic effects in cardiomyocyte cells [20]. Acute leptin pretreatment mediates antiapoptotic effects on H₂O₂-induced apoptosis in H9c2 rat cardiomyocytes [26]. Both studies imply leptin as a promising target in preventing heart failure in obesity. High-calorie “Western” diet-induced obesity CAN also cause cardiac dysfunction [27]. Diet-induced oxidative stress was reflected in reduced transcript levels of manganese superoxide dismutase, glutathione peroxidase 1, and higher protein levels of mitochondrial transcription factor A, suggesting compensatory mitochondrial biogenesis in the face of increased mitochondrial damage [27]. Besides, “Western” diet-induced obesity enhance cardiac apoptosis, as evidenced by TUNEL positivity, elevated mRNA transcript levels and activity of caspase 3 [27]. Cardiac apoptosis is a potential mechanism of myocardial dysfunction and early mortality in obesity.

5. Cardiac Fas receptor-dependent poptotic pathway in obesity

The ‘extrinsic’ Fas receptor-dependent (type I) apoptotic pathway is believed to be one major pathway directly triggering cardiac apoptosis [18,19]. This pathway is initiated by binding the Fas ligand and receptor, which causes receptors to cluster and initiates an extrinsic pathway [19]. Fas-ligand binding followed by Fas-receptor oligomerisation is known to spawn the formation of death-inducing signal complex, starting with

recruitment of the Fas-associated death domain (FADD) of the adaptor protein [19]. Fas-receptor oligomerization recruits FADD and procaspase 8 to the complex, thus activating caspase 8, which cleaves procaspase 3 and undergoes autocatalysis to form active caspase 3, a principle effector caspase of apoptosis [28,29]. Additionally, activated caspase 8 can cleave Bcl-2 homology domain 3 (BH3)-interfering domain death agonist (Bid), and cleaved Bid to t-Bid then causes release of mitochondrial cytochrome c, leading to activation of procaspase 9, which then activates procaspase 3 [19, 30]. The t-Bid is a key component involved in intracellular molecule signaling from Fas-dependent apoptotic to the mitochondria-dependent apoptotic pathway [19,30]. The Fas-receptor dependent apoptotic pathway was more active in obese rat hearts, which can be characterized by increases in cardiac Fas ligands, Fas death receptors, FADDs, and activated caspase-8 and -3 levels in the obese group relative to the lean group [23]. Our previous study suggests that cardiac Fas receptor-dependent apoptotic pathway was more active in obesity [23].

6. Cardiac mitochondria-dependent apoptotic pathway in obesity

The ‘intrinsic’ mitochondria-dependent (type II) apoptotic pathway is mediated by internal factors, especially in mitochondria [19]. The mitochondria is the main site of action for members of the apoptosis-regulating protein family exemplified by Bcl-2 family, e.g., Bcl-2 and Bax [19]. Commitment to apoptosis is typically governed by opposing factions of this family, including pro- versus antiapoptotic family members [31]. These Bcl2 family members can homo- or heterodimerize to each other, and they appear to interact with and neutralize each other; relative balance of these effectors strongly influences cytochrome c release and cell fate [32]. Bcl-2, an anti-apoptotic protein, prevents cytochrome c release, whereas Bax and proapoptotic proteins, enhance cytochrome c release from mitochondria [19]. When cytochrome c is released from mitochondria into cytosol, it is responsible for caspase 9 activation, which further activates caspase 3 and executes the apoptotic program [33]. In the obese animal model compared with the lean rat heart, proapoptotic Bcl2 family members, BNIP3 and Bad levels, significantly rose, while the antiapoptotic Bcl2 family member Bcl2 level dropped significantly [34]. Cytosolic cytochrome c indicating cytochrome c release from mitochondria was significantly increased in the obese rat heart. Upstream pro-caspase-9 and -3 also significantly decreased, while activated caspase-9 and activated caspase-3 significantly increased in obese versus lean rat hearts, implying proforms of caspase-9 and -3 cleaved into active-forms caspase-9 and -3 [34]. Our previous study [34] suggested cardiac mitochondrial-dependent apoptotic pathways more active in obese Zucker rats, which is one possible apoptotic mechanism for heart failure in obesity. “Western” diet-induced obesity starkly reduces antiapoptotic Bcl2 [27], indicating diet-induced obesity may activate mitochondrial-dependent pathway. Leptin reduced hypoxia/reoxygenation-induced translocation of the Bax pro-apoptotic protein to the mitochondrial membrane, which provides a mechanism to explain protective effects of the intrinsic apoptosis pathway

of in rat H9c2 cells [20]. This indirectly implies that leptin exerts an antiapoptotic effect on the mitochondrial-dependent apoptotic pathway in obesity.

7. Cardiac fibrosis in obesity

There is an association between turnover of collagens and remodeling of the rat ventricles [21,35], which progresses immediately after myocardial damage with an increased level of collagenases [36]. Collagens synthesized by fibroblasts invade and replace apoptotic myocytes [21,35,37,38]; myocardial interstitial changes resulting from increased collagen deposition lead to cardiac stiffness and cardiac dysfunction [37]. Accordingly, accumulated collagens will further contribute to the development of ventricular fibrosis and heart failure [38]. In our previous findings, abnormal myocardial architecture, increased interstitial space, and minor cardiac fibrosis in obese rat hearts suggest the development of cardiomyocyte death characterized by a distortion in the myocardium architecture and minor cardiac fibrosis in obesity [34]. A high-fat diet fed to normal female rats can elicit hypertensive response and induce perivascular fibrosis before the onset of overt obesity [39]. One study demonstrates that nutritional overfeeding and changes early in postnatal development with long-lasting effects on body weight and adiposity, along with cardiac fibrosis and heart structural, changes during adulthood [40]. This postnatal development of cardiac fibrosis may imply that cardiac fibrosis may be not easily

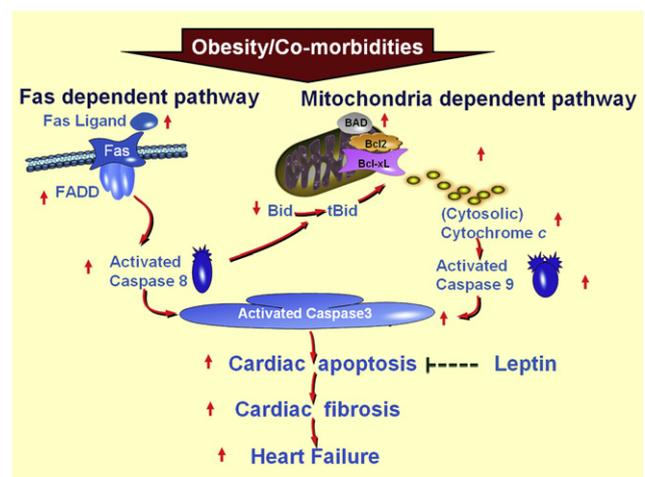


Fig. 1 – Hypothesized pathophysiology of heart failure in obesity. Cardiac Fas- and mitochondria-dependent apoptotic pathways are activated in obesity. The cardiac Fas-dependent apoptotic pathway appears to increase due to greater tumor necrosis factor-alpha, Fas ligand, Fas receptor, Fas-associated death domain, activated caspase-8, and activated caspase-3. The cardiac mitochondria-dependent apoptotic pathway increases in obesity, evidently due to increases in Bad, Bax, Bax/Bcl-2, cytochrome C release, along with activated caspase-3 and -9. Leptin pretreatment exerts an antiapoptotic effect on cardiomyocytes. Up and down arrows at the right represent increases and decreases, respectively.

reversible. After integrating previous findings into hypothesized pathophysiology, we hypothesize that cardiac Fas- and mitochondrial-dependent apoptotic pathways are more active in obesity. Apoptotic cardiomyocyte and accumulated collagens can also contribute to the development of cardiac fibrosis and heart failure (Fig. 1).

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Review article

Role of nonhomologous end-joining in oral cancer and personalized pharmacogenomics

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ABSTRACT

Recent years have witnessed the incidence of cancer rise worldwide, with no end to the war against it in sight. It is believed that cancer emanates from a series of genetic alterations leading to the progressive disorder of the normal mechanisms that control cell proliferation, differentiation, death, and/or genomic stability. With our genome under constant exogenous and endogenous assault, cellular capacity to maintain genomic stability by means of various DNA repair mechanisms looms vital to preventing tumor initiation and progression. In the same vein, the relative role of DNA repair as biomarker for prognosis, predictor of drug and therapy response, or indeed as target for novel gene therapy, has been recently patented and is very promising. This paper summarizes studies probing association among nonhomologous end-joining genes XRCC4, XRCC5, and XRCC6 vis-à-vis oral cancer susceptibility, then discusses their role in carcinogenesis and personalized pharmacogenomics.

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1. Introduction

The human genome is maintained by repair pathways that sense DNA damage and respond to exogenous and/or endogenous DNA insult. Pathways identified include: (a) direct reversal repair, (b) nucleotide excision repair, (c) base excision repair, (d) homologous repair (HR), (e) nonhomologous end-joining (NHEJ), and (f) mismatch repair. Normally, if these repair pathways fail to repair the DNA damage, the same molecular machinery can sense defects as a “threat” and trigger apoptosis. Yet, when DNA damage is neither repaired nor turned to induction of cell apoptosis terminating unhealthy cells, DNA defects remain and are propagated to

offspring cells. Under the later circumstances, carcinogenesis occurs. Decreased genomic integrity and stability in most cancer types and identification of cancer predisposition syndromes linked to defects in DNA repair pathways support the concept that DNA repair genes may play a critical role in opposing cancer initiation and progression [1–3].

One of the most deleterious DNA damaging types is double strand break (DSB). It should be repaired in eukaryotes by two major pathways mentioned above: HR and NHEJ. The former is a template-guided, error-free pathway predominantly operating in S and G2 phases of the cell cycle and involves RAD51, its paralogs RAD51B/C/D, XRCC2/3, and p53, replication protein A (RPA), BRCA 1/2, BLM DNA helicase (BLM), and

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MUS81 [4]. The latter, by contrast, is a potentially less accurate form of DSB repair, with both termini of a broken DNA molecule processed to form compatible ends directly joined. In most cases, NHEJ results in loss of a few nucleotides at broken ends, making this pathway error-prone. This article focuses on XRCC4, XRCC5, and XRCC6, which play crucial roles in NHEJ, which is considered the major repair pathway of DSBs in eukaryotic cells during most phases of the cell cycle, particularly G0/G1 phases [5]. NHEJ involves the XRCC4, XRCC5/XRCC6 (also known as Ku80/Ku70), XRCC7 [DNA-dependent protein kinase catalytic subunit (DNA-PKcs)], Artemis, XRCC4-like factor (XLF), DNA ligase 4, Ataxia telangiectasia mutated kinase (ATM), p53, and MDM2 proteins [6,7]. NHEJ deficiencies can increase genomic instability [8,9] as well as tumorigenesis [10–13]. Still, the exact roles of these genes and their protein products, such as XRCC4, XRCC5, or XRCC6, in oral and/or other cancers are neither well investigated nor revealed. Fig. 1 depicts the model for DSB repair via NHEJ, along with proteins involved.

XRCC4 is found to restore DNA DSB repair and the ability to support V(D)J recombination of transiently introduced

substrates in the XR-1 CHO cell line [14]. The XRCC4 interacts directly with XRCC5/ XRCC6; it is hypothesized to serve as a flexible tether between XRCC5/XRCC6 and associated protein ligase 4 [15]. XRCC4 is required for precise end-joining of blunt DNA DSBs in mammalian fibroblasts [16]. A gene-targeting mutation mouse model shows, XRCC4 gene inactivation spawning late embryonic lethality accompanied by defective lymphogenesis and neurogenesis, manifested by extensive apoptotic death of newly generated postmitotic neuronal cells [12,17]. Findings demonstrate that differentiating lymphocytes and neurons strictly require XRCC4 proteins.

XRCC5 and XRCC6 usually form heterodimer Ku; they are probably among the first proteins bound to DNA ends at a DSB. The XRCC5/6–DNA complex recruits and activates XRCC7 [18,19]. XRCC5/6 dimer and XRCC7 are proposed to act in a synapsis process [18,19]. XRCC5 and XRCC6 knockout mice are growth retarded, radiosensitive and are severely immunodeficient [20,21]. B-cell development is arrested at an early stage due to profound deficiency in V(D)J recombination [20,21]. Although XRCC5- or XRCC6-deficient mice are visible, their cells have defects in DNA end-joining, which manifest as irradiation sensitivity, growth defects, premature senescence, and inability to perform end-joining during V(D)J recombination. These defects may also happen during human embryonic development. Human cells absorb hundreds of thousands exogenous and endogenous DNA insults daily. If cells fail to repair DSB, accumulated genomic instability will lead to apoptosis and cause embryonic lethality. Beyond a doubt, XRCC5 and XRCC6 are critical to both genomic stability and human ontogenesis.

Since each NHEJ gene plays a critical and specific role in repairing DSBs, if any of them fails to finish its job correctly and immediately, NHEJ capacity is hindered and overall genomic instability raised. It is thus tempting to speculate that defects in the NHEJ pathway may be associated with human cancers. This makes it puzzling as to why no direct genetic linkage is found between defective NHEJ genes and cancers. Among them, mutations in two demonstrably predispose carriers to a higher rate of genetic disease, DNA ligase 4 and Artemis, both associated with Nijmegen breakage syndrome-like syndrome and severe combined immunodeficiency, respectively [22,23]. One explanation is that any severe defects (null mutants) in NHEJ-related genes result in great genomic instability and prove incompatible with life, hence no cancer observed. Crucial and irreplaceable roles of these gene products may raise difficulty of approaching their physiological function via knockout mice. Consequently, for these high-penetrance NHEJ genes, only subtle defects arising from low-penetrance alleles like hypomorphic mutant or polymorphic variant would escape the cell cycle checkpoint surveillance and allow the cell to survive, as well as to accumulate enough unrepaired genomic alteration required for tumor formation [24,25]. Studies applying single nucleotide polymorphism (SNP) technology, among the most powerful and subtle genetic analyses, to approach associations of high- and low-penetrance genes with various cancers are warranted to fulfill the overall scene in cancer research.

One aim of this article is to summarize the work in Terry Fox Cancer Research Lab of China Medical University and

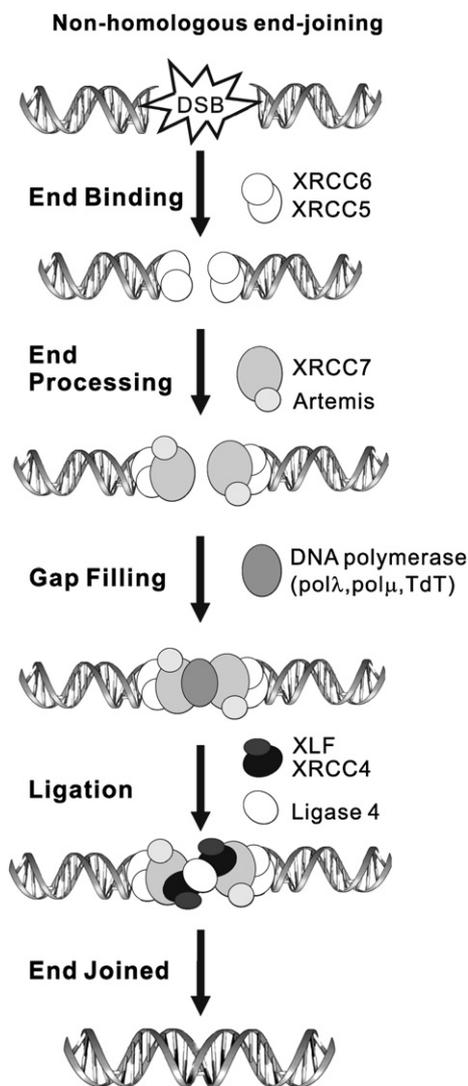


Fig. 1 – Model for repair of double-strand breaks by nonhomologous end-joining.

Hospital, Taichung, Taiwan, R.O.C., and worldwide, assessing correlation between SNPs of XRCC4, XRCC5, and XRCC6 regarding susceptibility to oral cancer. While rapid development of genome-wide association studies and bioinformatics help reveal secrets of human genome in cancer, knowledge of cancer genomics is still far from satisfying and in urgent need of further multi-approach studies. In addition, most SNPs have not been investigated for functional influence in cell or animal models, not to mention individual roles in cancer. It is very exciting that some SNPs of XRCC4, XRCC5, and XRCC6 have been associated with the susceptibility to various cancers. More interesting, specific gene-environment interaction appear in cancer patients who contact possible environmental carcinogens. Therefore, we infer risky SNP genotypes of XRCC4, XRCC5, XRCC6, and environmental carcinogens have some joint effects and increase susceptibility to cancer significantly. We hope this article provides novel useful markers for oncology early detection, prevention, along with some candidates for anticancer intervention.

2. Literature survey

We conducted MEDLINE, Current Contents, and Web of Science searches using *oral cancer*, *polymorphism*, XRCC4, XRCC5, or XRCC6 as keywords to search for articles published (January 1, 1966, to December 31, 2010). Additional papers were identified by references cited in the first series of selected articles; those included in meta-analysis were in any language, with human patients, published in primary literature and had no obvious overlap with patients of other studies. Case-control studies were eligible if they determined distribution of relevant genotypes in cancer cases and in concurrent controls using a molecular method for genotyping.

3. Oral cancer in Taiwan and worldwide

Oral cancer specifies a subgroup of head and neck malignancies developing at the lips, tongue, salivary glands, gingiva, mouth floor, oropharynx, buccal surfaces and other intraoral locations. Oral cancer incidence has increased through 2007, estimated in the United States as 10.3 cases per 100,000 persons, with mortality of 2.5 per 100,000 [26]. An estimated 36,540 new cases will be diagnosed in America during 2010, with 7,880 deaths due to this disease [27]. Oral cancer is more common in men than in women and accounts for 3% of new cancer cases in American men [27]. The World Health Organization estimates oral cancer as the eighth-most common cancer worldwide. Most important environmental risk factors in Western countries are consumption of tobacco and alcohol [28,29]. In Asian countries like Taiwan and India, betel quid accounts for a considerable percentage of such cases [30,31]. To date, genomic etiology is of great interest but largely unknown. In Taiwan, whose oral cancer frequency ranks highest worldwide, this disease looms as fourth greatest malignancy among men and sixth in women [32]; its relatively high prevalence in Taiwan stems mainly from the high-risk group of 2.5 million people with tobacco, liquor, and/or betel nut habits.

4. Role of XRCC4 in oral cancer genomics

Few papers investigated association of NHEJ genes with oral cancer worldwide; the two correlating XRCC4 genotypes with oral cancer risk were performed in Terry Fox Cancer Research Lab [33,34]. These studies screened five genetic variants of the XRCC4 gene, two of which showed strong correlation with oral cancer susceptibility: XRCC4 codon 247 (rs3734091) and intron3 (rs28360071). The XRCC4 codon 247 (rs3734091) locates on the exon of XRCC4; this SNP is in charge of shifting amino acids of XRCC4 gene products, which may also alter its biologic function. For this age- and sex-matched case-control study, control and patient groups each recruited 318 patients, with A allele assessed as a risk factor. Persons with it show 2.04-fold higher susceptibility. Another study's key variable was XRCC4 intron3 (rs28360071), deletion-insertion polymorphism (DIP) involved in 30 bp genetic variation. In all, 122 (38.4%) patients had D allele; the control group had only 91 (28.6%) such individuals. Statistically, people with D allele have a 1.55-fold higher risk than those with I allele.

Reports also checked joint effect of risky genotype and environmental exposure in oral cancer susceptibility. Briefly, risky genotype XRCC4 codon 247 (rs3734091) has synergistic effect with smoking on oral cancer risk, as does risky genotype intron3 (rs28360071) with both smoking and betel quid chewing. Joint effects indicate not only the XRCC4 gene but also the NHEJ DSB repair system as involved. Genetic factors indeed interact with environmental factors in overall cancer susceptibility.

5. Role of XRCC5 and CRXX6 in cancer genomics

In 2008, Terry Fox Cancer Research Lab found C allele of XRCC6 rs5751129 a marker for oral cancer risk; those of rs2267437, rs132770 and rs132774 were not [35]. Our team enlarged their population control/case from 318/318 to 600/600, reporting XRCC5 rs828907, but not rs11685387 or rs9288518, as associated with susceptibility [36]. Patients carrying GT and TT genotype at rs828907 showed 1.6-fold risk if they habitually chewed betel quid. A 2008 study of oral premalignant lesions found no correlation between XRCC5 rs1051685 genotypes and susceptibility [37].

6. XRCC4, XRCC5, and XRCC6 for individualized cancer pharmacogenomics

This article has reviewed association of XRCC4, XRCC5, and XRCC6 genotypes with susceptibility described oral cancer in literature, as summarized in Table 1. Clinical observation hints individual differences in response to drugs, variations that could be genetic [38,39]. Medical practice based on population response did not reflect ideal treatment for individuals [40]. Not until the Human Genome Project and advances in genomic epidemiology, along with systematic bioinformatics, have individual and/or ethnic genetic variations come to light step by step. Although the SNP and haplotype analyzing

Table 1 – Summary of the results of the investigations for oral premalignant lesion, oral cancer and the polymorphic genotype of XRCC4, XRCC5 and XRCC6 genes.

Disease	Author, year (ref number)	Gene	rs number	Location	Study subjects			Statistical Significance	Brief description
					Ethnic Country	Cases	Controls		
Oral Premalignant Lesion	Yang, 2008 (37)	XRCC5	1051685	3'UTR	America	147	147	NS	
Oral Cancer	Chiu, 2008 (33)	XRCC4	6869366 28360071	Promoter	Taiwan	318	318	NS	Allele deletion is risky (vs insertion), and had joint effects with smoking and betel quid chewing habits
				Intron 3				S	
	Tseng, 2008 (34)	XRCC4	3734091	Intron 7	Taiwan	318	318	NS	
				Intron 7				NS	
				Codon 247				S	
	Hsu, 2009 (36)	XRCC5	828907 11685387 9288518	Promoter	Taiwan	600	600	S	
				Promoter				NS	
				Intron 19				NS	
	Bau, 2008 (35)	XRCC6	5751129	Promoter	Taiwan	318	318	S	
				Allele T is risky, and had joint effects with betel quid chewing habits					
		2267437	Promoter				NS		
		132770	Promoter				NS		
		132774	Intron 3				NS		

S: statistically significant; NS: not statistically significant.

technology has grown ever more mature and complex, personalized cancer therapy and medicine heavily dependent on knowledge of susceptibility, treatment outcome, response to commonly used or gene-targeted anticancer drugs, and toxicities clustered among specific groups of patients in specific geographical regions, still need help from pharmacogenomics. Inter-individual variability in drug response cannot be satisfactorily explained by ambiguous renal or liver functional differences, patient age, morbidity, lifestyle, or comedication and patient compliance. Any solution is doomed to fail via individual genomics; polymorphisms lead to wide variation in how individuals respond to medication, either by changing pharmacokinetics (absorption, distribution, metabolism, and/or elimination) of anticancer drugs or by altering cellular response to therapeutic agents like radiotherapy.

As shown in Table 1, cancer molecular epidemiologists devote themselves to the description of subtle differences among patients in genetic polymorphism distribution that affected DNA-repair enzymes, drug-metabolizing enzymes, cell-cycle controlling proteins, oncogenes, tumor suppression genes, and cellular transporters of cytotoxic chemotherapy. With DNA repair enzymes as correctives for DNA damage induced by carcinogens and even some anticancer drugs and

radiotherapy, SNPs in DNA repair genes may very likely play important roles in all processes from cancer susceptibility to anticancer treatment outcome. This review summarizes SNPs of XRCC4, XRCC5, and XRCC6 genes specifically critical in NHEJ, plus their contribution to common cancers around of living. Among them, SNPs like XRCC5 rs828907, may merit our attention since they serve as biomarkers for early detection and prediction of various cancers (to date, oral, breast, and bladder cancers) [30,41,42]. The rs5751129 of XRCC6 was similar to such cases as rs828907 of XRCC5, associated with oral cancer and pterygium [35,43]. Involvement of these SNPs in other human cancers and related diseases warrant further investigation and may serve as pharmacogenomic targets for concocting personalized drugs.

Some DNA repair genes in the same and other subpathways, such as O(6)-methylguanine-DNA methyltransferase (MGMT) in direct removal pathway [44,45], XRCC1 in base excision repair [46], ERCC 1 and 2 in nucleotide excision repair (NER) [47,48], hMSH2 in mismatch repair [46], and hHR23A in HR [47] are all seen as anticancer candidate targets. Henceforth, XRCC4, XRCC5 and XRCC6 may be added to the list above. Association studies of XRCC4, XRCC5, and XRCC6 genotypes in other cancers and cancer-related diseases all supported the concept of NHEJ and DNA repair system playing a key role in

human carcinogenesis, such as in glioma [49,50], skin cancer [51], head and neck cancers [33–37,52,53], colorectal cancer [54], lung cancer [55,56], breast cancer [41,47–49,57–59], renal cell carcinoma [60,61], bladder cancer [42,62–64], and myeloma [65]. It may also be of importance for anticancer drugs inducing DSBs in feasibility of cotherapy. Alternatively, cotreatments of DNA-damaging agents and radiation play a central role besides other cancer treatment modalities. Balance between damage and capacity of repair mechanisms determines the final therapeutic outcome. The capacity of cells to complete DNA repair mechanisms is vital to therapeutic resistance and impacts therapeutic efficacy. Thus, pharmacologic inhibition of recently detected DNA repair targets with small-molecule compounds has the potential to enhance cytotoxicity of anticancer agents. Futami and colleagues [66] found inhibited gene expression associated with chromosome stabilization inducing cancer cell-specific apoptosis and inhibit cell proliferation.

7. Current and future works in the war against cancer

The story of “one size fits all” should be never spread; pharmacogenomics is the most fundamental element for individualized therapy and medicine. It is promising to know that the potential of translational medical science has become reality in the field of pharmacogenomics, with classical examples UGT1A1 and irinotecan, thiopurine methyltransferase (TMPT) and thiopurine, CYP2D6 and tamoxifen. Yet the fight against cancer is barely underway. Terry Fox Cancer Research Lab at the China Medical University Hospital teamed with many outstanding scientists and surgeons in establishing and perfecting an oral cancer mouse model by Dr. N.W. Chang, unique pairs of oral cancer patient primary cultured cells from both tumor and portal sites (as far apart as possible) of experimental animal and human patients by ourselves. We have deeply devoted ourselves at all times to the phenotype and genotype correlation study, e.g., individual phenotypic NHEJ capacity and XRCC4, XRCC5, or XRCC6 haplotype analysis. A promising genotype-phenotype correlation platform could reveal the network of all biosignaling from genomics, transcriptomics, and proteomics to functional levels. We visited the M. D. Anderson Cancer Center in Dallas, Texas, to gain experience in genome-wide, high-level gene-gene and gene-environment analyzing system, contributing all of our efforts to make cancer history. We believe that further incorporation and integration of genotype-phenotype analysis, population-based tissue and blood functional measurements, clinical outcome records, and especially in chemo- and radiotherapy response, are highly respected for international studies of ethnic variation using these pharmacogenomic biomarkers. The integration of pharmacogenomic, phenotypic, and pathologic biomarkers is pivotal in cancer risk prediction, along with personalized medicine and therapy evaluation.

The war against cancer is far from won; all oncologists from bench to bed face colossal tasks. All the above knowledge of these pharmacogenomic biomarkers may provide new

directions and practical tools for personalized medicine. Learning about specific/critical SNPs, especially those in exons and genetic polymorphisms, may lead to alterations in protein structures (the so-called nonsynonymous SNPs). Scientists in bioinformatics may perform molecular dynamic simulation of docking sites between target proteins, gaining insight into the impact of these SNPs on structural changes. Quantitative structure-activity relationships can aid in quantitative analyzing impact of non-synonymous polymorphisms on functions of target protein. These methods could supply powerful and practical tools for high-speed screening of synthetic and natural compounds; deduced data can be applied to molecular design of new drugs.

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Editorial

Biomedicine offers advanced medical findings

It is well established that traditional Chinese medicine (TCM) not only plays a key role in Eastern societies, but also looms as a major medical alternative in the West. Furthermore, mounting evidence indicates that the herbs used in TCM are capable of important pharmacological effects. This has led to the development of Chinese herbal medicines, which are also known as Chinese materia medica (CMM). The database regarding the molecular targets, metabolic pathways, and herb-drug interactions in CMM has recently been enriched and improved, making investigations of CMM for current and future clinical therapies both evident and attractive. It is a great pleasure to introduce the second issue of this journal that focuses on the contributions of CMM.

Several articles herein cite new findings or summarize recent evidence that bolsters the significance of CMM. Medicinal herbs or plants like *Allium sativum* L., *Angelica sinensis*, *Plantago major* L., *Thymus vulgaris*, and *Ligustrum lucidum* F. have been widely applied to prevent and remedy infections, the common cold, heart disease, diabetes, immune disorders, and cancer. The multiple medical benefits of these herbs and plants emanate from components such as allyl methyl trisulfide, butylidenephthalide, diallyl trisulfide, ursolic acid, and pentacyclic triterpenes. The mechanisms of these compounds can be partially explained by, but not limited to, antioxidative, -inflammatory, -glycative and -proliferative activities. Butylidenephthalide, a major component of *A. sinensis*, can cross the blood-brain barrier and could potentially be used to combat brain cancer. *A. sativum* L. contains organosulfur compounds that modulate drug-metabolizing enzymes and membrane transporter activities; thus, it could play a leading or auxiliary role in the metabolism of medicinal herbs. Triterpenes, via their antiglycative activities, may be effective agents that could prevent or attenuate pathological progression in glycation-associated diseases such as diabetes and aging. These review articles elucidate the active components, functions, possible modes of action, and potential applications of these herbs and plants.

In addition, the whole genome expression profiles of some herbs have been explored in past decades, yielding solid biomedical evidence in support of the functions and value of

these herbs; most notably, studies on genomic expression could explain their pharmacological effects and mechanisms (i.e., why and how an herb works). Furthermore, the genetic profiles of herbs could be also used for species selection, quality control, and cultivation management when they are planted. Thus, a concise review regarding the development of the microarray-based gene expression database of CMM is welcomed as a bridge linking traditional CMM knowledge and new scientific evidence.

Obesity a crucial risk factor in the development of heart failure. The activation of cardiac Fas- and mitochondria-dependent apoptotic pathways deteriorates molecular, cellular, and even cardiac tissue functions, which in turn promote cardiovascular mortality. Obviously, management of excessive weight requires more medical attention. Susceptibility to oral cancer is higher in certain persons with nonhomologous end-joining genes. The development of personalized medicine is increasingly focused on special diagnostic techniques, while genetic therapies are currently undergoing redesigns to satisfy patient requirements. Because these topics are so vital to personalized medicine, experts in these fields are invited to discuss their thoughts.

This journal aims to supply the most up-to-date information in the field of biomedicine. All of the members of our editorial board strive to attain this goal. We appreciate all suggestions and comments. Finally, researchers, scholars, and experts are welcome to share their research and clinical findings with us. This journal looks forward to receiving valuable manuscripts.

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INSTRUCTIONS TO AUTHORS

BioMedicine aims to publish high quality scientific research in the field of translational and personalized medicine, with the goal of promoting and disseminating medical science knowledge to improve global health.

Articles on clinical, laboratory and social research in translational and personalized medicine and related fields that are of interest to the medical profession are eligible for consideration. Review articles, original articles, case reports, short communications, and letters to the editor are accepted. The journal is published quarterly, with a total of four issues a year.

The Editorial Board requires authors to be in compliance with the *Uniform Requirements for Manuscripts Submitted to Biomedical Journals* (URMs); current URMs are available at <http://www.icmje.org>.

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1.1. Important Information

- Articles submitted should be in Microsoft Word document format and prepared in the simplest form possible. We will add in the correct font, font size, margins and so on according to the journal's style.
- You may use automatic page numbering, but do NOT use other kinds of automatic formatting such as footnotes, headers and footers.
- Put text, references, and table/figure legends in one file.
- Figures must be submitted separately as picture files, at the correct resolution. The files should be named according to the figure number, e.g., "Article1_Fig1", "Article1_Fig2". Also see Section 9.7. below.

1.2. Supporting Documents

The following documents must be included (refer also to the Checklist that follows these author instructions):

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- (6) Consolidated Standards of Reporting Trials (CONSORT) flow chart for randomized controlled trials submitted for publication. Also see Section 4 below.
- (7) Articles where human subjects can be identified in descriptions, photographs or pedigrees must be accompanied by a signed statement of informed consent to publish (in print and online) the descriptions, photographs and pedigrees from each subject who can be identified. Also see Section 5 below.
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2. Disclosure of Conflicts of Interest

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3. Ethical Approval of Studies and Informed Consent

For human or animal experimental investigations, appropriate institutional review board or ethics committee approval is required, and such approval should be stated in the methods section of the manuscript. For those investigators who do not have formal ethics review committees, the principles outlined in the Declaration of Helsinki should be

followed (World Medical Association. *Declaration of Helsinki: ethical principles for medical research involving human subjects*. Available at: <http://www.wma.net/en/30publications/10policies/b3/index.html>).

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4. Reporting Clinical Trials

All randomized controlled trials submitted for publication should include a completed Consolidated Standards of Reporting Trials (CONSORT) flow chart (available at <http://www.consort-statement.org>). This Journal has adopted the proposal from the International Committee of Medical Journal Editors (ICMJE) that require, as a condition of consideration for publication of clinical trials, registration in a public trials registry. Purely observational studies (those in which the assignment of the medical intervention is not at the discretion of the investigator) do not require registration. Further information can be found at <http://www.icmje.org>.

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A signed statement of informed consent to publish (in print and online) patient descriptions, photographs and pedigrees should be obtained from all subjects (parents or legal guardians for minors) who can be identified (including by the subjects themselves) in such written descriptions, photographs or pedigrees. Such persons should be shown the manuscript before its submission. Omitting data or making data less specific to de-identify patients is acceptable, but changing any such data is not acceptable.

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Submitted manuscripts are considered with the understanding that they have not been published previously in print or electronic format (except in abstract or poster form) and are not under consideration in totality or in part by another publication or electronic medium.

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Articles should be written in English (using American English spelling) and meet the following basic criteria: the material is original, the information is important, the writing is clear and concise, the study methods are appropriate, the data are valid, and the conclusions are reasonable and supported by the data.

8. Article Categories

8.1. Review Articles

These should aim to provide the reader with a balanced overview of an important and topical subject in the field, and should be systematic and critical assessments of literature and data sources. They should cover aspects of a topic in which scientific consensus exists as well as aspects that remain controversial and are the subject of ongoing scientific research. All articles and data sources reviewed should include information about the specific type of study or analysis, population, intervention, exposure, and tests or outcomes. All articles or data sources should be selected systematically for inclusion in the review and critically evaluated.

By invitation only. The format for review articles will be jointly decided by the Editors and the contributing author. Typical length: no more than 4000 words, 50–100 references.

8.2. Original Articles

These may be randomized trials, intervention studies, studies of screening and diagnostic tests, laboratory and animal studies, cohort studies, cost-effectiveness analyses, case-control studies, and surveys with high response rates, which represent new and significant contributions to the field.

Section headings should be: Abstract, Introduction, Methods, Results, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

The Introduction should provide a brief background to the subject of the paper, explain the importance of the study, and state a precise study question or purpose.

The Methods section should describe the study design and methods (including the study setting and dates, patients/participants with inclusion and exclusion criteria, or data sources and how these were selected for the study, patient samples or animal specimens used, explain the laboratory methods followed), and state the statistical procedures employed in the research.

The Results section should comprise the study results presented in a logical sequence, supplemented by tables and/or figures. Take care that the text does not repeat data that are presented in tables and/or figures. Only emphasize and summarize the essential features of any interventions, the main outcome measures, and the main results.

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8.3. Case Reports

These are short discussions of a case or case series with unique features not previously described that make an important teaching point or scientific observation. They may describe novel techniques, novel use of equipment, or new information on diseases of importance. Section headings should be: Abstract, Introduction, Case Report, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

The Introduction should describe the purpose of the report, the significance of the disease and its specificity, and briefly review the relevant literature.

The Case Report should include the general data of the case, medical history, family history, chief complaint, present illness, clinical manifestation, methods of diagnosis and treatment, and outcome.

The Discussion should compare, analyze and discuss the similarities and differences between the reported case and similar previously reported cases. The importance or specificity of the case should be restated when discussing the differential diagnoses. Suggest the prognosis of the disease and possibility of prevention. Typical length: no more than 1500 words, 20–40 references.

8.4. Short Communications

These should be concise presentations of clinical or preliminary experimental results. Section headings should be: Abstract, Introduction, Methods, Results, Discussion, Acknowledgments (if applicable), Conflicts of Interest (if any), and References.

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Letters are welcome in response to previously published articles, and may also include interesting cases that do not meet the requirement of being truly exceptional, as well as other communications of general interest. Letters should have a title and include appropriate references, and include the corresponding author's mailing and e-mail addresses. Letters are edited, sometimes extensively, to sharpen their focus. They may be sent for peer review at the discretion of the Editors. Letters are selected based on clarity, significance, and space. Typical length: no more than 600 words, 5–10 references; 1 table and/or 1 figure may be included.

8.6. Editorials

Editorials are invited articles or comments concerning a specific paper in the Journal or a topical issue in the field. While normally invited, unsolicited editorials may be submitted. Typical length: no more than 1500 words, 15–30 references.

9. Manuscript Preparation

Text should be typed double-spaced on one side of white A4 (297 × 210 mm) paper, with outer margins of 2.5 cm. A manuscript should include a title page, abstract, text, acknowledgments (if any), conflicts of interest statement (if any), references, and figures and tables as appropriate. Each section of the manuscript should begin on a new page. Pages should be numbered consecutively, beginning with the title page.

9.1. Title Page

The title page should contain the following information (in order, from the top to bottom of the page):

- category of paper
- article title
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Abstracts should be no more than 300 words in length. Abstracts for Original Articles should be structured, with the section headings: Background/Introduction, Purpose(s)/Aim(s), Methods, Results, Conclusion. Abstracts for Case Reports are unstructured, but should include the significance and purpose of the case presentation, the diagnostic methods of the case, the key data, and brief comments and suggestions with regard to the case. Abstracts for Review Articles and Short Communications should also be unstructured. No abstract is required for Letters to the Editor and Editorials. For the article categories that require an abstract, 3–5 relevant keywords should also be provided in alphabetical order.

9.3. Main Text

The text for Original Articles should be organized into the following sections: Background/Introduction, Purpose(s)/Aim(s), Methods, Results and Discussion. Sections for Case Reports are: Introduction, Case Report, and Discussion. Each section should begin on a new page.

9.3.1. Abbreviations

Where a term/definition will be continually referred to, it must be written in full when it first appears in the text, followed by the subsequent abbreviation in parentheses. Thereafter, the abbreviation may be used. An abbreviation should not be first defined in any section heading; if an abbreviation has previously been defined in the text, then the abbreviation may be used in a subsequent section heading. Restrict the number of abbreviations to those that are absolutely necessary.

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Système International (SI) units must be used, with the exception of blood pressure values which are to be reported in mmHg. Please use the metric system for the expression of length, area, mass, and volume. Temperatures are to be given in degrees Celsius.

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9.3.4. Statistical requirements

Statistical analysis is essential for all research papers except case reports. Use correct nomenclature of statistical methods (e.g., two sample t test, not unpaired t test). Descriptive statistics should follow the scales used in data description. Inferential statistics are important for interpreting results and should be described in detail.

All p values should be expressed to 2 digits to the right of the decimal point, unless $p < 0.01$, in which case the p value should be expressed to 3 digits to the right of the decimal point. The smallest p value that should be expressed is $p < 0.001$, since additional zeros do not convey useful information; the largest p value that should be expressed is $p > 0.99$.

9.3.5. Personal communications and unpublished data

These sources cannot be included in the references list but may be described in the text. The author(s) must give the full name and highest academic degree of the person, the date of the communication, and indicate whether it was in oral or written (letter, fax, e-mail) form. A signed statement of permission should be included from each person identified as a source of information in a personal communication or as a source for unpublished data.

9.4. Acknowledgments and Conflicts of Interest Statement

General acknowledgments for consultations, statistical analysis, etc., should be listed concisely at the end of the text, including the names of the individuals who were directly involved. Consent should be obtained from those individuals before their names are listed in this section. All financial and material support for the research and work from internal or external agencies, including commercial companies, should be clearly and completely identified. Ensure that any conflicts of interest (financial and/or non-financial) are explicitly declared.

9.5. References

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- References should be indicated by numbers in square brackets in line with the text, and numbered consecutively in order of appearance in the text.
- References cited in tables or figure legends should be included in sequence at the point where the table or figure is first mentioned in the main text.
- Do not cite uncompleted work or work that has not yet been accepted for publication (i.e., "unpublished observation", "personal communication") as references. Also see Section 9.3.5. above.
- Do not cite abstracts unless they are the only available reference to an important concept.

9.5.2. In the references section

- References should be limited to those cited in the text and listed in numerical order, NOT alphabetical order.
- References should include, in order, author surnames and initials, article title, abbreviated journal name, year, volume and inclusive page numbers. The last names and initials of all the authors up to 6 should be included, but when authors number 7 or more, list the first 6 authors only followed by "et al". Abbreviations for journal names should conform to those used in MEDLINE.
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Examples are given below.

Standard journal article

Chen Z, Fan M, Bian Z, Zhang Q, Zhu Q, Lu P. Immunolocalization of heat shock protein 70

during reparative dentinogenesis. *Chin J Dent Res* 2000;3:50–5.

Journal supplement

Kaplan NM. The endothelium as prognostic factor and therapeutic target: what criteria should we apply? *J Cardiovasc Pharmacol* 1998;32(Suppl 3):S78–80.

Journal article not in English but with English abstract

Nakayama H, Ishikawa T, Yamashita S, Fukui I, Mutoh T, Hikichi K, et al. CSF leakage and anosmia in aneurysm clipping of anterior communicating artery by basal interhemispheric approach. *No Shinkei Geka* 2011;39:263–8. [In Japanese, English abstract]

Book

Bradley EL. Medical and surgical management. Philadelphia: Saunders; 1982, p. 72–95.

Book chapter in book with editor and edition

Greaves M, Culligan DJ. Blood and bone marrow. In: Underwood JCE, editor. General and systematic pathology. 4th ed. London: Churchill Livingstone; 2004, p. 615–72.

Bulletin

World Health Organization. World health report 2002: reducing risk, promoting healthy life. Geneva, Switzerland: World Health Organization; 2002.

Company/manufacture publication/pamphlet

Eastman Kodak Company, Eastman Organic Chemicals. Catalog no. 49. Rochester, NY: Eastman Kodak; 1977, p. 2–3.

Electronic publications

Duchin JS. Can preparedness for biological terrorism save us from pertussis? *Arch Pediatr Adolesc Med* 2004;158:106–7. Available from: <http://archpedi.ama-assn.org/cgi/content/full/158/2/106>. Accessed June 5, 2004.

Smeeth L, Iliffe S. Community screening for visual impairment in the elderly. *Cochrane Database Syst Rev* 2002(2):CD001054. doi:10.1002/14651858.CD1001054.

Items presented at a meeting but not yet published

Durbin D, Kallan M, Elliott M, Arbogast K, Cornejo R, Winston F. Risk of injury to restrained children from passenger air bags. Paper presented at: 46th Annual Meeting of the Association for the Advancement for Automotive Medicine; September 2002; Tempe, AZ.

Greenspan A, Eerdeken M, Mahmoud R. Is there an increased rate of cerebrovascular events among dementia patients? Poster presented at: 24th Congress of the Collegium Internationale Neuro-Psychopharmacologicum (CINP); June 20–24, 2004; Paris, France.

Khuri FR, Lee JJ, Lippman SM. Isotretinoin effects on head and neck cancer recurrence and second primary tumors. In: Proceedings from the American Society of Clinical Oncology; May 31–June 3, 2003; Chicago, IL. Abstract 359.

Item presented at a meeting and published

Cionni RJ. Color perception in patients with UV- or blue-light-filtering IOLs. In: Symposium on Cataract, IOL, and Refractive Surgery. San Diego, CA: American Society of Cataract and Refractive Surgery; 2004. Abstract 337.

Material accepted for publication but not yet published

Carrau RL, Khidr A, Crawley JA, Hillson EM, Davis JK, Pashos CL. The impact of laryngopharyngeal reflux on patient-reported quality of life. *Laryngoscope*. In press.

Ofri D. Incidental findings: Lessons from my patients in the art of medicine. Boston, MA: Beacon Press. In press.

Theses and dissertations

Undeman C. Fully automatic segmentation of MRI brain images using probabilistic diffusion and a watershed scale-space approach [master's thesis]. Stockholm, Sweden: NADA, Royal Institute of Technology; 2001.

Ayers AJ. Retention of resin restorations by means of enamel etching and by pins [dissertation]. Indianapolis: Indiana University; 1971.

Website

American Association of Oral and Maxillofacial Surgeons. Wisdom teeth. AAOMS Web site. http://www.aaoms.org/wisdom_teeth.php. Published January 23, 2008. Updated March 9, 2009. Accessed November 15, 2009.

9.6. Tables

Tables should supplement, not duplicate, the text. They should have a concise table heading, be self-explanatory, and numbered consecutively in the order of their citation in the text. Information requiring explanatory footnotes should be denoted using superscripted lowercase letters in alphabetical order (a, b, c, etc.). Asterisks (*, **) are used only to indicate the probability level of tests of significance. Abbreviations used in the table must be defined and placed after the footnotes. If you include a block of data or table from another source, whether published or unpublished, you must acknowledge the original source.

9.7. Figures

9.7.1. General guidelines

The number of figures should be restricted to the minimum necessary to support the textual

material. They should have an informative figure legend and be numbered in the order of their citation in the text. All symbols and abbreviations should be defined in the legend. Patient identification should be obscured. All lettering should be done professionally and should be in proportion to the drawing, graph or photograph. Photomicrographs must include an internal scale marker, and the legend should state the type of specimen, original magnification and stain.

Figures must be submitted as separate picture files at the correct resolution (see Section 9.7.2. below). The files should be named according to the figure number, e.g., "Article1_Fig1", "Article1_Fig2".

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Regardless of the application used, when your electronic artwork is finalized, please "save as" or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

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