Protection Against Cancer by Plant Phenylpropenoids: Induction of Mammalian Anticarcinogenic Enzymes

A.T. Dinkova-Kostova*

Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

Abstract: Chemoprotection has established itself as a "major arm" in the "war against cancer" and induction of phase 2 detoxification enzymes as an effective strategy. Prominent among inducers are Michael reaction acceptors. Such functionalities are intrinsic to many phenylpropanoids present in edible plants, where they play roles in plant defense. This minireview focuses on the ability of such plant metabolites to elevate phase 2 enzymes in various cell culture and animal models and ultimately to protect against carcinogenesis.

INTRODUCTION

Exposure to electrophiles and reactive oxygen and nitrogen intermediates that arise during carcinogen metabolism and endogenous cellular processes can damage biological macromolecules. The subsequent selection and expansion of clones with increased autonomy and growth advantage can ultimately lead to malignancy. Parallel to the increase in our understanding of carcinogenesis, the belief that cancer is a preventable disease has been embraced by an increasing number of scientists [1]. It is now clear that the discrete events during which a normal cell becomes malignant are several and in most cases take years to develop [2, 3]. This could allow intervention at multiple stages and targets with the ultimate goal of prevention of a neoplastic outcome. Treatment of the established disease, on the other hand, has been proven extremely difficult and clinical progress in this field disappointingly slow. Consequently, chemoprotection directed toward interrupting or even reversing the events leading to neoplasia has emerged as a major "arm" in the "war against cancer" [2, 4-6]. Due to the extensive efforts of many investigators and as pointed out in the Report of the Chemoprevention Working Group, chemoprotection is now not only a basic science, but clinical science as well, and the development of more effective chemoprotective agents is a "principal need" [7].

The evaluation (and use) of plant products as potential chemoprotective agents is particularly attractive, since many of them are already present in the human diet and consumed in substantial quantities. Plants are the primary source (and/or base) of biologically active natural products. Such compounds are often abundant in their tissues. In order to be able to survive and coevolve with other competing organisms, plants have acquired or elaborated distinct "secondary" metabolic pathways, which enabled them to synthesize a large array of compounds with various protective functions, e.g., antioxidant (curcuminoids), cytotoxic (alkaloids), UV-screening (flavonoids), insecticidal, parasiticidal, etc [8, 9]. In the course of evolution mammalian cells have also developed their own defense strategies. The principal cellular nonprotein thiol, glutathione (GSH), which occurs in every living cell at millimolar concentrations, and phase 2 detoxification enzymes represent the two primary lines of defense against acute and chronic toxicities of xenobiotics, as well as against reactive oxygen and nitrogen species Fig. (**1**). In contrast to phase 1 enzymes (e.g., cytochrome P450s), which often catalyze reactions leading to activation of procarcinogens, phase 2 enzymes and GSH actively participate in their detoxification. The fact that the levels of phase 2 enzymes can be coordinately upregulated under certain conditions clearly shows that these systems normally do not operate at their full capacity and constitutes a logical strategy towards chemoprotection. Talalay and Fahey have recently designated these inductions as the "*Phase 2 response*"[10], which is "defined by the following features: (a) coordinate induction by several representatives of the same chemical classes of compounds that also induce classical phase 2 enzymes (e.g., glutathione *S*-transferases, UDP-glucuronosyltransferases); (b) regulation by mechanisms that are similar and may involve common promoters and transcription factors (e.g., ARE and Nrf2, respectively); (c) catalysis of a broad range of other chemical reactions that protect cells against the toxic and neoplastic effects of electrophiles and reactive oxygen species." Many animal models have clearly demonstrated a correlation between dietary intake of plant-derived phase 2 enzyme inducers and protection against toxicity and carcinogenicity. Among plant inducers are isothiocyanates [11], coumarins [12, 13], indoles [14-16]. Attaining chemoprotection by dietary modulation of the activities of these enzymes has been the main focus of this and other laboratories [7, 17-19].

A characteristic feature of the *Phase 2 response* is the participation of an array of proteins catalyzing versatile reactions that are essential for the overall cellular defense (Table 1). In this minireview all enzymes listed in Table 1 will be referred to as phase 2 enzymes, even though some classically have been known as detoxification and others as

*Address correspondence to this author at the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205, USA, Tel: 410-955-3503, Fax: 410-502-6818, email: adkostov@jhmi.edu

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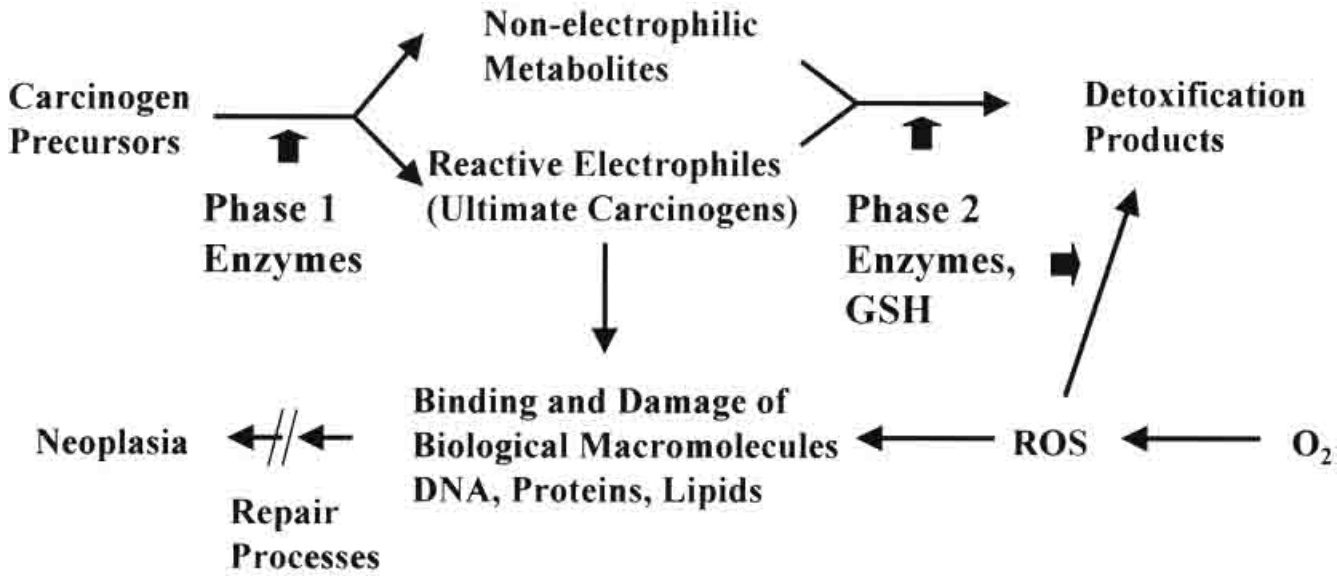


Fig. (1). Role of metabolism in carcinogenesis: activation and detoxification processes.

antioxidant enzymes. Such distinction is now inappropriate, because many perform both functions. Moreover, the antioxidant aspect of phase 2 enzyme induction is now attracting increasing attention [38, 39]. The diverse glutathione transferases (GSTs) constitute a typical example of phase 2 enzymes playing both detoxification, as well as direct antioxidant roles. These enzymes efficiently catalyze the detoxification of various exogenous as well as endogenous electrophiles. Thus, human GST A4-4 inactivates a wide range of nonenals, genotoxic products of lipid peroxidation [40-42]. GST M2-2 can detoxify *ortho*-quinones, some of which are formed during the oxidation of catecholamines [43, 44]. The glutathione peroxidase activity of human GSTs of the classes Alpha, Mu and Theta towards hydroperoxides demonstrates the direct antioxidant activity of these enzymes [45, 46]. This could become especially important under condition of low dietary selenium when the activity of other antioxidant enzymes, such as glutathione peroxidase is reduced.

NAD(P)H:quinone oxidoreductase (QR1, NQO1), which gene expression is coordinately regulated with other phase 2 enzymes, is another enzyme with both detoxification, as well as antioxidant functions. This flavoprotein can directly

Table 1. The Phase 2 Response and its Protective Functions

The Phase 2 response	Protective Function	Reference
Glutathione *S*-transferases	Conjugate electrophiles, detoxify oxidants Reduce peroxides, alkenals	13, 14, 20
Quinone Reductase 1	Prevents quinone redox cycling; Lowers levels of electrophilic quinones	13, 21
UDP-glucuronosyltransferase	Conjugates reactive species	20, 22
Epoxide hydrolase	Hydrolyzes damaging epoxides	14, 20, 23
Dihydrodiol dehydrogenase	Detoxifies epoxides	24
Aflatoxin aldehyde reductase	Detoxifies reactive carcinogen metabolites	13, 25
γ-Glutamylcysteine synthetase	Increases GSH levels	12, 26-28
Glutathione *S*-conjugate efflux pumps	Eliminate glutathione conjugates	29-32
Glutathione reductase	Regenerates reduced glutathione	33
Thioredoxin reductase	Regenerates reduced thioredoxin	34
Heme oxygenase	Generates antioxidants (bilirubin, CO)	35, 36
Ferritin	Sequesters ferrous ions	36
Manganese superoxide dismutase	Detoxifies superoxide	28
Catalase	Detoxifies H_2O_2	28
Leukotriene B_4 dehydrogenase	Suppresses inflammation	37

detoxify quinones by catalyzing their obligatory two-electron reduction, thus diverting them from oxidative cycling [47]. The resulting hydroquinones can be further inactivated, e.g., by glucuronidation, or sulfation [48]. The direct antioxidant function of QR1 has been clearly demonstrated in a quantitative experiment by Prochaska and colleagues who showed that the light emission produced by oxidative cycling of menadione could be quantitatively extinguished by direct addition of crystalline quinone reductase [49]. In addition, QR1 maintains physiologically important quinones, such as coenzyme Q (ubiquinone), vitamin K and vitamin E in their reduced hydroquinone state [50-52]. Furthermore, QR1 and GST can be coordinately induced in human neuroblastoma cells [53] and the involvement of the Nrf-2-dependent activation of the antioxidant response element (see below) in the mechanism of induction has been shown recently [54]. Similar response was observed in glial cultures [55]. When primary cultures of rat astrocytes were exposed to L-Dopa (catecholamine used in the treatment of Parkinson's disease) increases of the levels of QR1 and cellular glutathione were observed [56]. Furthermore, this upregulation was proposed to be a potential strategy for protection against neurotoxicity of such drugs. Induction of QR (and possibly other phase 2 enzymes) and ARE-mediated gene expression in astrocytes has been suggested to be one of the mechanisms by which these cells play neuroprotective role and behind the observation that mild ischemia protects against subsequent severe ischemia [57, 58]. GST M2-2 catalyzes detoxification of the dopamine metabolite, the *ortho*-quinone aminochrome by conjugation with glutathione [44]. Thus, the possibility that QR1 and GST may play roles in protecting the nervous system against oxidative stress is also emerging. Finally, the finding that the same players take part in the regulation of the gene expression of enzymes, classically known as antioxidant (e.g., heme oxygenase, γ-glutamylcysteine

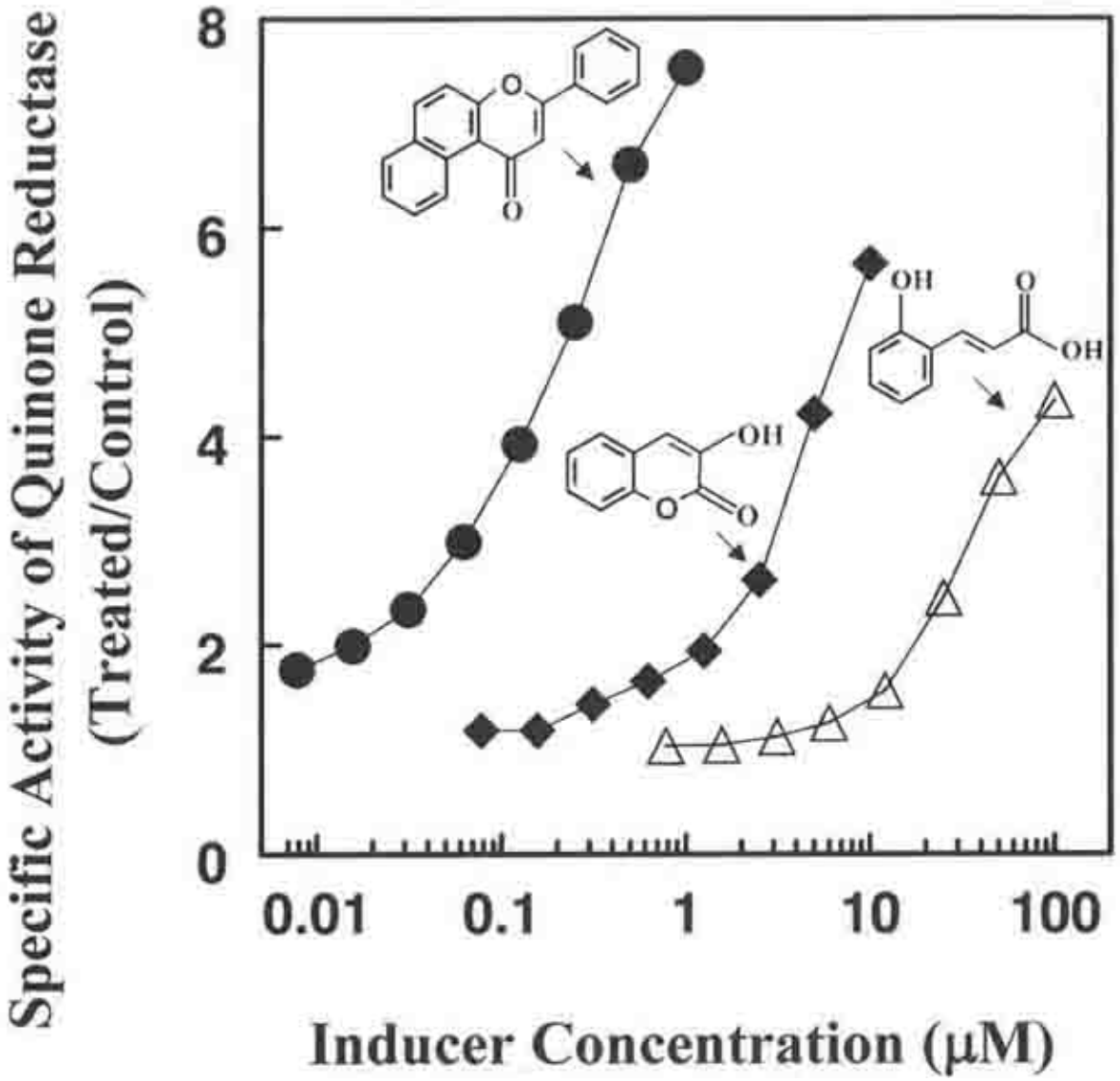


Fig. (2). Induction of quinone reductase in Hepa 1c1c7 murine hepatoma cells by a range of concentrations of a: (·) flavonoid; (♦) coumarin; and (Δ) coumaric acid. Cells were cultured and assays were performed in microtites plates [42, 43]. The specific activities of quinone reductase are expressed as the ratios of the values obtained from treated and control cells. The identities and potencies (CD values) of these inducers are also shown in Tables 2, 4 and 6.

synthetase), have also placed these enzymes in the category of phase 2 proteins [26, 27, 35, 59]. A wide variety of stimuli can lead to the coordinate induction of phase 2 proteins and the genes that encode them share common regulatory elements. The 5'-upstream antioxidant (electrophile) responsive element (ARE/EpRE), with the consensus sequence TGACNNNGC, has been shown to be responsible for both basal and inducible expression of many of these genes [27, 60-63]. The transcription factors involved are now being identified and Nrf2, a member of the basic leucine zipper family, has been shown to play a central role [59, 64-68]. This conclusion is supported by three new lines of evidence, obtained in experiments using *nrf2*-deficient mice, in which in contrast to wild-type mice: (a) little if any phase 2 enzyme induction is observed; (b) susceptibility to carcinogenesis is increased; and (c) are not protected by a phase 2 enzyme inducer [69-71]. Importantly, *nrf2*-deficient mice demonstrate higher sensitivity to acetaminophen [72, 73] and butylated hydroxytoluene [74]. Nrf2 is normally localized in the cytosol, where it is kept through protein-protein interactions with the chaperone Keap1. This is a 624-amino acid protein, containing 25 cysteine residues, 9 of which are expected to have increased reactivities, since they have basic amino acid(s) as immediate neighbor(s) [75]. Furthermore, all the 25 cysteine residues are conserved in the amino acid sequences of the mouse, the rat and the human homologues of Keap 1 [76]. The importance of this fact is highlighted since the only common feature among all inducers (see below) is their ability to react with sulfhydryl groups and thus the biological "sensor" (e.g., Keap1-Nrf2 complex) is expected to possess highly reactive sulfhydryl group(s). The presence of an inducer disrupts the Keap1-Nrf2 interactions, allowing Nrf2 to migrate to the nucleus and act as a transcription factor (in conjunction with its partners) [66]. The observation that *keap1*-deficient mice exhibit elevated basal expression of phase 2 gene products provides further support to these findings [Itoh *et al.*, personal communications].

For many years quinone reductase 1 (QR1) has been used in this laboratory as a prototype of phase 2 enzymes. A quantitative rapid microtiter plate assay for evaluating the activity of this enzyme was developed by the late Hans Prochaska, permitting efficient screening of a large number of pure compounds, as well as plant extracts and even more complex mixtures [77, 78]. It involves exposing the murine hepatoma cell line Hepa 1c1c7 in culture to serial dilutions of potential inducers and subsequent determination of the enzymatic activity of QR1. The **C**oncentration that **D**oubles the specific activity of QR1 (**CD** value) is used as a highly sensitive and quantitative measure of inducer potency. As can be seen in Fig. (**2**), the CD values can be quite different, reflecting inducer potency: e.g., compare β-naphthoflavone (CD 15 nM), 3-hydroxycoumarin (CD 1.5 μM) and *o*-coumaric acid (CD 19 μM). A further refinement of the microtiter plate assay involves replacement of Hepa 1c1c7 cells with its mutant cell lines Bp^{r}c1 (lacking intact *Ah* receptor), or c1 (lacking functional cytochrome P4501A1 gene). This allows one to distinguish between monofunctional inducers (that selectively elevate phase 2 proteins) and bifunctional inducers (that induce both phase 1 and phase 2 enzymes) [79]. The ability to make this distinction is important, since phase 1 enzymes participate in the activation of many procarcinogens Fig. (**1**) and therefore monofunctional inducers are preferred candidates for chemoprotective agents.

The microtiter plate assay was first developed in this laboratory and subsequently adopted by many others for "activity guided fractionation" of plant extracts in the search for cancer chemoprotective natural products [78, 80, 81]. Using this assay it was shown that a wide range of natural and synthetic compounds can induce phase 2 enzymes. At least nine distinct classes of inducers with potencies spanning more than four orders of magnitude have been identified: (a) oxidizable diphenols, phenylenediamines, and quinones; (b) other Michael reaction acceptors; (c) isothiocyanates; (d) hydroperoxides; (e) vicinal dimercaptans; (f) trivalent arsenicals; (g) 1,2-dithiole-3-thiones; (h) divalent heavy metals; and (j) carotenoids and other conjugated polyenes [82, 83].

It is now timely to discuss the Michael reaction acceptor concept. In 1887 it was reported by A. Michael that olefins or acetylenes conjugated with electron-withdrawing groups, i.e., electrophiles, are succeptible to attack by nucleophiles Fig. (**3**). Such compounds are designated as Michael reaction acceptors [84]. A series of experiments directed towards identification of common chemical feature(s) among seemingly unrelated inducers revealed that many of them have, or aquire by metabolism, a Michael reaction acceptor functionality [85]. Moreover, it was found that inducer potency correlates with reactivity in the Michael reaction.

Many phytochemicals contain Michael reaction center(s) in their molecules and the ubiquitously distributed and diverse phenylpropenoid metabolites are a prominent example. This review describes the ability of plant phenylpropenoids to modify the activities of phase 2 proteins and its correlation to their chemoprotective activity. Induction of such proteins represents a major cellular response. It should be pointed out that many of the compounds to be mentioned exhibit significant antioxidant activities (e.g., the ones bearing phenolic hydroxyl groups) in various systems, which probably also contribute to their chemoprotective activity. However, this review does not discuss such activities in detail and instead focuses on their phase 2 protein inducing capability.

While demonstration of a causal relation between phase 2 enzyme induction and chemoprotection in humans is extremely complex and challenging, numerous studies in the field of cancer epidemiology have shown a link between high consumption of vegetables and fruits and reduced cancer risk [86-88]. This has led to the proposal that certain plant products and their derivatives may serve as natural cancer-protective chemicals, a view that is consistent with their versatile effects and many possible molecular targets. Animal models have shown repeatedly and convincingly various biological activities of such substances, including antitumor effects and modulation of toxicities of xenobiotics. The ability to affect enzymes that participate in drug metabolism has often been implicated in the mechanisms of action [2, 89]. These biological effects are quite complex and depend on the chemical structure of the

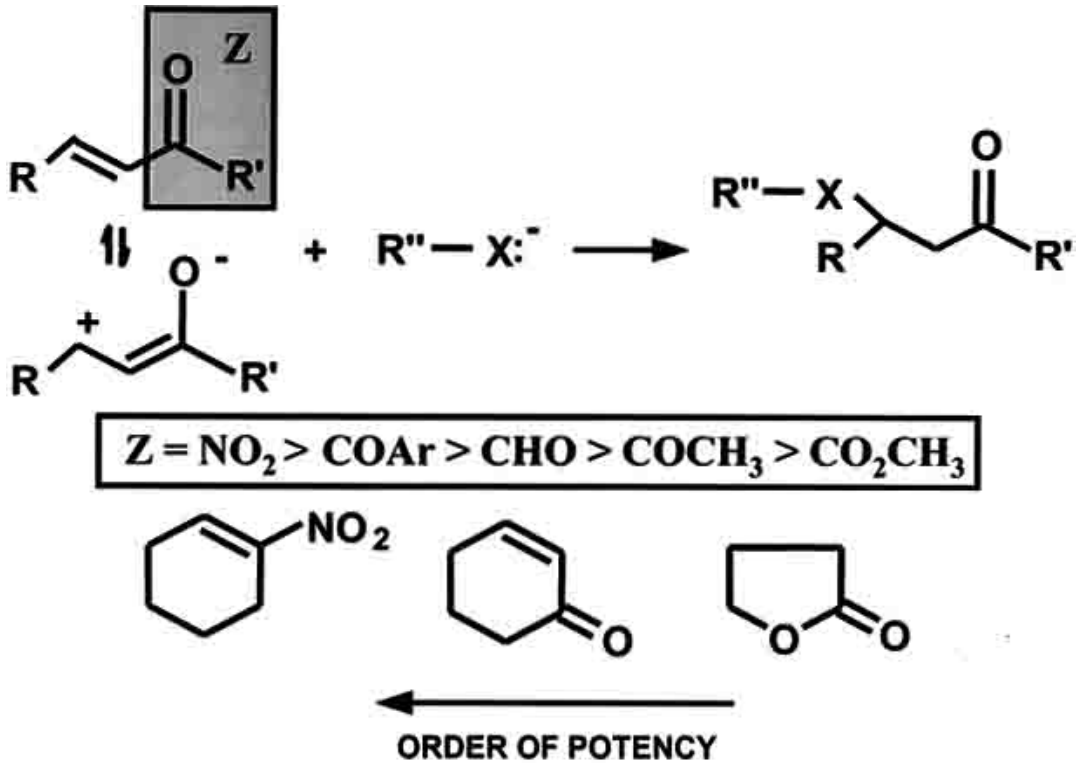


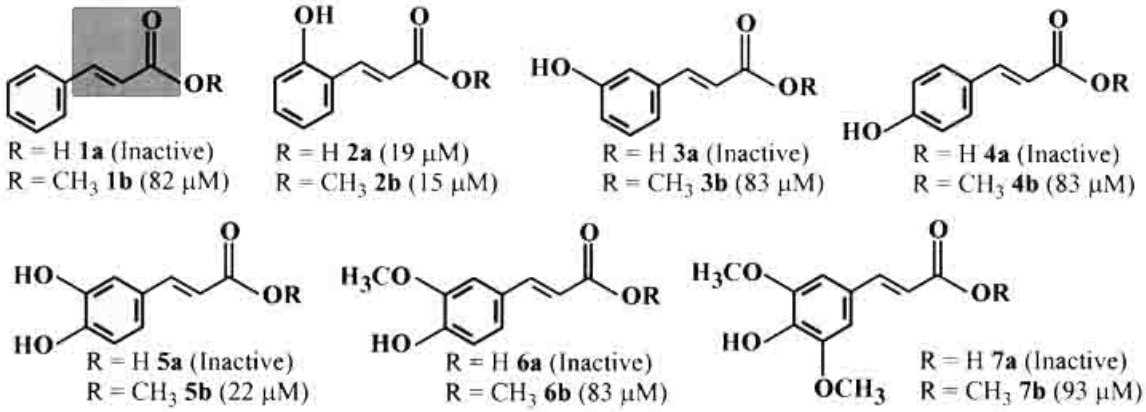
Fig. (3). The Michael reaction acceptor concept.

compound and its metabolites, the function of the specific enzyme(s), as well as on the biological system studied.

CINNAMIC ACIDS AND THEIR METHYL ESTERS

The plant biosynthesis of phenylpropanoids originates from the aromatic amino acids, phenylalanine and tyrosine Fig.(**4**), which are themselves products of the shikimate/chorismate metabolism. The phenylpropanoid and the acetate-malonate pathways join to give rise to an astonishing variety of structures with equally diverse biological functions. Some give colors to reproductive organs, others act as UV-protective compounds, phytoalexins, insecticides, allelochemicals [9, 90]. The biogenetic relationship between the major classes of phenylpropanoid metabolites is shown in Fig.(**4**). The Michael reaction acceptor containing cinnamic acids constitute early products of the phenylpropanoid pathway. They are subsequently further metabolized to give rise to a

Table 2. Structures of Cinnamic Acid Derivatives and their Inducer Potencies (CD Values) in the Quinone Reductase Microtiter Plate Assay in Hepa 1c1c7 Murine Hepatoma Cells. The Michael Reaction Acceptor Functionality of the Basic Skeleton is Highlighted



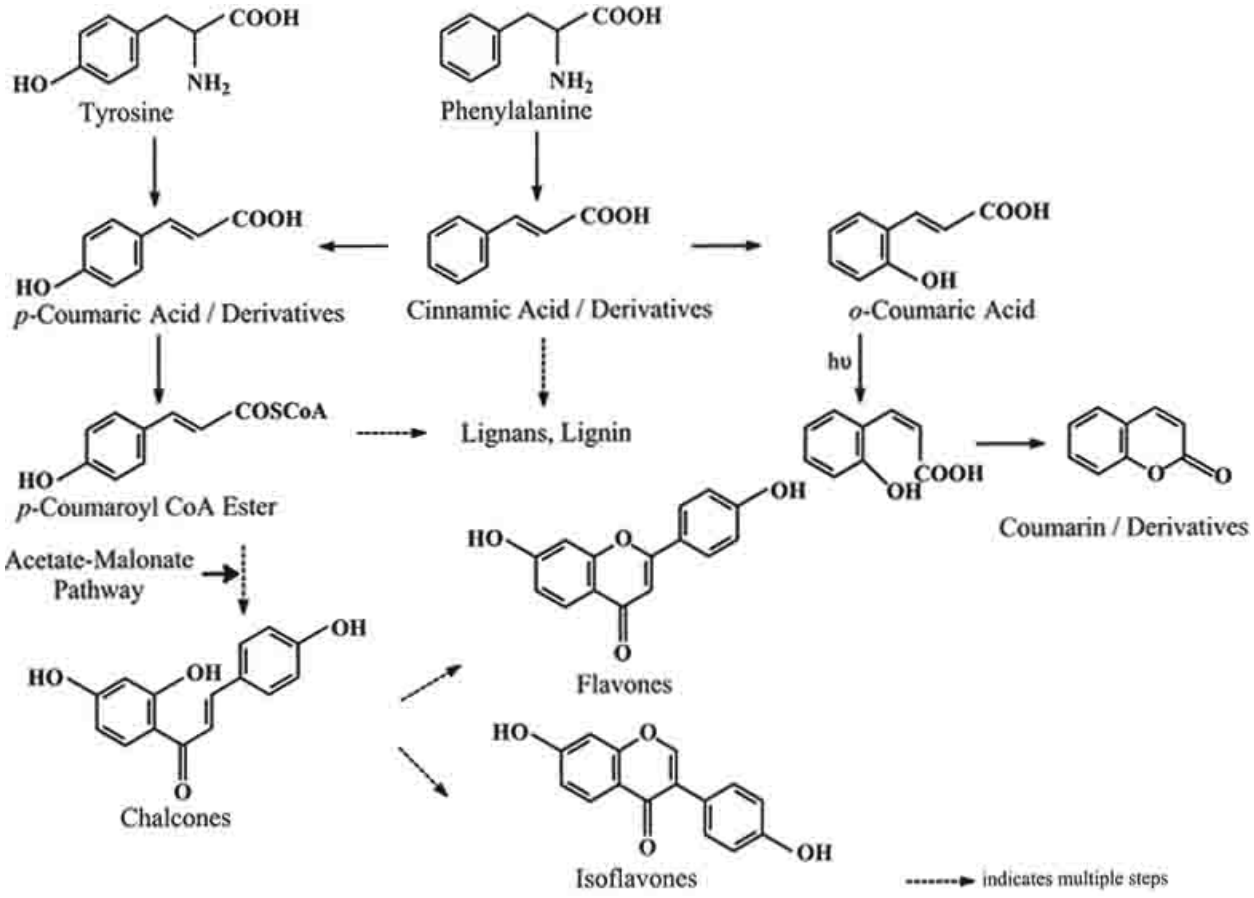


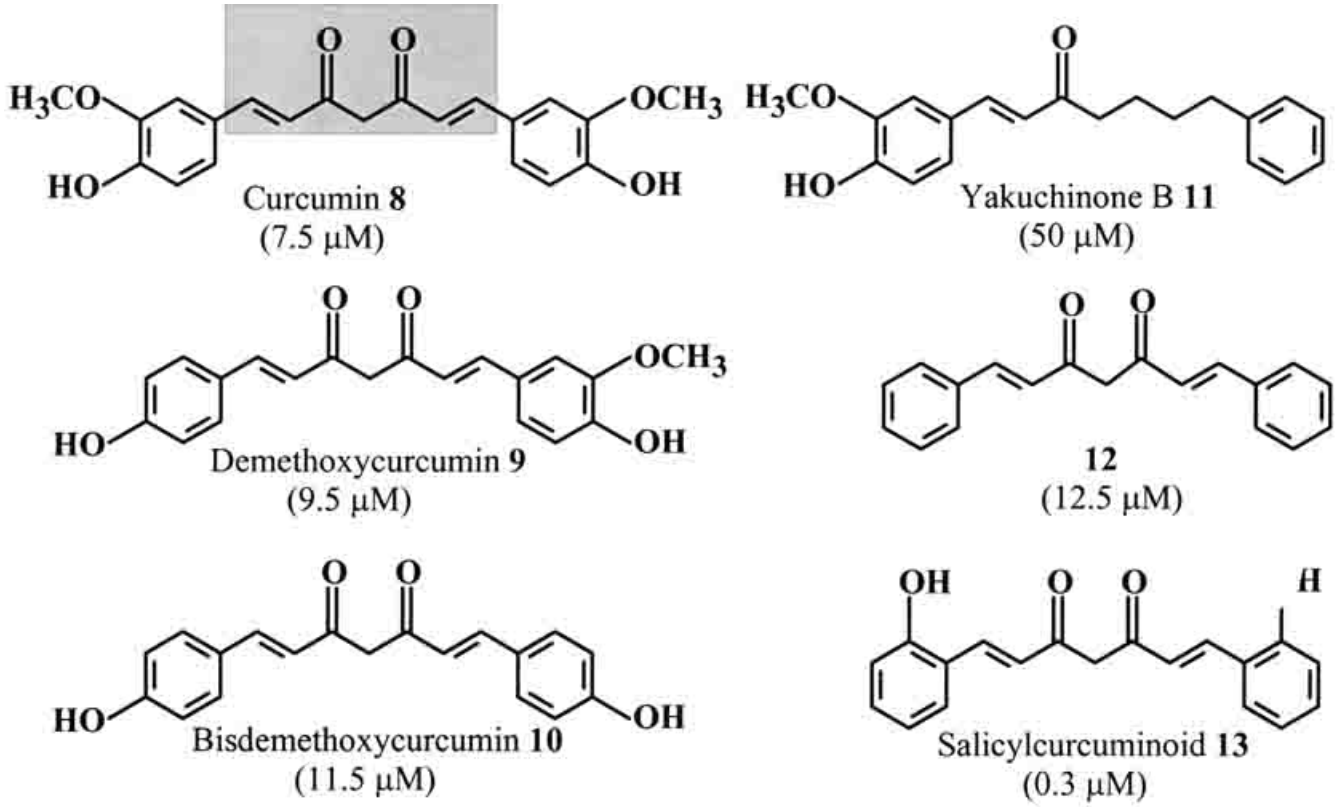
Fig. (4). Major classes of plant phenylpropanoid metabolites.

wide variety of phytochemicals, e.g., coumarins, lignans, lignins, suberins, flavonoids. Many are known to exhibit various biological activities in both homologous (plant) and heterologous (animal) systems.

Examination of the ability of a series of cinnamic acid derivatives and their methyl esters to induce phase 2 proteins revealed that the methyl esters are invariably more potent than the corresponding acids [91, 92]. This was expected on the basis of their better reactivity in the Michael reaction Fig.(**3**) [85]. In addition, substitutions (hydroxyl or methoxyl) at *meta*- and/or *para*-positions of the aromatic ring have no effect on inducer potency, e.g., compare compounds **1**, **3**, **4**, **5**, **6** and **7** (Table 2). In contrast, hydroxyl substitution at *ortho*-position invariably increased the inducer potency markedly. Thus, *o*-coumaric acid (**2a**) induces QR in Hepa 1c1c7 cells (CD 19 μM), while the corresponding *meta*- (**3a**) and *para*- (**4a**) derivatives are devoid of activity. Similarly, methyl *o*-coumarate (**2b**) (CD 15 μM) is a much more potent inducer than either methyl *m*-coumarate (**3b**) (CD 83 μM), or methyl *p*-coumarate (**4b**) (CD 83 μM). Thus, this study showed for the first time that hydroxyl group(s) on the aryl ring(s) and only at *ortho*-position(s) profoundly increase inducer potency. Subsequently, this effect was observed repeatedly when other phenylpropenoid Michael reaction acceptors were examined (see below) and served as the basis for the design and synthesis of some of the most potent inducers known to date [92].

Although it is not known at present whether the same structure-activity relationship translates into inhibition of tumorigenesis *in vivo*, the antitumor effects of plant cinnamates have been demonstrated in several models of chemical carcinogenesis. Thus, L. Wattenberg and P. Lesca reported about 20 years ago that ferulic acid (4-hydroxy-3-methoxycinnamic acid) (**6a**) suppressed benzo(*a*)pyrene-induced neoplasia of the forestomach [93] and lung [94] of mice. More recently, chemoprotection by this and related phenolic acids was also demonstrated in other rodent models, i.e., ferulic acid in the diet was protective against direct-acting carcinogens such as 4-nitroquinoline-1-oxide (4-NQO)-induced oral carcinogenesis [95, 96], as well as against azoxymethane (AOM)-induced colon carcinogenesis [97, 98]. Ferulic acid is widely distributed among plants. It is a cell wall component of wheat and barley and consequently often consumed by humans. Importantly, ferulic acid is well absorbed [99, 100]. It should be noted that antitumor activities have been described in various *in vitro* systems for other plant cinnamates and related synthetic derivatives, e.g., antiproliferative [101], cell differentiation promoting [102], anti-tumor promoting [103], cytotoxic [104]. The Michael reaction acceptor (propenal) group of a series of cinnamaldehydes was identified as a key

Table 3. Structures of Curcuminoid Derivatives and their Inducer Potencies (CD Values) in the Quinone Reductase Microtiter Plate Assay in Hepa 1c1c7 Murine Hepatoma Cells



functional group, since the effect of the saturated derivatives was much weaker [85, 104].

CURCUMINOIDS

The double Michael reaction acceptor curcumin (**8**) (Table 3) and the closely related demethoxy- (**9**) and bisdemethoxycurcumin (**10**) are major constituents of the powdered dry rhizome of turmeric (*Curcuma longa*, Zingiberaceae) and the principal coloring and flavoring compounds of the spice curry. In Asia, traditional medicine has used curcumin for centuries for the treatment of a plethora of diseases [105]. A great variety of biological effects have been extensively documented for this natural product. While it is not possible to describe in this minireview all of the effects of curcumin, a few should be mentioned: antioxidant [106], antimutagenic [107], antitumor [108-112], antiangiogenic [113], apoptosis inducing [114, 115], protein kinase suppressing [116]. Curcumin can be viewed as a dimer of two molecules of ferulic acid (**6a**) bridged by a methylene group. Of note, the effects of curcumin are consistently found to be more powerful that the effects of ferulic acid itself [117-119]. Curcumin is a very attractive candidate for a chemoprotective agent because of its long history of use and low toxicity.

Examination of a series of naturally occurring and synthetic curcuminoids (Table 3) for their ability to induce QR revealed that all were more potent than their corresponding monomeric cinnamates even adjusting for the "double" molarity [120]. As expected, yakuchinone B (**11**) (CD 50 μM), which is the only single Michael reaction acceptor among the curcuminoid derivatives tested, was the least potent QR inducer (unpublished results). In contrast, the *ortho*-hydroxylated double Michael reaction acceptor salicylcurcuminoid (**13**) (CD 0.3 μM) was remarkably and far more potent than the other curcuminoids tested. In this case too, while hydroxyl groups at *ortho*-positions had a profound effect (>30-fold increase in potency), hydroxyl and/or methoxyl substitutions at other positions on the aryl rings did not affect the inducer potency significantly (compare compounds **8** through **13**). Importantly, using a two-stage mouse tumor promotion model, Anto *et al.* [110] demonstrated that among a series of curcuminoids, salicylcurcuminoid (**13**) was the most potent inhibitor of tumorigenesis. It completely prevented the appearance of papillomas in the treated animals at a time point when 90% of the control mice had tumors (10 weeks).

Curcumin inhibits carcinogenesis in a number of animal models. Topical application of curcumin or demethoxycurcumin potently inhibits tumor promotion by TPA in a two-stage initiation-promotion model in the epidermis of CD-1 mice, while bisdemethoxycurcumin or tetrahydrocurcumin are less active [117, 121, 122]. Dietary curcumin was inhibitory in several rodent models of chemical carcinogenesis of the gastrointestinal tract: *N*-nitrosomethylbenzylamine-induced esophageal carcinogenesis in F344 rats [123], benzo[*a*]pyrene-induced forestomach carcinogenesis in A/J mice, *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine-induced duodenal carcinogenesis in

C57BL/6 mice, azoxymethane-induced colon carcinogenesis in CF-1 mice [109] and azoxymethane-induced aberrant-crypt foci formation in the colon of F344 rats [124]. Dietary curcumin reduced both tumor incidence and multiplicity in *N*-diethylnitrosamine-induced hepatocarcinogenesis in C3H/HeN mice [125]. It is also effective against 4-nitroquinoline 1-oxide-induced oral carcinogenesis in F344 rats [126]. Dietary curcumin decreases the intestinal tumor burden in Apc^{min} mice, a model of human familial adenomatous polyposis [127]. Furthermore, it significantly inhibits 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced tumorigenesis in proximal (but not middle or distal) small intestine of Apc^{min} mice [128].

The ability of curcumin to inhibit chemical carcinogenesis in animal models is probably due to its dual effects: (a) direct, as an extremely efficient free radical scavenger; and (b) indirect, as an inducer of phase 2 enzymes. The possible molecular mechanisms of the chemoprotective effects of curcumin and related diarylheptanoids from the ginger family have been extensively reviewed [129]. Although out of the scope of the present review, it should be emphasized that in numerous *in vitro* systems curcumin behaves as one of the most potent antioxidants known [130 and our unpublished observations]. In addition, this double Michael reaction acceptor induces phase 2 enzymes and raises cellular glutathione levels not only in cell cultures, but also *in vivo*. Piper *et al.* [131] have demonstrated that curcumin administration to rats by gavage increases the liver levels of glutathione, γ-glutamylcysteine synthetase, glutathione *S*-transferases and glutathione peroxidase in a dose-dependent manner. The same group has subsequently shown that exposure of human leukemia cells (K562) to 1 μM curcumin increases the levels of glutathione, γ-glutamylcysteine synthetase and glutathione *S*-transferases [132]. Concentration- and time-dependent induction of heme oxygenase-1 (mRNA, protein expression and enzymatic activity) was recently demonstrated when bovine aortic endothelial cells were exposed to curcumin [133]. Furthermore, prolonged exposure to 5 μM curcumin protected these cells against oxidative stress (of hydrogen peroxide generated by glucose oxidase treatment). This effect was significantly attenuated by the presence of tin protoporphyrin IX, a heme oxygenase inhibitor.

COUMARINS

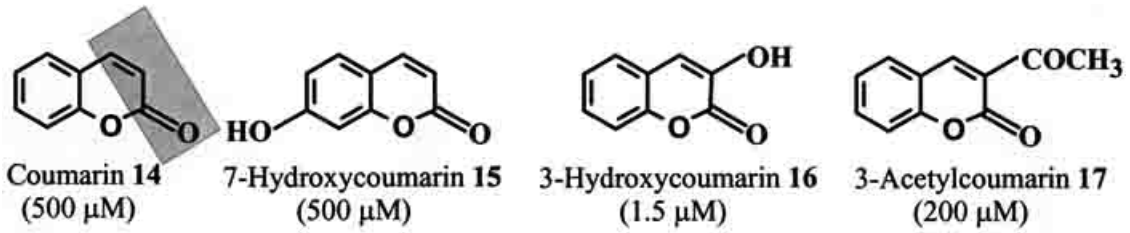
During the biosynthetic pathway leading to the plant coumarins the side-chain double bond of *o*-coumaric acid (**2a**) undergoes light-catalyzed *trans-cis* isomerization Fig.(**4**) and subsequent lactonization gives rise to the benzopyran-2-one group of the basic coumarin skeleton [134]. Coumarin (**14**) (Table 4), an "old" antitumor agent [135, 136], is a very weak inducer of QR (CD ~ 500 μM). Its major metabolite in humans, 7-hydroxycoumarin (**15**) has the same weak inducer activity. Remarkably, 3-hydroxycoumarin (**16**) is approximately 300-fold more potent and has a CD value of 1.5 μM. The specific impact of the *ortho*-hydroxyl group was further confirmed by the fact that its replacement with acetyl group (compound **17**) decreases the inducer potency dramatically to a level comparable to that of the unsubstituted coumarin (**14**) (CD 200 μM). Two recent *in vivo* chemoprevention studies clearly showed that inclusion of coumarin in the diet leads to coordinate induction of several phase 2 enzymes in rat liver, i.e., aflatoxin aldehyde reductase, a number of glutathione *S*-transferases, quinone reductase and γ-glutamylcysteine synthetase [12, 13]. Notably, GST P1 protein levels were induced by ~20-fold (as demonstrated by Western blots) [12]. Furthermore, dietary administration of coumarin prior to aflatoxin B_1 treatment was protective against the development of hepatic preneoplastic lesions in the rat [13].

CHALCONES, FLAVONES, FLAVONOLS, FLAVANES AND ISOFLAVONES

The flavonoids, which also originate from the phenylpropanoid pathway Fig.(**3**), are among the most ubiquitous phenolic compounds in nature. In plants, they perform various physiological functions, e.g., screen UV-light, provide color to reproductive organs, even behave as insect antifeedants. Their participation in plant defense strategies as antioxidant, antifungal and antimicrobial agents is widely recognized. More than 4000 related structures have been identified [137]. Many are constituents of edible plants and consequently ingested in substantial quantities by humans. However, their bioavailability is limited.

Chalcones are open chain flavonoids, in which the two aromatic rings (A and B) are bridged by an α, β-unsaturated carbonyl moiety, and thus can be regarded as another class of naturally-occurring Michael reaction acceptors (Table 5). Many chalcones exhibit anti-inflammatory and antitumor activities [138-140], others serve as "lead compounds" for the development of potent cytotoxic and anticancer agents [141]. Importantly, structure-activity studies have pinpointed

Table 4. Structures of Coumarin Derivatives and their Inducer Potencies (CD Values) in the Quinone Reductase Microtiter Plate Assay in Hepa 1c1c7 Murine Hepatoma Cells



the absolute requirement for the olefinic function for the anti-inflammatory [142] and antifungal [143] activities of chalcones. In accord with our previous observations, evaluation of the phase 2 enzyme inducer activity of a series of hydroxylated chalcone derivatives in comparison with the unsubstituted parent compound (**18**) confirmed that hydroxyl substitution at *para*-position did not affect inducer potency, since 4-hydroxychalcone (**19**) and chalcone (**18**) had similar CD values (Table 5). In contrast, hydroxylation at *ortho*-position of either aromatic ring improved the inducer potency approximately 3 times, i.e., 2-hydroxychalcone (**20**) (CD 12 μM) and 2'-hydroxychalcone (**21**) (CD 9.8 μM). 4,2',4'-Trihydroxychalcone (**22**) has a CD of 11 μM, confirming that hydroxyl substitutions at *para*-positions do not improve further the inducer potency. The effect of the simultaneous presence of two *ortho*-hydroxyl groups on both aromatic rings is additive and 2,2'-dihydroxychalcone (**23**) has a CD of 4.7 μM. As expected, 2,2',4'-trihydroxychalcone (**24**) (CD 4.8 μM) is equal in potency. Interestingly, in the course of identification of phase 2 enzyme inducers from natural sources Chang *et al.* [81] reported the isolation of a potent QR inducer from the pantropical coastal shrub *Tephrosia purpurea*, the chalcone derivative (+)-tephropurpurin (**25**) (CD 0.15 μM), bearing an *ortho*-hydroxyl group on the A-ring.

The synthetic flavonoid β-naphthoflavone (**26**) (Table 6) was one of the first (bifunctional) QR inducers examined in this laboratory [85]. Exposure of Hepa 1c1c7 cells to this compound increases the levels of QR in a dose-dependent manner and by more than 10-fold at a concentration of 1.0 μM. Structure-activity studies showed that the position of the additional aromatic ring in relation to the basic flavonoid skeleton affects the inducer activity enormously, i.e., the order of potency is: β-naphthoflavone (**26**) (CD 15 nM) > α-naphthoflavone (**27**) (CD 80 nM) > γ-naphthoflavone (**28**) (CD 500 nM). The reasons for these differences are unclear. Breinholt et. al. [144] showed that dietary administration of β-naphthoflavone to female Wistar rats induces glutathione *S*-transferase enzyme activity, protects against oxidative stress (induced by the a food mutagen, the heterocyclic amine PhIP) and inhibits PhIP-DNA adduct formation in the colon. However, despite the high potency of β-naphthoflavone, its use as a chemoprotecive agent is not desirable since it is a bifunctional inducer [79]. It binds to the *Ah* (*A*ryl *H*ydrocarbon) receptor and induces both phase 1

Table 5. Structures of Chalcone Derivatives and their Inducer Potencies (CD Values) in the Quinone Reductase Microtiter Plate Assay in Hepa 1c1c7 Murine Hepatoma Cells

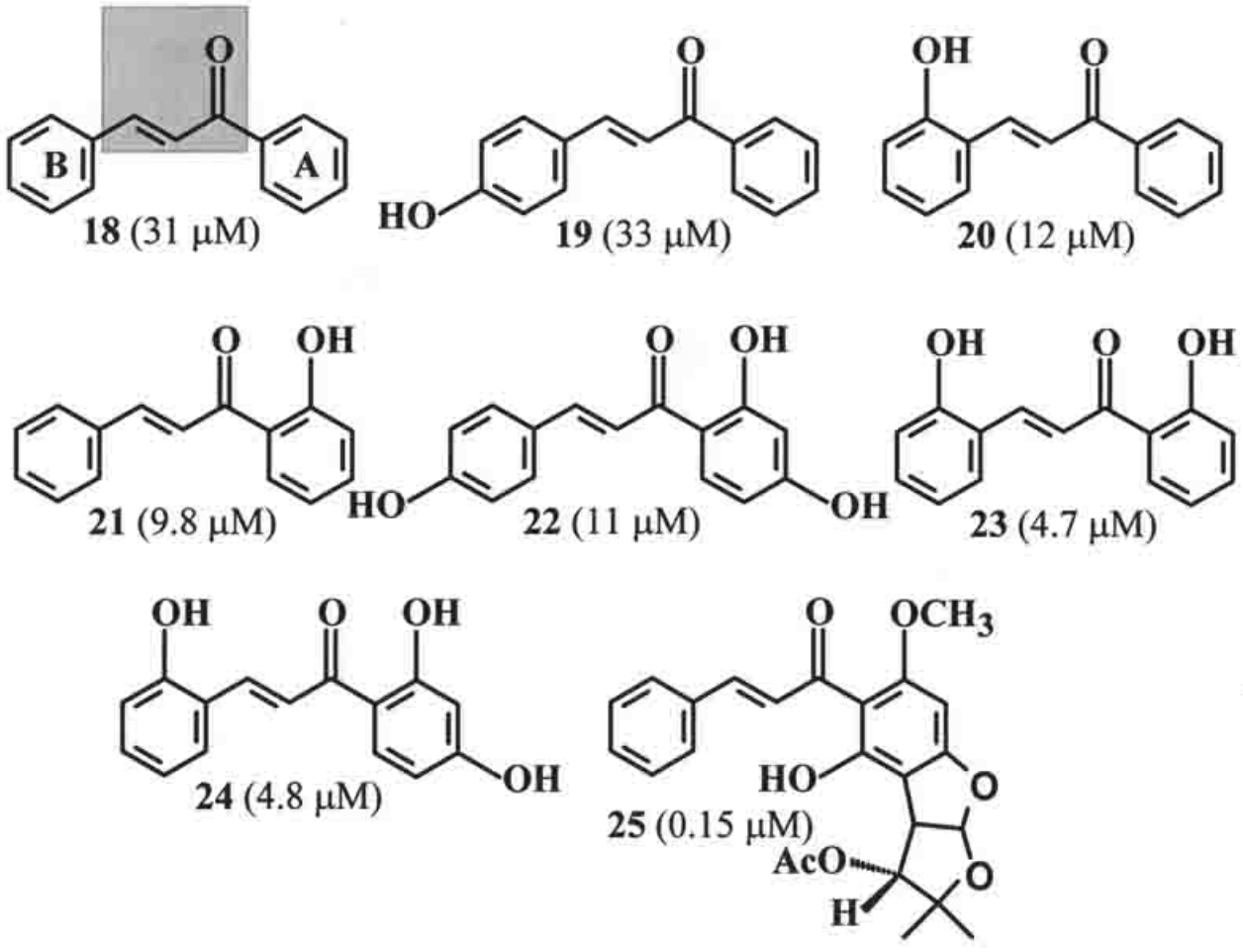
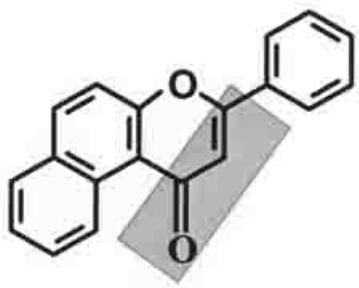
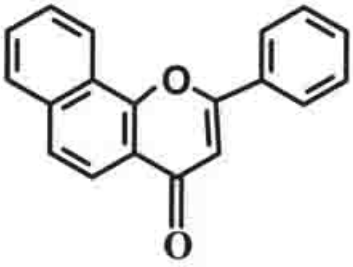


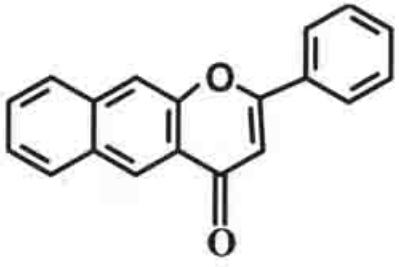
Table 6. Structures of Naphthoflavones and their Inducer Potencies (CD Values) in the Quinone Reductase Microtiter Plate Assay in Hepa 1c1c7 murine hepatoma cells



β-Naphthoflavone **26**
(15 nM)



α-Naphthoflavone **27**
(80 nM)



γ-Naphthoflavone **28**
(500 nM)

and phase 2 detoxification enzymes. Phase 1 enzymes participate in the activation of many procarcinogens to their ultimate carcinogenic species. Pretreatment with β-naphthoflavone reduced the tumor burden caused by benzo[*a*]pyrene [145] or 3-methylcholanthrene [146] in a number of tissues in several different *Ah*-responsive mouse strains, while non-responsive strains were not protected. However, when *N*-nitrosoethylurea was used as a carcinogen, the opposite effect of β-naphthoflavone pretreatment (enhancement of carcinogenicity) was observed [147].

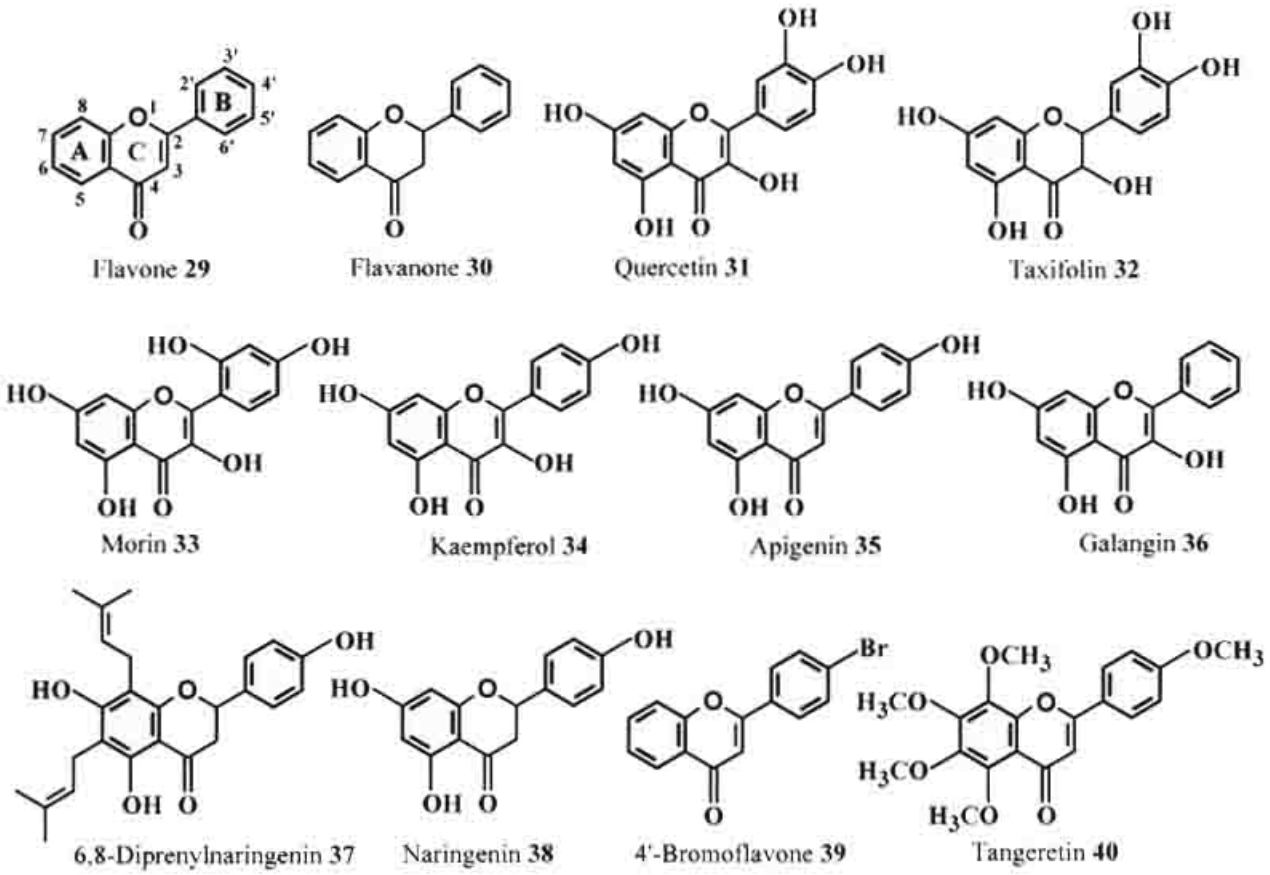
Evaluation of various flavonoids showed that although most of them behave as bifunctional inducers in murine hepatoma cells, there are some, which appear to be monofunctional [148]. Moreover, the patterns of flavonoid glucuronidation in wild type cells (Hepa 1c1c7) and *Ah* receptor deficient cells (Bp^rc1) are different. There are no apparent differences in their uptake in the two cell types. This observation points out that in addition to the importance of the chemical structure of a flavonoid, its metabolism and the possible role of the metabolites deserve careful consideration.

Examination in our laboratory of a number of flavonoids for their ability to induce QR and comparison between their potencies showed that the Michael reaction acceptor-containing flavones are inducers (CD values between 3 and 12.5 μM) with a high efficacy (total level of induction) and low toxicity (not detectable at concentrations up to 100 μM) (Table 7). Remarkably, maximal induction by the unsubstituted flavone (**29**) was observed at 50 μM, at which concentration the QR specific enzymatic activity was more than 11 times that of controls. Nijhoff *et al.* [149, 150] showed that dietary administration of flavone increases glutathione *S*-transferase activities (Alpha- and Mu-isoforms) and the levels of glutathione in many tissues of male Wistar rats and thus, enhancement of detoxification capacity was proposed to contribute to the observed chemoprotective action of these compounds. We found that in contrast to flavone (**29**), flavanone (**30**), which has a saturated 2,3-double bond in its heterocyclic C-ring, is inactive in the QR1 bioassay, indicating the absolute requirement for a Michael reaction center for inducer activity. Similarly, quercetin (**31**) is a good inducer (CD 5.4 μM), while taxifolin (**32**), which has the same substitution pattern, but lacks the Michael reaction center, is inactive [151].

Importantly, dietary administration of morin (**33**) to F344 rats led to significant increases in the activities of QR and GST in the liver, large bowel and tongue and was protective against azoxymethane (AOM)-induced adenocarcinoma of the large intestine [152], as well as against 4-nitroquinoline 1-oxide-induced tongue carcinogenesis. [153]. This flavone bears a hydroxyl group at position 3. While not required, such substitution increases significantly inducer activity, e.g., kaempferol (**34**) is approximately 6 times more potent than apigenin (**35**) in inducing QR in Hepa 1c1c7 cells [151]. Glycosylation at this position reverses the effect of hydroxylation, as exemplified by the lack of activity of quercetin-3-glucoside, quercetin-3-rutinoside and quercetin-3, 4'-diglucoside [154]. In contrast, quercetin-4'-glucoside is an inducer, indicating that substitutions on the B-ring are not essential for inducer activity. This finding was further confirmed by the same group, who showed that galangin (**36**) (no hydroxyl substitutions on the B ring) and kaempferol (**34**) (1-hydroxyl substitution on the B ring) are inducers with equal potencies. Interestingly, prenylation appears to suppress inducer potency, since the prenylated naringenins from hops, e.g., 6,8-diprenylnaringenin (**37**), exhibit weak inducer activity, while the parent naringenin (**38**) is devoid of any activity [155].

Using the microtiter plate assay Pezzuto's group examined the ability of a number of naturally occurring and synthetic flavonoids to induce quinone reductase and identified a very potent inducer, i.e., the synthetic 4'-bromoflavone (**39**) (CD 10 nM) [156]. Enhanced expression of the Alpha- and Mu-isoforms of glutathione *S*-transferase by 4-50 μM 4'-bromoflavone in cultured rat hepatoma cells (H4IIE) was also observed with no detectable toxicity within this dose range. Furthermore, dietary administration of 4'-bromoflavone significantly delayed and reduced the incidence and multiplicity of mammary tumors in the DMBA-induced rat mammary carcinogenesis model. Although **39** is a bifunctional inducer in cell culture, no significant changes in the P450 1A1 activity *in vivo* were found. The reasons for this apparent discrepancy are unclear.

Table 7. Structures of Flavonoid Derivatives Tested as Inducers of Quinone Reductase and/or Inhibitors of Carcinogenesis



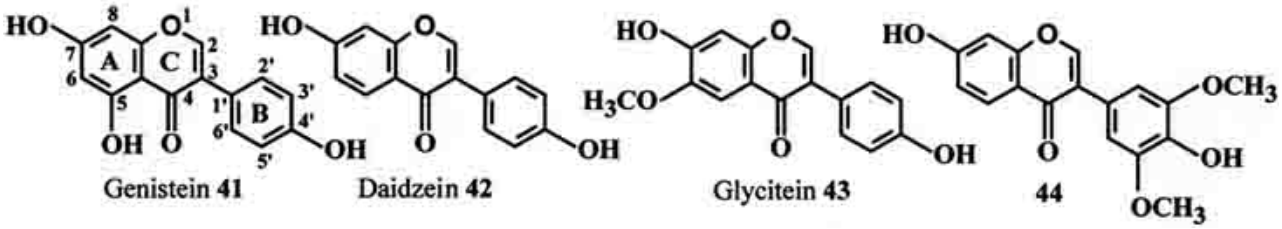
In contrast, Siess *et al.* [157] showed that dietary administration of flavone (**29**) to male Wistar rats elevated the activities of phase 2 enzymes (glutathione *S*-transferase and UDP-glucuronosyltransferase), as well as phase 1 enzymes (P450 1A1/2 and P4502B1/2). Importantly, the activities of P450 1A1/2 started to increase as early as 6 h after the first dose and reached maximal induction (~5-fold) after 4 days, while the earliest elevation of the activities of P450 2B1/2 and phase 2 enzymes was observed 24 h after feeding. This difference in the time course of induction suggests that the ultimate inducers of the second group of enzymes are probably flavone metabolites and not the parent compounds themselves.

Indeed, Nielsen *et al.* [158] showed that initial biotransformations of flavonoids by rat liver microsomes involve demethylation and/or hydroxylation reactions on the B-ring and are most likely catalyzed by P450 1A1 isozymes. Moreover, although these reactions depend on the nature, number and positions of the substituents, the major end-product is a 3',4'-dihydroxy- (catechol) derivative of the parent flavonoid. The catechol so obtained can be further oxidized to semiquinone or quinone species, thus giving rise to potentially damaging free radicals.

This situation is even more complex *in vivo*. The same group has identified and quantified 10 different metabolites, each bearing an intact flavan skeleton, in rat urine and faeces after repeated administration of tangeretin (**40**) (abundant in citrus peel), again primarily demethylation and/or hydroxylation products [159]. The recent development of a highly sensitive method (column switching liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry) for determination and quantification of flavonoids in human urine will permit detailed evaluation of their metabolites in humans [160]. This is important because although flavonoids are generally considered to be beneficial as anticarcinogens and cardioprotective agents, the potential risk of their excessive intake is still unknown [161].

The isoflavonoids differ from the flavonoids only by the position on the heterocyclic pyrane C ring (carbon 3) at which the aromatic B ring is attached (Table 8). These metabolites play a critical role in plant defense and their biosynthesis is induced upon pathogen attack in Leguminosae [9]. Traditionally, leguminous plants have been an important nutritional component for many cultures, and soybeans in particular constitute an especially rich source of isoflavonoids. Epidemiological studies have linked the large consumption of soy products in China and Japan to low incidence of breast, prostate and colon cancers in these countries [162]. This has directed attention to genistein (**41**) (4',5,7-trihydroxyisoflavone), daidzein (**42**) (4',7-dihydroxy-isoflavone) and glycitein (**43**) (4',7-dihydroxy-6-methoxyisoflavone), which together constitute >90% of the total soybean isoflavone content. Genistein was first isolated as a phytoestrogen [163]. Later genistein was shown to bind

Table 8. Structures of Some Isoflavonoids from Leguminosae



to the estrogen receptor β (ERβ) with an affinity equal to that of 17β-estradiol, and with a 20-times lower afiinity to the estrogen receptor α (ERα) [164]. The crystal structure of the ERβ-genistein complex is now available [165]. Genistein and daidzein belong to the class of natural selective estrogen-receptor modulators (SERMs). Hundreds of studies have been carried out showing that genistein suppresses cancer cell growth *in vitro* and inhibits tumorigenesis in rodents *in vivo* [166, 167]. Several distinct activities have been implicated in the mechanism(s) of action [168-171], the most prominent being: (a) inhibition of enzymes involved in signal transduction cascades, e.g., tyrosine protein kinases, MAP kinase; (b) induction of the expression of an epithelial cell growth inhibitor, transforming growth factor β (TGFβ); (c) inhibition of angiogenesis and metastasis; (d) inhibition of topoisomerase II; and (e) estrogen-like effects. As in the case with the flavonoids, metabolism of isoflavones and the possible role of their metabolites should be also considered [172].

Messina and colleagues [173] have reviewed the available data (cell culture, animal and epidemiological) regarding correlation between soy intake and cancer risk and although not conclusive, it encourages further investigations. Soy diet was shown to induce phase 2 enzymes in various mouse tissues, to elevate reduced glutathione and decrease oxidized glutathione levels in plasma [174, 175]. Importantly, these effects were associated with a tendency of decrease in tumor multiplicity in the dimethylbenz[*a*]anthracene rat mammary carcinogenesis model. When genistein and daidzein were evaluated as phase 2 enzyme inducers, they were found to raise quinone reductase in Hepa1c1c7 cells, as well as in its mutant Bp^rc1 cells (lacking intact *Ah* receptor), suggesting that these isoflavones are monofunctional inducers [148]. Parallel to quinone reductase induction was the potent inhibition of benzo[*a*]pyrene metabolite-DNA binding by genistein [176]. Induction of quinone reductase by genistein was also observed in the human colon cancer cell line Colo205 [177]. When the quinone reductase bioassay was used for "activity-guided fractionation" of plant extracts, a novel compound, 7,4'-dihydroxy-3',5'-dimethoxyisoflavone (**44**), was isolated from *Tephrosia purpurea* (Leguminosae) with moderate inducer potency (CD 17.2 μM) [81].

Both genistein and daidzein increase intracellular total glutathione levels in rat vascular smooth muscle cells and this was attributed to induction of γ-gluamylcysteine synthetase, the enzyme which catalyzes the rate-limiting step for glutathione biosynthesis. In addition, and probably as a consequence, in the presence of genistein or daidzein these cells were protected against oxidative stress, as judged by the decreased formation of 8-hydroxy-2'-deoxyguanosine [178]. This antioxidant enzyme constitutes an important part of the phase 2 response and induction of both of its catalytic and regulatory subunits is regulated, at least in part, by the same regulatory mechanism(s).

Finally, it should be noted that an increasing number of studies shows that flavonoids can behave as antimutagens/promutagens and antioxidants/prooxidants depending on the conditions [see 161 for a recent review]. Careful considerations should be given to potential excessive intake of these biologically active molecules, especially when used as dietary supplements.

CONCLUDING REMARKS

Studies from a number of laboratories dedicated to chemoprotection have demonstrated that a variety of phenylpropanoid metabolites, which play a role in plant defense, induce mammalian phase 2 enzymes and protect animals against toxicity and carcinogenicity. All of these phenylpropanoid inducers contain Michael reaction acceptor functionalities in their structures. The induction represents a major cellular response, involves a battery of defense enzymes, is generally independent of the organ or tissue type, and represents a major protective strategy against electrophiles and oxygen toxicity. In addition to chemical structure, metabolism of these molecules is also a critical factor in determining the potencies both as phase 2 enzyme inducers and chemoprotective agents.

ACKNOWLEDGEMENTS

The author is grateful to Professor Paul Talalay (Johns Hopkins University) for numerous helpful discussions and advice. The work from this laboratory was supported by a program-project Grant CA44530 form the National Cancer Institute, Department of Health and Human Services, by postoctoral fellowships from the Cancer Research Foundation of America, and by generous gifts from the Lewis B. and Dorothy Cullman Foundation.

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