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Biological Reactive Intermediates (BRIs) Formed from Botanical Dietary Supplements

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Abstract

The use of botanical dietary supplements is increasingly popular, due to their natural origin and the perceived assumption that they are safer than prescription drugs. While most botanical dietary supplements can be considered safe, a few contain compounds, which can be converted to reactive biological reactive intermediates (BRIs) causing toxicity. For example, sassafras oil contains safrole, which can be converted to a reactive carbocation forming genotoxic DNA adducts. Alternatively, some botanical dietary supplements contain stable BRIs such as simple Michael acceptors that react with chemosensor proteins such as Keap1 resulting in induction of protective detoxification enzymes. Examples include curcumin from turmeric, xanthohumol from hops, and Z-ligustilide from dang gui. Quinones (sassafras, kava, black cohosh), quinone methides (sassafras), and epoxides (pennyroyal oil) represent BRIs of intermediate reactivity, which could generate both genotoxic and/or chemopreventive effects. The biological targets of BRIs formed from botanical dietary supplements and their resulting toxic and/or chemopreventive effects are closely linked to the reactivity of BRIs as well as dose and time of exposure.

Keywords

Biological reactive intermediates; botanical dietary supplements; chemoprevention; electrophiles; epoxides; carbocations; nitrenium ions; quinones

Introduction

The use of botanical dietary supplements has increased exponentially in recent years. Approximately 60% of Americans use some form of supplements regularly and 1 in 6 report using botanical dietary supplements [1]. They are often perceived to be safer than prescription drugs due to their “natural” origin, long-standing ethnomedical use, and over-the-counter availability. However, unlike pharmaceuticals that must undergo extensive clinical trials prior to FDA approval, dietary supplements have little regulation of either efficacy or safety in the USA [2, 3]. Over-the-counter availability often leads to over dosing and to severe interactions when taken together with other medications. In addition, several of these botanicals are currently prepared using different procedures than traditional extraction methods often leading to higher concentrations of certain potentially toxic compound classes. In particular, a significant number of these herbal products have been associated with hepatotoxic effects, resulting from consumption of high doses of the supplements and/or when used in combination with other medications [4-6]. The ultimate

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toxin is often a biological reactive intermediate (BRI) either an electrophile or free radical formed by oxidative metabolism. Alternatively, some botanical dietary supplements contain weak BRIs, which could be responsible for their chemopreventive properties. Several examples of botanicals, which form BRIs will be discussed in this review.

Types of BRIs

The most common types of BRIs are shown in Figure 1. The reactivity/selectivity principle can be used to make general predictions on the biological targets of BRIs and their resulting biological effects [7]. BRIs range in reactivity from highly reactive positively charged electrophiles such as carbocations and nitrenium ions, which have fleeting lifetimes to more stable neutral electrophiles such as epoxides, quinoids, and simple Michael acceptors. Highly reactive electrophiles are not selective and react indiscriminately with the most convenient biological nucleophiles, which are usually those present at the site of formation of the BRI (e.g., synthesizing enzyme) and/or those present in highest concentration (e.g., water). The carbocations and nitrenium ions fall into this category. On the other hand, more stable electrophiles are more selective and generally prefer reaction at sulfur nucleophilic groups on proteins and with GSH.

The reactivity/selectivity of BRIs has a major influence on their toxicity and/or chemopreventive properties (Table 1, Figure 2). While BRI's can cause toxicities, BRI's with modest reaction rates, at low concentrations are able to activate natural detoxification responses leading to chemoprevention/cytoprotection [8]. Since reactivity, dose, time and the resulting chemopreventive or toxic effects are closely related (Table 1, Figure 2), predictions about the biological effects of BRIs can be difficult. What follows are specific examples of BRIs formed from botanical dietary supplements, which may be responsible for their toxic and/or chemopreventive properties.

Sassafras oil

The main constituent of sassafras oil is safrole, which is also present in a number of herbs and spices, such as nutmeg, mace, cinnamon, anise, black pepper, and sweet basil [4]. Sassafras oil is extracted from the root bark of the tree *Sassafras albidum* [9, 10]. It has had a traditional and widespread use as a natural diuretic, as well as a remedy against urinary tract disorders until safrole was discovered to be hepatotoxic and weakly carcinogenic [4, 11]. In 1960 the FDA banned the use of sassafras oil as a food and flavoring additive because of the high content of safrole and its proven carcinogenic effects [12]. Oil of sassafras that is safrole free can still be used as flavoring agent in food [13]. Two bioactivation pathways of safrole to potentially hepatotoxic intermediates have been reported (Figure 3) [14-16]. The first one involves P450 catalyzed hydroxylation of the benzyl carbon producing 1'-hydroxysafrole and conjugation with sulfate generating a reactive sulfate ester. This ester undergoes a S_N1 displacement reaction creating a highly reactive carbocation, which alkylates DNA (Figure 3) [14, 17]. The second pathway involves P450 catalyzed hydroxylation of the methylenedioxy ring and formation of the catechol, hydroxychavicol, which is a natural product found in betel leaf (Figure 3) [14, 18, 19]. Hydroxychavicol can easily be oxidized to the α -quinone which isomerizes nonenzymatically to the more electrophilic p -quinone methide [14]. Both pathways could explain the genotoxic effects of safrole and DNA adducts consistent with the carbocation pathway (Figure 3) have been identified *in vitro* and *in vivo* [20-25]. Recently, studies with betel quid chewers demonstrated that betel quid containing safrole induced DNA adducts are highly associated with the development of oral squamous cell carcinoma (OSCC) in Taiwan [26]. These experiments confirm the genotoxic effects of safrole and thus justify the restrictions made by the FDA and other health authorities. Interestingly, when topically

applied betel leaf extract and hydroxychavicol were evaluated for their preventive activity on 7,12-dimethylbenz(a)anthracene (DMBA) induced skin tumors in two mice strains, the extract as well as hydroxychavicol reduced DMBA induced tumor formation [27]. These studies demonstrate that toxic and/or chemopreventive activities of quinones, such as the quinones formed from hydroxychavicol, might be closely related.

Aristolochia fangchi

Aristolochic acid (Figure 4), a nitrophenanthrene carboxylic acid, is a natural product found in the plant *Aristolochia fangchi*. The roots of this plant were found in a number of products sold as “traditional herbal medicines”, as dietary supplements, or weight-loss remedies [28]. In Belgium in the early 1990s, the use of slimming products including Chinese herbs lead to an endemic nephropathy with permanent kidney damage, including end-stage kidney failure requiring dialysis or kidney transplantation [29, 30]. The same symptoms occurred in patients, who were exposed to botanical preparations containing aristolochic acid, in other European and Asian countries as well as in the United States [31]. In addition, aristolochic acid can induce cancer primarily in the urinary tract as observed in some of the patients taking the slimming products [32, 33]. Further analysis of the dietary supplements revealed that at least in some of these products the roots of *Stephania tetrandra* were inadvertently substituted with the roots of *Aristolochia fangchi*, because of the close similarity of the Chinese names [34]. It has been reported that the first step of metabolic bioactivation of aristolochic acid involves reduction of the NO₂ group catalyzed by P450 reductase (Figure 4) [31, 35-37]. The amino- and carboxyl groups cyclize producing an aristolactam, which is N-hydroxylated. The N-hydroxylactam intermediate could be further metabolized by Phase II enzymes, such as sulfotransferases, leading to the formation of reactive sulfate esters, which undergo S_N1 displacement to produce the ultimate carcinogenic and electrophilic nitrenium ion [38]. These highly reactive BRIs forms stable covalent DNA adducts at the exocyclic amino groups of adenine and guanine [29, 39, 40]. These mutagenic lesions lead to AT – TA transversions *in vitro*, which were identified in high frequency in codon 61 of the c-Ha-ras oncogene in tumors of rodents [41]. Based on all these findings the International Agency of Research on Cancer classified *Aristolochia* species as human carcinogens [42] and the FDA issued warnings about the use of products that may contain aristolochic acid [43].

Pennyroyal oil

Pennyroyal oil derived from *Mentha pulegium* or *Hedeoma pulegoides* has been used as an abortifacient and as an insect repellent since Roman times [44]. Despite reports of hepatotoxicity, pennyroyal products have been purported to purify blood, alleviate menstrual symptoms, feverish colds, and to act as a digestive [45, 46]. While no apparent evidence exists for these claimed beneficial activities, there is strong data supporting the hepatotoxic effects of pennyroyal oil [47-50]. Small quantities can cause acute liver and lung damage and 10-15 mL of the oil have resulted in death [51]. As a result, most pennyroyal preparations, including the oil, contain warnings of possible toxicity. The oil primarily contains pulegone plus smaller amounts of several other monoterpenes (menthone, iso-menthone, and neomenthone) that are encountered in mint species [52]. *In vivo* metabolism of pulegone is extremely complex, generating dozens of metabolites in urine and bile of treated animals [47]. The major bioactivation step involves the oxidation of pulegone by cytochrome P450s to its proximate hepatotoxic metabolite menthofuran (Figure 5) [53-56]. Subsequently, menthofuran is oxidized to an epoxide which is likely the ultimate toxic BRI (Figure 5) [48]. It has been shown that the epoxide was capable of covalently bind to GSH and proteins causing hepatic injury [48, 52, 57, 58]. Evidence from animal experiments suggests that N-acetylcysteine provides at least partial protection from the hepatotoxic effects of pennyroyal oil [52].

Black Cohosh

Black cohosh root extract have a long tradition in native American Indian culture [59]. Extracts have been used against snake bites, women complaints, malaria, fever, rheumatism, cough, inflammation. Black cohosh dietary supplements are currently the most frequently used dietary supplements for the alleviation of menopausal symptoms, in particular hot flashes [60, 61]. However, recently hepatotoxic reactions have been described [62]. Thirty case reports on use of black cohosh products concerning liver damage were reported and analyzed [63]. Only possible causality, but no probable or certain causality have been described for liver toxicities observed in patients taking black cohosh dietary supplements.

Black cohosh extracts have been screened for their potential to form electrophilic BRIs which could be trapped as their GSH conjugates and analyzed using an ultrafiltration mass spectrometric assay [64]. On the basis of their propensity to form GSH adducts following metabolic activation by hepatic microsomes and NADPH in vitro, a total of eight electrophilic metabolites of black cohosh were detected, including quinoid metabolites of fukinolic acid, fukiic acid, caffeic acid (Figure 6), and cimracemate B. Mercapturates (N-acetylcysteine conjugates) corresponding to the GSH conjugates identified during screening were synthesized and characterized using LC-MS/MS with product-ion scanning. During a phase I clinical trial of black cohosh in perimenopausal women, urine was collected from seven subjects, each of whom took a single oral dose of either 32, 64, or 128 mg of the black cohosh extract. These urine samples were analyzed for the presence of mercapturate conjugates using positive-ion electrospray LC-MS and LC-MS/MS. However, mercapturate conjugates of these black cohosh constituents were not detected in urine samples from women even at the highest dose. Therefore, for moderate doses of a dietary supplement containing black cohosh, this study found no cause for safety concerns over the formation of quinoid metabolites from black cohosh in women.

Kava

Kava (*Piper methysticum*) has been used by the South Pacific Islanders for centuries for spiritual services and as an intoxicating beverage [65]. Kava is marketed in North America as a sedative, muscle relaxant, anesthetic, and anticonvulsant [66, 67]. Kava lactones, such as kawain and methysticin (Figure 7), are considered to be the active constituents of kava extracts responsible for their sedative and anxiolytic effects [66]. However, largely due to reports of hepatotoxic effects, several European countries and Canada have banned or severely restricted the sale of kava containing products [51, 68]. In the USA, the FDA has issued warnings although no restrictions have been placed on the sale of Kava dietary supplements [68].

The mechanism of kava hepatotoxicity is not completely understood; however, it likely involves bioactivation of the methylenedioxy kava lactones to electrophilic α -quinones, which can react with glutathione (GSH) and/or covalently modify proteins [68]. Incubation of a methanol extract of kava roots with hepatic microsomes in the presence of GSH showed the formation of two GSH conjugates consistent with trapping of the α -quinones from methysticin and 7,8-dihydromethysticin (Figure 7) [68]. This and other reports show that kava extracts rich in kava lactones can cause GSH depletion and therefore put undue stress on the liver [69]. Interestingly, it has been reported that the traditional extraction method, which involves maceration of the roots in a water and coconut milk solution, extracts less kava lactones from the rhizomes than observed in commercial products, which are usually 96% ethanol or acetone extracts [65, 69]. This suggests that a reduction of kava lactones in kava products and an optimization of extract preparation could reduce the hepatotoxic potential of kava. Alternatively, kava quinones could be responsible for potential

chemopreventive properties of Kava [70] through induction of detoxification enzymes and/or through anti-inflammatory pathways (see below).

Hops

The strobiles of hops (*Humulus lupulus* L.), Cannabaceae, (hops) are popular as a flavoring agent in beer and have been traditionally used as remedies for mood and sleep disturbances in Europe [71]. Recently, hops extracts have been studied as botanical alternatives to hormone replacement therapy for the relief of menopausal symptoms mainly due to the potent phytoestrogen, 8-prenylnaringenin [72-78]. In addition, the cytoprotective properties of hops and its most abundant prenylated chalcone, xanthohumol, have also been analyzed (Figure 8A) [79-82]. XH is a pleiotropic agent with multiple biological activities, for example induction of NAD(P)H quinone oxidoreductase 1 (NQO1) [83]. The expression of NQO1 and other detoxifying enzymes are known to be upregulated through the Keap1/Nrf2 pathway in response to weak BRIs and/or oxidative stress. In unstressed cells, Keap1 forms a complex with Nrf2 and facilitates the degradation of Nrf2 in the cytoplasm (Figure 8A). It has been shown that the Michael acceptor xanthohumol in hops alkylates human Keap1 protein and increases ARE reporter activity in a dose-dependent manner [83, 84]. The three most readily modified cysteines of human Keap1 by xanthohumol were identified as C151, C319, and C613 [85]. Taken together, the modification of cysteines by Michael addition reaction acceptors such as xanthohumol in Keap1 seem to play a critical role in the Keap1-Nrf2 signaling system leading to upregulation of ARE regulated detoxification enzymes (Figure 8A).

Dang gui

The dried roots of dang gui (*Angelica sinensis*) (Oliv.) diels, Apiaceae have been used for centuries as “women’s tonic” especially for alleviating menstrual disorders or menopausal symptoms in Asia [86-92]. Cytoprotective properties of dang gui have been described including antioxidative, cancer preventive, and overall reduction in oxidative stress [88, 93]. For example, it has been reported that lipophilic extracts, as well as *n*-butyridenephthalide isolated from the chloroform extract exert antiproliferative effects on tumor cells *in vitro* and *in vivo* [94-97]. In addition, recent data revealed that *Z*-ligustilide, the major lipophilic constituent in *A. sinensis*, reduces oxidative stress in brain tissue possibly through increasing antioxidative enzymes, such as glutathione peroxidase and superoxide dismutase, and reducing apoptotic markers [91, 98].

Recent mechanism of action studies revealed that *A. sinensis* extracts contain the simple Michael acceptor *Z*-ligustilide that can induce the detoxification enzyme NQO1 [99] (Figure 8B). Incubation of *Z*-ligustilide with GSH and subsequent LC-MS/MS analysis revealed that *Z*-ligustilide covalently modified GSH. In addition, using MALDI-TOF mass spectrometry and LC-MS/MS, it was demonstrated that the lipophilic extracts and ligustilide alkylated important cysteine residues in human Keap1 protein, such as C151, thus activating Nrf2 and transcription of ARE regulated genes. *Z*-ligustilide’s activity can be explained by the $\alpha,\beta,\gamma,\delta$ -unsaturated lactone moiety with the cross-conjugated alkene system (Figure 8B) reacting in a Michael fashion with nucleophiles, such as sulfhydryl groups in Keap1 protein.

Conclusions

Although most botanical dietary supplements have health benefits and can be considered safe, a few contain compounds, which can be converted to BRIs causing toxicity in some circumstances or chemoprevention in others. Examples shown here illustrate the formation of BRIs of differing reactivity including carbocations (safrole), nitrenium ions (aristolochic acid), epoxides (pennyroyal oil), quinoids (safrole, black cohosh, kava), and simple Michael

acceptors (hops, dang gui) (Table 1). The botanical dietary supplements, which have been associated with mutagenic/carcinogenic effects, all contain compounds, which are metabolized to BRIs that can alkylate DNA (safrole, aristolochic acid) (Figure 9). On the other hand, the weak electrophiles represented by the simple Michael acceptors in hops (xanthohumol), dang gui (Z-ligustilide), turmeric (curcumin) [100] are likely responsible for the chemopreventive effects of these botanicals through induction of detoxification enzymes such as NQO1 through the Keap1/Nrf2 pathway (Figure 10). Quinoids (quinones, quinone methides) such as those formed from compounds in sassafras (Figure 3), black cohosh (Figure 6), and/or kava (Figure 7), could represent BRIs with both mutagenic effects through DNA alkylation and/or chemopreventive properties through modification of chemosensor proteins. The overall risk/benefit of botanical dietary supplements could be due to BRI formation and interaction with resulting biological targets, which likely depends on the reactivity/selectivity of the BRI, as well as the dose and time of exposure. Adverse effects and/or chemopreventive effects attributed to consumption of dietary supplements could be the result of oxidative metabolism of some constituents to BRIs. Evaluation of the efficacy and safety of botanical dietary supplements especially for standardization of active or potentially toxic compounds and good manufacturing processes with recommendations for dosing regimens, are warranted.

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Figure 1. Types of BRIs

Figure 2. Reactivity/selectivity principle and BRIs

The reactivity/selectivity of BRIs has a major influence on their toxicity and/or chemopreventive properties [7]. BRIs range in reactivity from highly reactive electrophiles such as carbocations (R^+) and nitrenium ions, which have fleeting lifetimes to more stable neutral electrophiles such as epoxides, quinoids, and simple Michael acceptors. Reactive electrophiles are not selective and react indiscriminately with nucleophiles potentially causing toxicity. Moderately reactive electrophiles, such as epoxides or quinoids, may react with DNA leading to DNA adducts causing mutagenesis and carcinogenesis. Finally, weak electrophiles, such as simple Michael acceptors at low concentrations are able to activate natural detoxification responses leading to chemoprevention/cytoprotection [8].

Figure 3. BRIs formed from safrole in sassafras oil

Figure 4. BRIs formed from aristolochic acid

Figure 5. BRIs formed from pulegone in pennyroyal oil

Figure 6. BRIs formed from black cohosh

Figure 7. BRIs formed from Kava

Z-LIGUSTILIDE

Figure 8. A: BRI formed from Hops and schematic of ARE upregulation of detoxifying enzymes by Michael addition acceptors, such as xanthohumol

In unstressed cells, Keap1 forms a complex with Nrf2 and facilitates the degradation of Nrf2 in the cytoplasm [106]. Michael acceptors, such as xanthohumol, can alkylate Keap1 causing Nrf2 nuclear accumulation [83, 84]. In the nucleus, Nrf2 binds to the ARE with association of small MAF proteins and increases transcription of ARE regulated detoxifying enzymes [107].

B: BRI formed from Dang gui

Figure 9. Targets of mutagenic BRLs

Figure 10. Targets of chemopreventive BRIs

Table 1

Summary of botanical BRIs, toxicity concerns, and resulting FDA actions.

Botanical dietary supplement	Main compound that forms BRIs ^a	Type of BRI ^b	Predicted Toxicity/chemoprevention	FDA	Availability in the USA	Reference
Sassafras oil	Safrole	R ⁺ , Q, QM	Carcinogenic, hepatotoxic	Safrole prohibited	Safrole free sassafras extracts are available	[13, 101]
Aristolochia	Aristolochic acid	N ⁺	Carcinogenic, nephrotoxic	Prohibited	Not available	[43]
Pennyroyal oil	Pulegone	Epoxide	Hepatotoxic	Warning, restricted	Available	[45, 102]
Black cohosh	Caffeic acids	Q	Hepatotoxic/chemoprevention	Some side effects reported	Available	[64, 103]
Kava	Methysticin	Q, MA	Hepatotoxic/chemoprevention	Safety alert	Available	[104]
Hops	Xanthohumol	MA	Chemoprevention	Essential oil recognized as safe by FDA	Available	[81, 83, 105]
Dang gui	Ligustilide	MA	Chemoprevention	No reports	Available	[99]

^aExamples of compounds that form BRIs.

^bR⁺: Carbocation, Q: Quinone, QM: Quinone methide, N⁺: Nitrenium ion, MA: Michael acceptor.