Effects of Hydroxyl Group Numbers on the B-Ring of 5,7-Dihydroxyflavones on the Differential Inhibition of Human CYP 1A and CYP1B1 Enzymes

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Flavonoids are polyphenols composed of two aromatic rings (A, B) and a heterocyclic ring (C). In order to determine the effects of the number of hydroxyl groups in the B-ring of the flavonoids on human cytochrome P450 (CYP) 1 family enzymes, we evaluated the inhibition of CYP1A-dependent 7-ethoxyresorufin O-deethylation activity by chrysin, apigenin and luteolin, using bacterial membranes that co-express human CYP1A1, CYP1A2, or CYP1B1 with human NADPH-cytochrome P450 reductase. Chrysin, which possesses no hydroxyl groups in its B-ring, exhibited the most pronounced inhibitory effects on CYP1A2-dependent EROD activity, followed by apigenin and luteolin. On the contrary, CYP1A1-mediated EROD activity was most potently inhibited by luteolin, which is characterized by two hydroxyl groups in its B-ring, followed by apigenin and chrysin. However, all of the 5,7-dihydroxyflavones were determined to similarly inhibit CYP1B1 activity. Chrysin, apigenin, and luteolin exhibited a mixed-type mode of inhibition with regard to CYP1A2, CYP1B1, and CYP1A1, with apparent Ki values of 2.4, 0.5, and 2.0 μM, respectively. These findings suggested that the number of hydroxyl groups in the B-ring of 5,7-dihydroxyflavone might have some influence on the degree to which CYP1A enzymes were inhibited, but not on the degree to which CYP1B1 enzymes were inhibited.

Key words: CYP1 enzymes, Ethoxyresorufin O-deethylase, Chrysin, Apigenin, Luteolin

INTRODUCTION

Cytochrome P450 (CYP) comprises a superfamily of hemoproteins, which are capable of the catalysis not only of the biotransformation of a variety of xenobiotics and endogenous substances, but also of the bioactivation of a host of procarcinogens and toxins (Guengerich et al., 1995; Rendic and Di Carlo, 1997). In humans, the CYP1 enzymes, CYP1A1, CYP1A2, and CYP1B1, appear to be involved in the oxidation of a variety of aryl hydrocarbon procarcinogens to their reactive electrophilic intermediates, which then interact with cellular nucleophiles, culminating in chemical carcinogenesis (Guengerich and Shimada, 1998; Gonzalez and Gelboin, 1994). The CYP1 enzymes manifest overlapping substrate specificity, but are also characterized by both differential tissue distri-

bution and substrate preference. CYP1A1 and CYP1B1 are expressed primarily in extrahepatic tissues, and may perform some function in the metabolic activation of the carcinogenicity of polycyclic aromatic hydrocarbons in these sites. In the human liver, CYP1A2 is known to be responsible for the activation of heterocyclic aromatic amines to genotoxic species (Guengerich and Shimada, 1998; Gonzalez and Gelboin, 1994). The modulation of CYP1 enzyme activity by dietary flavonoids may bear important implications with regard to cancer prevention and drug metabolism.

Flavonoids are naturally occurring polyphenolic compounds found in fruits and vegetables. They are, therefore, important constituents of a healthy human diet (Ross and Kasum, 2002). Epidemiological studies and *in vivo* animal studies have both shown that flavonoids inhibit the development of carcinogen-induced tumors in a variety of organs (Frank et al., 1998; Tanaka et al., 1998; So et al., 1996). One of the mechanisms by which flavonoids may exert their chemopreventive effects

involves interactions with the CYP system, *via* the attenuation of the activation of procarcinogens into ultimate carcinogens. Thus, the inhibitory capacity of flavonoids with regard to the activity of CYP is currently receiving a considerable amount of attention, as it has some potential with regard to the development of agents that can disrupt carcinogenesis at the initiation stage (Kanazawa *et al.*, 1998; Lautraite *et al.*, 2002; Kang *et al.*, 2004). Studies have determined that both synthetic and naturally occurring flavonoids function as effective CYP1 enzyme inhibitors (Lee *et al.*, 1998; Lautraite *et al.*, 2002).

More than 8000 different flavonoid structures have, thus far, been identified (Pietta, 2000). However, the basic chemical structure of a flavonoid involves two benzene rings (A and B), which are linked by a heterocyclic ring (C) (Fig. 1). Regardless of the overall structural commonalities observed to exist between flavonoids, their biological and biochemical properties vary substantially, even with only minor modifications of the basic flavonoid structure (Breinholt, 2002; Hodek, 2002). The cytotoxic, antioxidant, estrogenic, mutagenic, or antimutagenic potentials of the flavonoids tend to vary according to the substitution pattern. A host of studies have focused on the inhibitory capacities of different flavonoids, as well as their structure-relationship with regard to CYP 1 activity (Lee *et al.*, 1998; Zhai *et al.*, 1998; Dai *et al.*, 1998). The active site of CYP1A1 binds preferentially with 7-hydroxyl-substituted flavones. The presence of multiple hydroxyl groups (preferably two, in positions 5 and 7) on the

flavone skeleton constitutes a prerequisite for binding to CYP1A2. The binding sites of CYP1A1 and 1B1 are more similar to each other, than either one of them is to those of CYP1A2 (Hodek P, 2002). However, little remains known with regard to the relative effects of the hydroxylation numbers of the B-rings of flavonoids on human CYP1 enzymes. In this study, in order to evaluate the effects of the number of hydroxyl groups on the B-rings of flavonoids on the functions of human CYP 1A1, CYP1A2, and CYP1B1, we selected three 5, 7-dihydroxyflavones: chrysin, apigenin, and luteolin. These substances exhibit either no hydroxyl groups, one hydroxyl group, or two hydroxyl groups on their respective B-rings (Fig. 1). We then attempted to evaluate the effects of these compounds on 7-ethoxyresorufin O-deethylation (EROD) activity, using recombinant human CYP1 enzymes as well as human microsomes.

MATERIALS AND METHODS

Materials

The apigenin, chrysin, luteolin, β-NADPH, δ-aminolevulinic acid, 7-ethoxyresorufin, and resorufin used in this study were purchased from the Sigma Chemical Co. (St, Louis, MO). The isopropyl-beta-D-thiogalactopyranoside (IPTG) was acquired from Calbiochem (La Jolla, CA, U.S.A.). The bactotryptone, bactopeptone, yeast extract, and bactoagar were purchased from Difco-Becton Dickinson (Sparks, MD, U.S.A.). All other chemicals employed were of the highest grade available.

Expression of recombinant human P450s

Bicistronic plasmids containing human CYP1B1, CYP1A1, or CYP1A2 with human NADPH-cytochrome P450 reductase (hNPR) cDNAs were expressed according to the previously described method (Parikh *et al.*, 1997; Shimada *et al.*, 1998). In brief, these bicistronic plasmids were introduced into *Escherichia coli* DH5α cells (New England Biolabs, U.S.A.) *via* electrophoration. A single ampicillin-resistant colony of transformed cells was picked up and grown overnight in LB medium containing 100 μg ampicillin/mL, until saturation was achieved. A 10 mL aliquot of this was then subsequently inoculated into each liter of modified TB media (Parikh *et al.*, 1997) and grown for an additional at 28°C for 48 h, with agitation (220 rpm). Membrane fractions containing the recombinant human family 1 P450s and hNPR were prepared from the cultured bacteria, according to the previously described method (Parikh *et al.*, 1997), then suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% (v/v) glycerol. The levels of CYP and NPR expression in the *E. coli* membranes used in this experiment are shown in Table I.
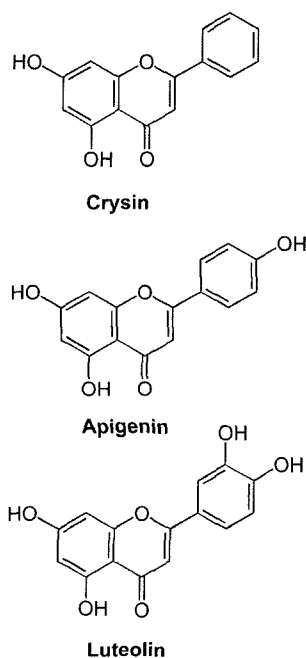

Crysin

Apigenin

Luteolin

Fig. 1. Chemical structures of the 5,7-dihydroxyflavones used in this study

Table I. Kinetic parameters for the *O*-deethylation of 7-ethoxy-resorufin by recombinant CYP1 enzymes

	Molar ratio of reductase/ CYP in bacterial membrane	Vmax (nmol/min/nmol P450)	Km (μM)	Vmax/Km
CYP1A1	0.30	2000	13.8	145
CYP1A2	0.19	714	12.4	58
CYP1B1	0.48	3333	15.5	218

0.1-2 μM 7-Ethoxyresorufin was incubated with membranes from *E. coli* co-expressed human cytochrome P450s and NADPH-P450 reductase cDNAs (50 pmol mL⁻¹ CYP) at 37°C for 10 minutes. Results are derived from triplicate determinations.

Enzyme assays

7-Ethoxyresorufin *O*-dealkylation assays were conducted using methods which have been described elsewhere (Dong *et al.*, 2002). The enzyme reaction mixture (0.5 mL) contained 100 mM potassium phosphate buffer (pH 7.4), 25 pmol P450 of *E. coli* membranes, or 0.2 mg of human liver microsome proteins (kindly donated by Prof. Y.N. Cha of Inha University, Inchon, Korea), as well as varying concentrations of 7-ethoxyresorufin. The reaction mixture was preincubated at 37°C for 3 minutes, and oxidation was elicited *via* the addition of 0.5 mM NADPH. After 10 minutes of incubation, the enzyme reaction was discontinued *via* the addition of 2 mL of ice-cold methanol, and the amount of resorufin generated during the incubation period was determined fluorometrically, using a Tecan SPECTRAFluor Plus set (Tecan Ltd., Switzerland) at 520 nm (excitation) and 590 nm (emission). The rates were calculated on the assumption that this production had occurred in a linear fashion over the incubation period. The CYP content in the isolated *E. coli* membranes was determined by the spectral methods described by Omura and Sato (1964), using a Cary 300 Bio UV-visible spectrophotometer (Beckman, Australia) at ambient temperature, and was calculated with an extinction coefficient of $\Delta\varepsilon_{450-490} = 91$ mM⁻¹cm⁻¹. The inhibitory effects of the flavonoids on the rate of NADPH-driven cytochrome c reduction (NPR) were determined in accordance with the method described by Yasukochi and Masters (1976), with an extinction coefficient of $\Delta\varepsilon_{550} = 21$ mM⁻¹ cm⁻¹.

NADPH dependence of inhibition

The bacterial membranes co-expressing CYP and NPR containing 50 pmol of CYP were preincubated in 100 mM potassium phosphate buffer (pH 7.4), and the IC₅₀ of each 5,7-dihydroxyflavone, in the presence or absence of 200 μM NADPH, at 37°C. After differing incubation times, the 7-ethoxyresorufin was added, with or without NADPH. The amount of resorufin remaining after incubation was then evaluated, as described above.

Data analysis

The IC₅₀ values for the activity-concentration curves from each of the individual experiments were calculated with Sigma-plot software (SPSS Science, Chicago), using a non-linear regression equation. The inhibitory mode of the flavonoids on the CYP1-catalyzed EROD activity was then determined *via* a Lineweaver-Burk plot, and the K*i* values were calculated from the replots of slope versus inhibitor concentrations. All results shown were obtained from at least 3 separate experiments.

RESULTS

Effects of the 5,7-dihydroxyflavones on CYP1-dependent ethoxyresorufin *O*-deethylation activities

During the initial studies, we determined the 7-ethoxyresorufin concentration-dependent EROD activity in the bacterial membranes that coexpressed human CYP1A1, CYP1A2, or CYP1B1 with NPR (Table I). The Vmax of EROD, as calculated by Linewever-Burke plot, was determined to occur in decreasing order, as follows: CYP1B1, CYP1A1, and CYP1A2. However, the Km values did not differ significantly among the three.

The inhibitory effects of the 5,7-dihydroxyflavones on recombinant human CYP1 enzymes expressed in *E. coli* with NPR are shown in Fig. 2. The control activity levels of resorufin formation by CYP1A1, CYP1A2, and CYP1B1 were 42.03 ± 3.78, 20.66 ± 5.11, and 64.28 ± 8.05 nmol min⁻¹ nmol⁻¹ P450, respectively. CYP1B1-catalyzed EROD activity was determined to have been inhibited by all of the 5,7-dihydroxyflavones at lower concentrations (IC₅₀ less than 0.4 μM), to a greater degree than was observed for either CYP1A1 or CYP1A2-dependent EROD activities (Table II). Chrysin, which features a non-hydroxyl group on its B-ring, was the most potent inhibitor of CYP1A2-catalyzed EROD activity, among the three tested compounds. Luteolin, which features two hydroxyl groups on its B-ring, was determined to be the most effective CYP1A1-catalyzed EROD inhibitor. Increases of the hydroxyl group number on the B-ring of the interacting flavonoid resulted in a more profound inhibition of CYP1A1, in contrast to CYP1A2. However, we determined there to be no observable correlation with regard to the inhibition potency of CYP1B1 by the 5,7-dihydroxy-flavones, regardless of the hydroxyl group numbers on the B-rings.

Effects of 5,7-dihydroxyflavones on hNPR activity

As NPR transfers electrons from NADPH to the CYPs, CYP should be inhibited as the result of chemical inhibition of the NPR. The effect of 5,7-dihydroxyflavones was tested on human NPR activity at 10 μM, but we
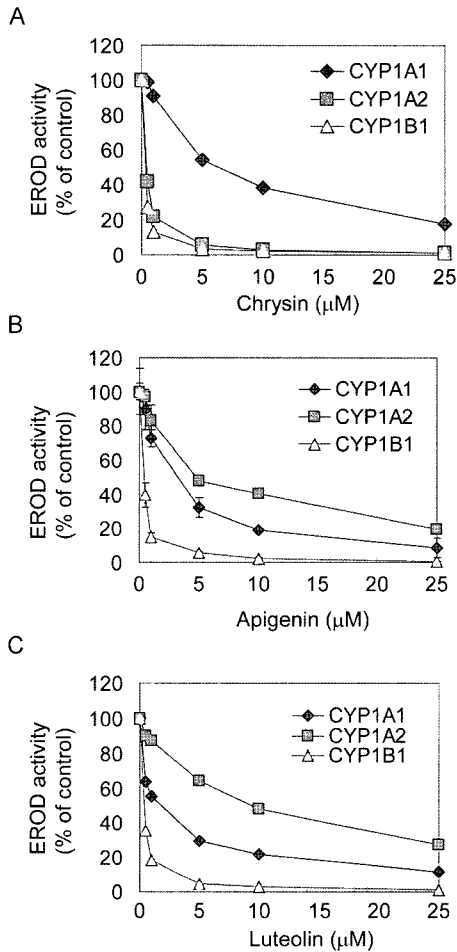
Fig. 2. Effect of 5,7-dihydroxyflavones on human CYP 1A1, CYP1A2, and CYP1B1 activities in CYP and NPR coexpression systems. Bacterial membranes coexpressing human CYP 1s and NPR were incubated with various concentrations of (A) crysin, (B) apigenin, and (C) luteolin, as described in the Materials and Methods section. Each data point represents the mean ± S.E.M. of three experiments.

Table II. IC_{50} values for 5,7-dihydroxyflavones on 7-ethoxyresorufin O-deethylation by recombinant CYP1 enzymes

CYP	5,7-dihydroxyflavones (mM)		
	Apigenin	Chrysin	Luteolin
CYP1A1	4.1	6.2	1.2
CYP1A2	5.1	0.42	8.9
CYP1B1	0.36	0.28	0.36

The IC_{50} values for CYP1 enzymes

observed no significant inhibitions of NPR (data not shown).

Inhibition pattern of human CYP1 activity by 5,7-dihydroxyflavones

In order to gain insight into the mechanisms underlying the inhibitory properties of the 5,7-dihydroxyflavones, we
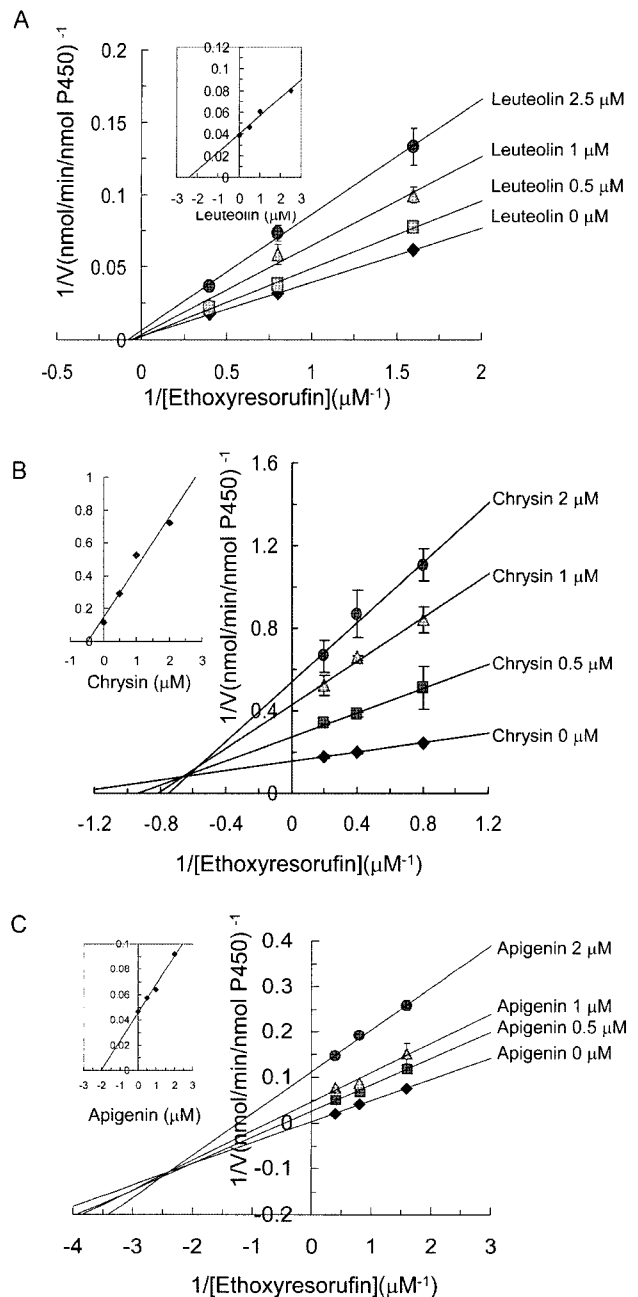

Fig. 3. Lineweaver-Burk plots representing the inhibition of CYP1A1, CYP1A2, and CYP1B1 activities by luteolin, chrysin and apigenin. The inhibition of (A) CYP1A1, (B) CYP1A2, and (C) CYP1B1 was conducted using the strongest inhibitor among three 5,7-dihydroxyflavonoids for each of the CYP isozymes. EROD activity was assessed using E. coli membranes coexpressing human CYP and NPR, in the presence of an inhibitor. The slopes were generated via linear regression analysis. The assays were performed at three different substrate concentrations and four different inhibitor concentrations. Each data point represents the mean ± S.E.M. of three experiments.

conducted kinetic analyses of the EROD in the E. coli membranes that coexpressed CYP1 and NPR (Fig. 3). In order to generate the Lineweaver-Burk plot, we used 0.5-

2.5 µM of 5,7-dihydroxyflavone, the most potent inhibitor of each of the CYP1 enzymes among our tested compounds, as well as 0.625, 1.25 or 2.5 µM of 7-ethoxyresorufin. Luteolin proved to be a competitive inhibitor of CYP1A1 at low concentrations. At luteolin concentrations above 1.0 µM, however, the inhibition mode switched from a competitive to a mixed-type mode. Chrysin and apigenin were found to be mixed-type inhibitors of CYP1A2 and CYP1B1, respectively. The apparent Ki values of luteolin, chrysin, and apigenin, vs. CYP CYP1A1, CYP1A2, and CYP1B1 were 2.4 µM, 0.5 µM, and 2.0 µM, respectively.

Mechanism of CYP1 activity inhibition by 5,7-dihydroxyflavones

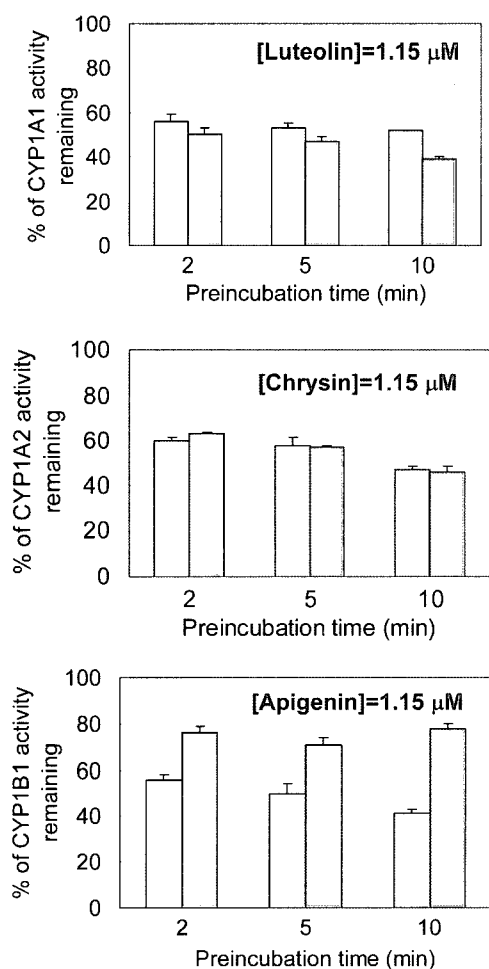In order to determine whether or not the catalytic

Fig. 4. Time- and NADPH-dependent inactivation of CYP activities by the 5,7-dihydroxyflavonoids. The 5,7-dihydroxyflavonoids, along with bacterial membranes coexpressing CYP and NPR, were preincubated either with or without NADPH, for 2, 5 or 10 minutes. The mixture was then diluted 10-fold, in order to conduct the catalytic assays, using 2.5 mM ethoxyresorufin and 0.5 mM NADPH. The control experiments used the same conditions, but without the flavonoids. Each data point represents the mean ± S.E.M. of three experiments.
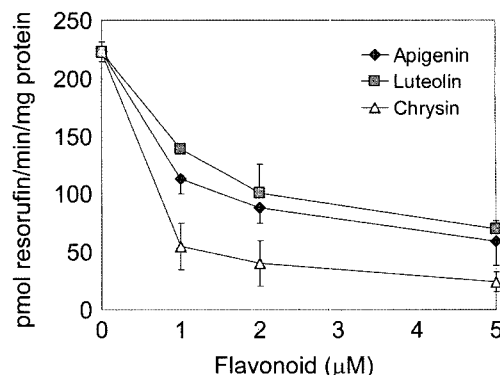
Fig. 5. EROD activity-inhibitory effects of the 5,7-dihydxoyflavonoids in human liver microsomes. The reaction mixture, containing 0.2 mg of human liver microsomes, was incubated for 20 minutes, as described in the Materials and Methods section. Each data point represents the mean ± S.E.M. of three experiments.

processing of the inhibitors was a prerequisite for the inactivation of CYP, we attempted to characterize the effects of NADPH. We conducted the preincubation step for differing durations (2 to 10 min), in the presence or absence of NADPH and an IC$_{50}$ of 5,7-dihydroxyflavones (Fig. 4). When the preincubation durations were increased in these samples, the presence of NADPH appeared to induce only minor changes in the activities of CYP1A1 and CYP1A2, when luteolin and chrysin, respectively, were used. However, when apigenin was employed, the level of CYP1B1 inhibition decreased as the result of preincubation with NADPH. This indicates that the inhibition of CYP1 was mediated by the 5,7-dihydroxyflavones, rather than by a metabolite. Therefore, the 5,7-dihydroxyflavones do not appear to be mechanism-based inhibitors.

Inhibitory effects of 5,7-dihydroxyflavones on human liver microsomal ethoxyresorufin O-deethylation activity

Although all three of the 5,7-dihydroxyflavones were shown to inhibit EROD in human microsomes, these compounds differed with regard to their inhibitory potencies (Fig. 5). Chrysin, among these, proved to be the most effective EROD inhibitor, with an IC$_{50}$ of 0.57 µM in human microsomes. Apigenin and luteolin also profoundly inhibited EROD activity, with IC$_{50}$s of 1.28 and 2.62 µM, respectively.

DISCUSSION

The flavonoid-mediated modulation of CYP activity has recently attracted a great deal of attention, as the result of individual differences in their susceptibility to chemical carcinogens. As a result of much of the early work regarding the oxidative metabolism of carcinogenic poly-

cyclic aromatic hydrocarbons and related chemicals, it is now generally accepted that the three human CYP1 enzymes (CYP1A1, CYP1A2, and CYP1B1) constitute effective molecular targets for the activities of cancer chemopreventive agents (9, 10). Therefore, the ability of flavonoids to regulate CYP1 has been studied extensively, as have the effects of the structural characteristics of flavonoids with regard to the inhibition of CYP1 activity. According to the findings of studies concerning CYP1A1 and CYP1A2 inhibition, the structure-function relationship of flavonoids has also been fairly exhaustively researched (Hodet et al., 2002; Lee et al., 1998). Flavanones and flavans (which lack the C2-C3 double bond), both of which possess phenyl groups (B-ring) that are situated nearly perpendicularly to the rest of the molecule, exhibited a much less pronounced inhibitory efficacy than did the flavones. Therefore, planar molecules with small volume/surface ratios also appear to exhibit high degrees of CYP1A2 inhibitory activity. Glycosylation, in addition to the presence of several hydroxyl groups and/or methoxy-group additions, result in drastic reductions in this inhibitory activity. On the basis of observations of the effects of the catechins, the oxo-group (position C4) in the C-ring would seem to be an essential factor with regard to enzyme inhibition (Lee et al., 1998; Moon et al., 1998).

Lee et al. (1998) reported that these inhibitory activities cannot be attributed solely to the numbers of hydroxyl groups in the B-rings of flavonoids. In this study, we selected the 5,7-dihydroxyflavones, which satisfy the structural requirements for profound CYP1 inhibition, in order to assess the actual effects of the hydroxyl group numbers in the B-rings of flavonoids on the inhibition of human CYP1 enzyme-dependent EROD activity. All three of the 5,7-dihydroxyflavones were known to be potent CYP1 enzyme inhibitors, at IC_{50} values of less than 10 μM. These results were consistent with the theory that the presence of a double bond at the C2, C3 location confers a co-planar conformation upon the B-ring, with respect to the benzopyran rings (rings A and C). Planar flavonoid molecules interact preferentially with CYP1 enzymes.

We observed that CYP1B1-catalyzed EROD activity was inhibited by all of the 5,7-dihydroxyflavones used in this study at lower concentrations (IC_{50} less than 0.4 μM), to a greater degree than were either CYP1A1 or CYP1A2-dependent EROD activities (Table II). It appears that no real correlation exists between the inhibitory potency of CYP1B1 by the 5,7-dihydroxyflavones and the hydroxyl group numbers of their respective B-rings. Chrysin, which possesses a non-hydroxyl group in its B-ring, was the most potent inhibitor of CYP1A2-dependent EROD activity, with an IC_{50} of 0.42 μM, followed by apigenin (5.2 μM), and finally luteolin (8.9 μM). Lee et al. (1998) reported similar results, such that in the N-

demethylation of caffeine in human microsomes, the order of CYP1A2 inhibition was as follows: chrysin (IC_{50} 0.2 μM) > apigenin (IC_{50} 1.35 μM) > luteolin (IC_{50} 13.4 μM). They suggested that the hydrophobic interactions between CYP1A2 and the flavonoids appeared to be a more relevant factor in the determination of inhibitory ability than did the electronic properties of the flavonoids. This would show that the 5,7-dihydroxyflavones, which possess the lowest numbers of hydroxyl groups on their B-rings, are actually more effective CYP1A2 inhibitors. Unlike what was seen with CYP1A2, luteolin, which possesses two hydroxy groups on its B-ring, was determined to be the most effective inhibitor of CYP1A1-catalyzed EROD, followed by apigenin, and finally chrysin. The IC_{50} of luteolin was 3.6- and 5.4-fold less than the IC_{50}s determined for apigenin and chrysin, respectively. Collectively, increases in the numbers of hydroxyl groups on the B-rings conferred more potent inhibitory abilities with regard to CYP1A1, and less potent inhibitory abilities with regard to CYP1A2. Thus, the numbers of hydroxyl substitutions appear to exert a regulatory effect on the isozyme selectivity inherent to flavonoid-induced inhibition.

In the present study, all of the tested 5,7-dihydroxy-flavones functioned as mixed-type inhibitors of CYP1 enzymes, and exhibited no inhibitory effects on NPR activity. These data showed that they are able to compete for substrate binding sites, and may also bind to regions which are not, in fact, directly involved in substrate binding. Other flavones, including 2',5,6',7-tetrahydroxy flavone (Kim et al., 2002), and apigenin (Lee et al., 1998), also exhibit mixed-type inhibitory activity with regard to CYP1A2. When the preincubation time was increased in the presence or absence of NADPH and 5,7-dihydroxy-flavones we noted no significant alteration of the inhibition of chrysin and luteolin-induced CYP activity, in the presence of NADPH. However, CYP1B1 inhibition by apigenin was notably reduced when the preincubation involved the presence of NADPH. Apigenin was metabolized to luteolin in both human and mouse hepatic microsomes, and this activity could be inhibited, albeit weakly, by fluvoxamine (IC_{50} > 250 μM) in humans, and by α-naphthoflavone (IC_{50} 25.4 μM) in rats (Breinholt et al., 2002). These findings show that CYP1 may not be actively involved in the metabolization of apigenin to luteolin. Although this has yet to be determined, the CYP1B1-dependent apigenin metabolite may not inhibit its activity.

In the human microsome experiments, chrysin was determined to be the most potent inhibitor of EROD activity, followed by apigenin and, finally, luteolin. Human CYP1A2 is expressed principally in the liver. This, however, is not the case with CYP1A1 and CYP1B1, which are expressed only in small amounts in the liver.

Thus, the inhibitory order of EROD by the 5,7-dihydroxy-flavones in human liver microsomes is identical to that of CYP1A2-mediated EROD activity.

Humans are estimated to ingest approximately 0.6-1 g of flavonoids daily, and flavones may be present in considerable amounts in leafy vegetables (Ross and Kasum, 2002). Variable dietary exposure to flavonoids may contribute not only to the inhibition of carcinogenic initiation, but also may facilitate some of the individual variations in the pharmacokinetics and pharmacological responses of medicines metabolized by CYP1A2 enzymes, including caffeine and theophyllin, as well as those observed for drugs which function as substrates for other CYP isozymes. Thus, depending on the medicines taken by an individual, it may prove beneficial to control the amounts of dietary flavonoids ingested by a given subject.

In summary, the numbers of hydroxyl groups on the B-rings of 5,7-dihydroxyflavones exert a regulatory effect on the selectivity of CYP1 enzymes. Higher hydroxyl group numbers result in more profound inhibitions of CYP1A1 activity, but less potent inhibitions of CYP1A2 activity. However, CYP1B1 activity does not appear to be affected by this variability.

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