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Inhibition of Extrahepatic Human Cytochromes P450 1A1 and 1B1 by Metabolism of Isoflavones Found in *Trifolium pratense* (Red Clover)

DEAN W. ROBERTS,† DANIEL R. DOERGE,† MONA I. CHURCHWELL,† GONÇALO GAMBOA DA COSTA,§ M. MATILDE MARQUES,§ AND WILLIAM H. TOLLESON*,†

Division of Biochemical Toxicology, National Center for Toxicological Research, 3900 NCTR Road, Jefferson, Arkansas 72079, and Centro de Química Estrutural, Complexo I, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

Biochanin A and formononetin are the predominant isoflavones in red clover. In a previous study (*J. Agric. Food Chem.* **2002**, *50*, 4783−4790), it was demonstrated that human liver microsomes converted biochanin A and formononetin to genistein and daidzein. This paper now shows CYP1B1-catalyzed *O*-demethylation of biochanin A and formononetin to produce genistein and daidzein, respectively, which inhibit CYP1B1. Recombinant human CYP1A1 or CYP1B1 was incubated with biochanin A or formononetin. CYP1A1 catalyzed isoflavone 4′-*O*-demethylation and hydroxylations with similar efficiency, whereas CYP1B1 favored 4′-*O*-demethylation over hydroxylations. Three of the biochanin A metabolites (5,7,3′-trihydroxy-4′-methoxyisoflavone, 5,7,8-trihydroxy-4′-methoxyisoflavone, and 5,6,7-trihydroxy-4′-methoxyisoflavone) were characterized by ^{1}H NMR spectroscopy and mass spectrometry. Daidzein (K_i = 3.7 μM) exhibited competitive inhibition of CYP1B1 7-ethoxyresorufin *O*-deethylase activity, and genistein (K_i = 1.9 μM) exhibited mixed inhibition. Biochanin A and/or formononetin may exert anticarcinogenic effects directly by acting as competitive substrates for CYP1B1 or indirectly through their metabolites daidzein and genistein, which inhibit CYP1B1.

KEYWORDS: Biochanin A; CYP1A1; CYP1B1; cytochrome P450; demethylation; enzyme inhibition; extrahepatic metabolism; formononetin; hydroxylation; isoflavones; *Trifolium pratense*

INTRODUCTION

Dietary consumption of foods and food additives containing isoflavone phytoestrogens has been associated with a variety of health benefits, including relief from symptoms of menopause (*1*) and reduced risk of hormonal cancer (*2*). Extracts of *Trifolium pratense* (red clover) have been proposed as an alternative to traditional hormone replacement therapy in postmenopausal women (*3*) even though clinical trials showed no effect (*4*). The isoflavones formononetin and biochanin A, and their corresponding 4′-*O*-demethylated derivatives daidzein and genistein (**Figure 1**), comprise approximately 52, 40, 2, and 5%, respectively, of the total isoflavone content of *T. pratense* extracts (*5*). Further oxidative metabolism of daidzein and genistein (*6*, *7*) and of formononetin and biochanin A (*8*, *9*) by rat and human liver microsomes to yield polyphenolic derivatives has recently been described.

Isoflavone metabolism in humans occurs stepwise (reviewed in ref *8*). Dietary isoflavonoids consist mainly of 7-β-D-glucoside and 6″-malonylglucoside conjugates (*10*). Both the glucoside conjugates and their isoflavone aglycons are bioavailable (*11*−*13*). Isoflavone glycosides in the intestinal tract are deconjugated to the corresponding aglycons by bacterial β-glucosidases and by membrane-bound β-glucosidase expressed by enterocytes of the small intestine. Isoflavone aglycons are taken up by enterocytes and converted to glucuronide or sulfate conjugates by phase II enzymes before their delivery into the circulation. Enterocytes also express cytochrome P450 (CYP) 3A4 abundantly, as well as CYP2C9 and CYP2C19 at moderate levels, and lower or variable amounts of the inducible isoforms CYP1A1, CYP1B1, and CYP2E1 (trace) (*14*−*16*). In addition, CYP2E1 mRNA expression has been reported in the human small intestine (*17*).

Previous studies in vitro showed that human liver microsomal enzymes catalyze isoflavone 4′-*O*-demethylation (*6*, *9*). However, the major hepatic CYP450 isoform, CYP3A4, does not catalyze biochanin A or formononetin demethylation, and recombinant human CYP2C9 and CYP2C19 are only weakly competent to catalyze this transformation (*9*). These findings suggest that other CYP450 isoforms, namely, the CYP1 family, catalyze the conversion of formononetin and biochanin A to

* Corresponding author [telephone (870) 543-7645; fax (870) 543-7136; e-mail wtolleson@nctr.fda.gov].
† National Center for Toxicological Research.
§ Instituto Superior Técnico.

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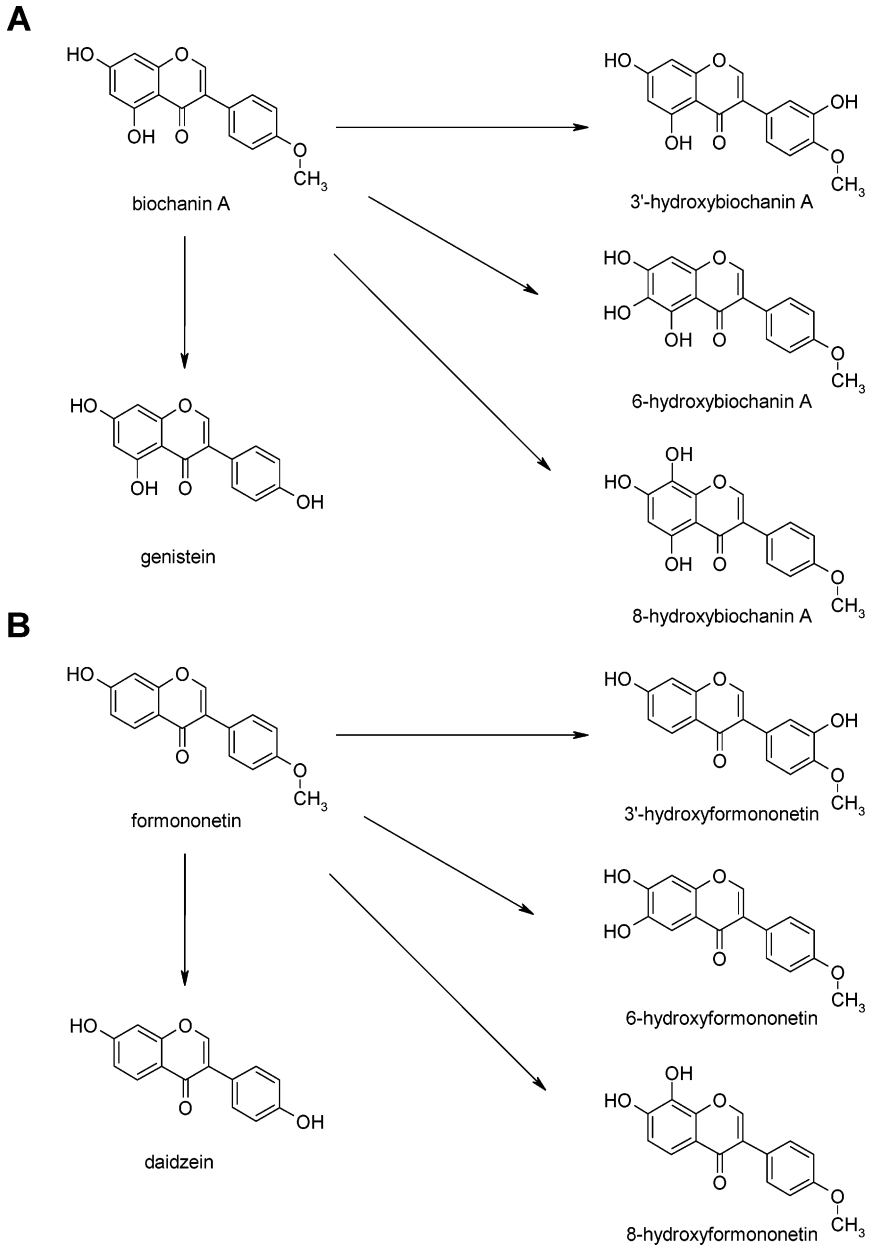


Figure 1. Hydroxylation and demethylation reactions catalyzed by cytochromes P450 1A1 and 1B1 for (**A**) biochanin A and (**B**) formononetin.

daidzein and genistein, respectively. In fact, human CYP1A2 is the primary hepatic CYP450 isoform responsible for this activity (*9*, *18*), provoking the hypothesis that extrahepatic CYP1 isoforms are also important for isoflavone metabolism.

Extensive demethylation of formononetin and biochanin A was indicated by transient low levels (<10 ng/mL) of formononetin and biochanin A and much higher and sustained levels of daidzein and genistein (>100 ng/mL) in the plasma of a human volunteer following consumption of a commercially available red clover extract dietary supplement containing 23.7 mg of biochanin A and 14.3 mg of formononetin (*10*). This evidence is consistent with efficient CYP450-dependent 4′-*O*-demethylation. However, it also suggests that consumption of a sufficiently large dose of formononetin and biochanin A may saturate metabolic capacity, enabling detectable amounts of the methylated isoflavones to accumulate in the peripheral circulation.

Consumption of foods containing flavonoids, the 2-substituted analogues of isoflavonoids, may inhibit gut enzymes that normally catalyze isoflavone *O*-demethylation. Doostdar and co-workers (*19*) demonstrated that six common flavonoids present in citrus juices at ∼70−200 mg/L are potent inhibitors of CYP1A1, CYP1A2, and CYP1B1 activities in vitro. Diosmetin (5,7,3′-trihydroxy-4′-methoxyflavone) and hesperetin (5,7,3′-trihydroxy-4′-methoxyflavanone) are potent inhibitors of the CYP1 family and share structural features with 3′-hydroxybiochanin A, a metabolite described in this study. Collectively, these observations suggest that consumption of fruits and vegetables containing flavonoids (e.g., acacetin, diosmetin, naringenin, hesperetin, eriodictyol, or homoeriodictyol) at the same time as food additives containing biochanin A or formononetin could inhibit CYP1 family enzymes in the gut and liver and increase the concentration of biochanin A or formononetin in blood.

In view of the putative health benefits associated with phytoestrogen consumption, the potential metabolic interaction between phytoestrogens and endogenous estrogens in target tissues for estrogen carcinogenesis is of particular interest. The major source of estrogen is a three-step reaction sequence catalyzed by CYP19 (aromatase), which converts androstene-

dione and testosterone to estrone and estradiol, respectively (*20*). Normal human breast and human breast tumor tissues are known to express various CYP450 isoforms, including CYP1B1, a catalytically efficient estrogen 4-hydroxylase (*21*). The co-localization of aromatase and CYP1B1 in breast tissue suggests that the local concentration of putatively carcinogenic 4-hydroxy-estrogen products may be higher than that indicated by the circulating levels of these metabolites (*21*). It is conceivable that alternate substrates or inhibitors of CYP1B1 could be associated with a reduced risk of estrogen-induced carcinogenesis.

In the work presented here we examine the metabolism of formononetin and biochanin A by CYP1A1 and CYP1B1 to yield daidzein, genistein, and hydroxylation products and their effects on the catalytic activities of both isoforms.

MATERIALS AND METHODS

Chemicals and Biochemicals. Human cytochrome P450 isoforms 1A1 and 1B1 and human cytochrome P450 oxidoreductase, expressed by a baculovirus microsomal expression system, and an NADPH regeneration system were obtained from Gentest Corp. (Woburn, MA). Genistein [CAS Registry No. 446-72-0], daidzein [486-66-8], formononetin [485-72-3], and biochanin A [491-80-5] were obtained from Indofine Chemical Co. (Somerville, NJ). Isoflavone stock solutions were prepared in DMSO and diluted to 0.1 mM with absolute ethanol (0.2% DMSO, final). The purity of isoflavone stock solutions was verified by HPLC with UV and electrochemical detection, and concentrations were determined by UV spectrophotometry using the molar absorptivity coefficients reported by Franke et al. (*22*).

Microsomal Incubations with Human CYP450 Isoforms. Each 0.5 mL reaction mixture included 0−10 nmol of isoflavones, microsomes containing 1 pmol of recombinant human CYP450 isoform 1A1 or 1B1, and microsomes expressing recombinant human cytochrome P450 oxidoreductase (0.09 mg). The protein concentration was adjusted to 0.1 mg/mL by adding control microsomes devoid of CYP450 activity (Gentest). Reactions also contained 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 1 mg/mL ascorbic acid, 1.33 mM $NADP^+$, 3.3 mM glucose-6-phosphate, and 3.3 mM magnesium chloride. Reactions were equilibrated for 3 min at 37 °C and then initiated by adding 0.4 unit/mL glucose-6-phosphate dehydrogenase. Incubations were conducted at 37 °C with gentle mixing for the times given in the appropriate figure legends. The reactions were quenched by extracting twice with 1.0 mL of ethyl acetate. The organic extracts were combined, dried in vacuo, and stored at −80 °C. The samples were reconstituted in 100 μL of methanol assisted by ultrasonic agitation, and 20 μL aliquots were injected into the HPLC detection system.

Rat Liver Microsome Reactions. Bulk reactions (100 mL) were used to produce sufficient quantities of isoflavone metabolites for ^{1}H NMR analysis. The incubation mixtures contained either 0.5 mM formononetin or biochanin A and 1.0 mg/mL β-naphthoflavone-induced rat liver microsomes (In Vitro Technologies, Baltimore, MD) with the NADPH generating system and buffer described above. The mixtures were incubated overnight at 37 °C and extracted with ethyl acetate (3 × 100 mL). The extracts were passed through a mixed bed ion-exchange resin (Bio-Rad AG 501 X8 resin; 5 g) preequilibrated with ethyl acetate to remove charged lipids and concentrated to 4 mL under vacuum. The volume of the concentrated extract was adjusted to 8 mL using methanol and then further diluted to 200 mL with high-purity water. The diluted extract was divided into 100 mL portions and applied to separate Waters Sep-Pak cartridges (C-18 resin, 6 mL) preequilibrated with aqueous 5% methanol. The cartridges were washed with 15 mL of 5% methanol and then eluted using 5 mL of 75% methanol. The eluted samples were dried under vacuum and reconstituted to 0.5 mL using methanol. Isoflavone metabolites were isolated by preparative reverse phase HPLC as described previously (*9*). Metabolite-containing HPLC fractions were diluted with high-purity water to a final concentration of 4% acetonitrile, adsorbed to C18 Sep-Pak cartridges as described above, and eluted with 4 mL of methanol. The solvent was evaporated under vacuum, and the samples were dissolved in 0.1 mL of methanol for further purification by HPLC until >90% purity was achieved. Unstable isoflavone metabolites were sealed under argon and stored at −80 °C until ^{1}H NMR analyses could be performed.

Isoflavone Analyses Using HPLC-EC and HPLC-MS. Analyses using high-pressure liquid chromatography with electrochemical detection (HPLC-EC) and HPLC with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) detection were conducted as described previously (*9*).

^{1}H NMR Analyses. ^{1}H NMR analyses of the isoflavone metabolites isolated from rat liver microsomal incubations were performed in dimethyl sulfoxide-d_6 solutions on a Varian Gemini 300 spectrometer operated at 300 MHz. Chemical shifts are reported in parts per million downfield from tetramethylsilane, and coupling constants are reported in hertz. Proton assignments were based on comparison with the spectra of the parent biochanin A or formononetin, as well as their previously characterized metabolites (*8, 9*). Whenever necessary, 2D ^{1}H−^{1}H-COSY sequences were run for additional confirmation of the assigned resonances.

Enzyme Kinetic Constants for Human Cytochromes P450 1A1 and 1B1. Michaelis−Menten enzyme kinetic constants were calculated using the isoflavone metabolite concentrations determined in the ethyl acetate extracts from 0.5 mL reactions. Nonlinear regression analysis was performed using the Prism software program (GraphPad Software Inc., San Diego, CA).

Inhibition of Cytochrome P450 1B1-Dependent 7-Ethoxyresorufin Deethylase (EROD) Activity by Genistein and Daidzein. EROD activity was determined as described by the enzyme supplier using a Fluoroskan 96-well plate reader (Thermo Labsystems Inc., Franklin, MD) equipped with 550 nm excitation and 590 nm emission filters. Each 250 μL reaction mixture contained 0.1 M potassium phosphate (pH 7.4), 2 mM EDTA, 1 mg/mL ascorbic acid, 4 nM CYP1B1, 0.1 mg/mL microsomal protein, 0.4 nM cytochrome P450 oxidoreductase, 5−1280 nM 7-ethoxyresorufin, 1.33 mM $NADP^+$, 3.3 mM glucose-6-phosphate, and 3.3 mM magnesium chloride. Genistein or daidzein (0−20 μM) was added to each mixture, which was then incubated for 3 min at 37 °C. The reactions were initiated by adding 0.4 unit/mL glucose-6-phosphate dehydrogenase, and the increase in fluorescence at 590 nm was recorded. Product formation was determined by comparing fluorescence measurements to those obtained from resorufin standard curves. Enzyme inhibition constants were calculated using Sigma Plot (SPSS, Inc., Chicago, IL).

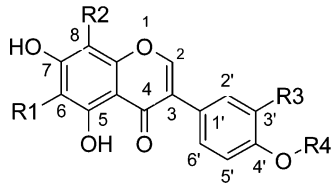
Statistical Analyses. Statistical analyses were performed using Prism software. Student's *t* test and one-way ANOVA were used to compare enzyme kinetic and inhibition parameters. The level of statistical significance was set at $P < 0.05$ for all data comparisons.

RESULTS

Identification of Metabolites Formed by *O*-Demethylation and Hydroxylation of Formononetin and Biochanin A. In a previous paper (*9*) we demonstrated that formononetin and biochanin A are *O*-demethylated by human hepatic microsomal enzymes to yield daidzein and genistein, respectively. In addition, we used HPLC-MS and ^{1}H NMR spectroscopy to identify 7,3′-dihydroxy-4′-methoxyisoflavone, 6,7-dihydroxy-4′-methoxyisoflavone, and 7,8-dihydroxy-4′-methoxyisoflavone as metabolites of formononetin formed by human liver microsomal enzymes, particularly cytochrome P450 isoforms 1A2 and 2C9*1. Besides genistein, three additional metabolites of biochanin A formed by human liver microsomal enzymes were detected. One of these metabolites was identified as 3′-hydroxygenistein. The other two metabolites had protonated molecular ions (*m*/*z* 301) consistent with hydroxylated biochanin A regioisomers, but insufficient quantities for detailed structural analyses were generated.

In this paper we show that extrahepatic human cytochrome P450 isoforms 1A1 and 1B1 also efficiently catalyze 4′-*O*-demethylation and hydroxylation reactions of formononetin and

Table 1. ^{1}H NMR Data for Biochanin A (BA) and BA Metabolites[a]



δ	biochanin A ($R_1 = R_2 = R_3 = H$; $R_4 = CH_3$)	genistein[b] ($R_1 = R_2 = R_3 =$ $R_4 = H$)	3′-OH-biochanin A ($R_1 = R_2 = H$; $R_3 = OH$; $R_4 = CH_3$)	8-OH-biochanin A ($R_1 = R_3 = H$; $R_2 = OH$; $R_4 = CH_3$)	6-OH-biochanin A ($R_1 = OH$; $R_2 = R_3 = H$; $R_4 = CH_3$)
CH_3	3.79 (3H, s)		3.79 (3H, s)	3.79 (3H, s)	3.79 (3H, s)
H2	8.38 (1H, s)	8.26 (1H, s)	8.32 (1H, s)	8.41 (1H, s)	8.35 (1H, s)
H6	6.24 (1H, s)	6.21 (1H, d, $J = 1.95$)	6.22 (1H, d, $J = 2.0$)	6.30 (1H, s)	
H8	6.40 (1H, s)	6.39 (1H, d, $J = 1.95$)	6.38 (1H, d,J 2.0)		6.50 (1H, s)
H2′,6′	7.50 (2H, d, $J = 8.6$)	7.38 (2H, d, $J = 8.2$)		7.51 (2H, d, $J = 8.8$)	7.51 (2H, d, $J = 8.7$)
H3′,5′	7.01 (2H, d, $J = 8.6$)	6.82 (2H, d, $J = 8.2$)		7.00 (2H, d, $J = 8.8$)	7.00 (2H, d, $J = 8.7$)
H2′			7.03 (1H, d, $J = 1.7$)		
H5′			6.96 (1H, bs)		
H6′			6.95 (1H, m)		
5-OH	12.93 (1H, s)	12.98 (1H, s)	12.97 (1H, bs)	12.35 (1H, s)	12.76 (1H, s)
6-OH					8.79 (1H, bs)
7-OH	11.04 (1H, bs)	10.92 (1H, s)	ND[c]	10.55 (1H, bs)	10.54 (1H, bs)
8-OH				8.76 (1H, bs)	
3′-OH			9.06 (1H, bs)		
4′-OH		9.60 (1H, s)			

[a] Spectra were recorded in DMSO-d_6. Abbreviations: bs, broad singlet; d, doublet; m, multiplet; s, singlet; *J*, coupling constant (Hz). [b] Data from Wang, H.; Nair, M. G.; Strasburg, G. M.; Booren, A. M.; Gray, J. I. Antioxidant polyphenols from tart cherries (*Prunus cerasus*). *J. Agric. Food Chem.* **1999**, *47*, 840–844. [c] Not detected.

biochanin A. We further show that daidzein and genistein are the major reaction products of CYP1A1- and 1B1-catalyzed reactions with formononetin and biochanin A, respectively, and that hydroxylation products of formononetin and biochanin A are formed at lower levels. In addition, we characterize three hydroxybiochanin A metabolites.

Large-scale incubations of biochanin A with β-naphthoflavone-induced rat liver microsomes were used to produce sufficient quantities of the hydroxybiochanin A metabolites for ^{1}H NMR analysis. In these bulk reactions, biochanin A was predominantly *O*-demethylated to form genistein (2−3% conversion), whereas each of the hydroxylated biochanin A products was produced in yields of 0.5−2%. Increased reaction times and increased ratios of NADPH regeneration system to enzyme did not markedly increase the yields of these products, suggesting that end-product inhibition limited reaction progress. Moreover, two of the three hydroxylated biochanin A isomers were unstable and were stored under argon to prevent further oxidation.

HPLC-ES-MS/MS analysis confirmed that the three hydroxybiochanin A metabolites exhibited protonated molecules (*m*/*z* 301), consistent with monohydroxylation. ^{1}H NMR spectroscopy (**Table 1**) was performed to assign the structures. All three metabolites exhibited a three-proton singlet at 3.79 ppm consistent with the presence of a 4′-methoxy substituent, and the H2 proton was detected as a sharp downfield singlet (8.32−8.41 ppm), indicating conservation of the chromone substructure.

The NMR spectra of 8-hydroxybiochanin A and 6-hydroxybiochanin A displayed very similar features. In both instances, the presence of two mutually ortho-coupled two-proton doublets in the aromatic region (7.00 and 7.51 ppm), characteristic of an AB coupling pattern and virtually identical to those observed in biochanin A (**Figure 2** and **Table 1**), indicated that no further substitution had occurred in the 3-(4′-methoxy)phenyl substituent. Similarly to biochanin A, both 8-hydroxybiochanin A and 6-hydroxybiochanin A exhibited two downfield one-proton resonances, one as a sharp singlet at $\delta > 12.3$ and the other as

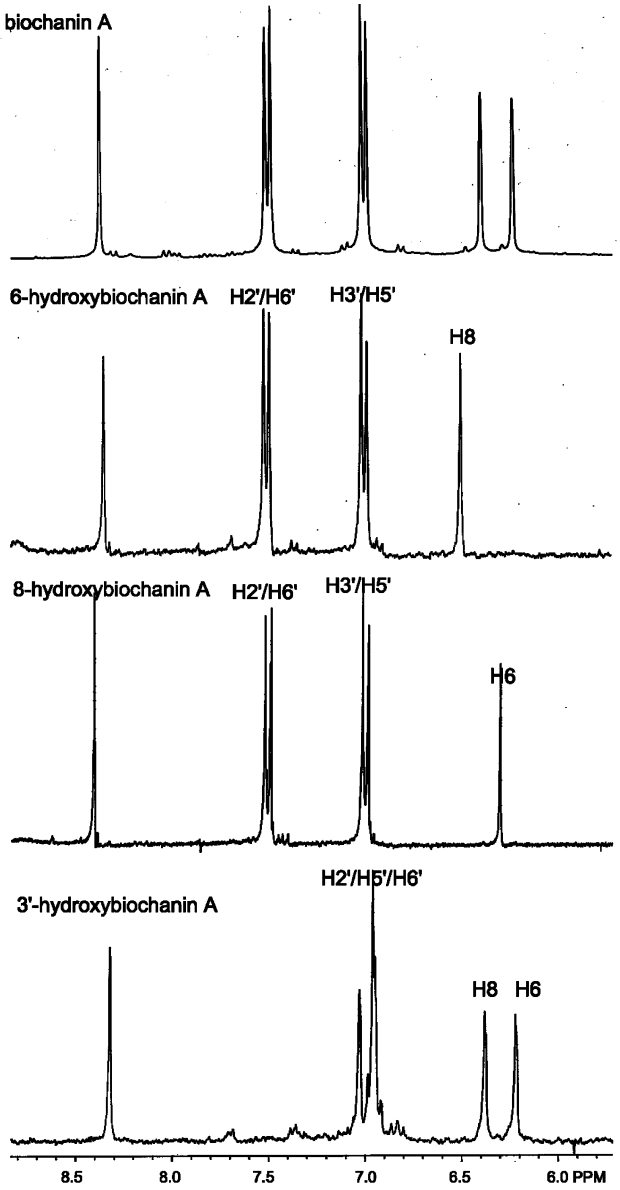


Figure 2. Aromatic region (δ 6–8.5) of the ^{1}H NMR spectra of biochanin A and the monohydroxylated biochanin A metabolites characterized in this study.

a broad singlet at $\delta \sim 10.5$. These signals were assigned to the 5-OH and 7-OH protons, respectively, on the basis of the expected deshielding caused by intramolecular hydrogen bonding of the 5-hydroxyl proton to the peri carbonyl oxygen. In contrast with biochanin A, for which two signals (H6 and H8) were observed in the upfield zone of the aromatic region (6.24 and 6.40 ppm, respectively), only one sharp singlet, accounting for one proton, was observed for each of the two metabolites (**Figure 2** and **Table 1**). This observation, combined with the presence, in both instances, of a broad singlet at approximately 8.8 ppm (**Table 1**), consistent with a phenolic proton, further corroborated that hydroxylation had occurred at either C6 or C8. On the basis of the relative chemical shifts of the upfield aromatic protons (**Table 1**), 8-hydroxybiochanin A [δ (H6) = 6.30] and 6-hydroxybiochanin A [δ (H8) = 6.50] were identified.

The ^{1}H NMR spectrum of the 3′-hydroxybiochanin A metabolite (**Figure 2** and **Table 1**) indicated the presence of the H6 and H8 protons as two mutually meta-coupled one-proton doublets (δ 6.22 and 6.38, respectively), implying that hydroxylation occurred in the 3-aryl substituent. This was confirmed by the absence of an AB coupling profile in that ring, which contained only three almost superimposed aromatic protons at approximately δ 7.0, in a pattern closely resembling that of 7,3′-dihydroxy-4′-methoxyisoflavone (*9*). Furthermore, two broad exchangeable resonances, assigned to phenolic protons, were detected at δ 12.97 (5-OH) and δ 9.06, with the latter being assigned to the additional hydroxyl proton in the 3-aryl substituent. The 7-OH resonance, expected at $\sim$10.5−11.0 by comparison with the spectrum of biochanin A, was presumably too broad to be detected as a result of the high dilution of the sample. Taken together, the ^{1}H NMR data are fully compatible with characterization of this metabolite as 3′-hydroxybiochanin A.

Table 2. Kinetic Constants for CYP1A1 and CYP1B1

enzyme, substrate, and products	K_m (μM)	k_0^{app} (min^{-1})	k_0^{app}/K_m (min^{-1} μM^{-1})
Incubations of Formononetin with CYP1A1			
daidzein	5.9 ± 1.4	13.7 ± 1.7	2.3 ± 0.7
3′-hydroxyformononetin	7.5 ± 1.9	1.6 ± 0.2	0.21 ± 0.08
8-hydroxyformononetin	5.8 ± 1.1	6.7 ± 0.5	1.2 ± 0.3
6-hydroxyformononetin	4.5 ± 0.8	7.3 ± 0.5	1.6 ± 0.4
Incubations of Biochanin A with CYP1A1			
genistein	5.2 ± 0.9	8.4 ± 0.6	1.6 ± 0.4
3′-hydroxybiochanin A	7.3 ± 0.8	1.2 ± 0.06	1.6 ± 0.03
8-hydroxybiochanin A	6.0 ± 0.7	2.9 ± 0.2	0.48 ± 0.08
6-hydroxybiochanin A	2.7 ± 0.4	2.6 ± 0.1	0.96 ± 0.18
Incubations of Formononetin with CYP1B1			
daidzein	0.27 ± 0.06	3.6 ± 0.2	13 ± 4
3′-hydroxyformononetin	nd[a]	nd	nd
8-hydroxyformononetin	nd	nd	nd
6-hydroxyformononetin	nd	nd	nd
Incubations of Biochanin A with CYP1B1			
genistein	1.3 ± 0.1	4.9 ± 0.1	3.8 ± 0.4
3′-hydroxybiochanin A	1.5 ± 0.1	0.1 ± 0.01	0.6 ± 0.01
8-hydroxybiochanin A	40 ± 13	1.2 ± 0.3	0.03 ± 0.02
6-hydroxybiochanin A	nd	nd	nd

[a] Not determined. Iterative nonlinear regression methods failed to converge on enzyme kinetic constants for these metabolites.

Enzyme Kinetics for the Metabolism of Formononetin and Biochanin A Catalyzed by Human Cytochrome P450 Isoforms 1A1 and 1B1. Human cytochromes 1A1 and 1B1 catalyzed the 4′-*O*-demethylation of formononetin and biochanin A to yield daidzein and genistein, respectively. **Figures 3** and **4** show that the *O*-demethylated metabolites daidzein and genistein accumulated more rapidly than the hydroxylation products, particularly for CYP1B1-catalyzed reactions.

Figure 5 and **Table 2** show that K_m values are similar for the formation of daidzein (5.9 ± 1.4 μM) and genistein (5.2 ± 0.9 μM) catalyzed by CYP1A1. Comparison of the apparent rate constants, k_0^{app}, for these CYP1A1-catalyzed reactions (**Table 2**) reveals that the rate of formation of daidzein (13.7 ± 1.7 min^{-1}) is greater than that for genistein formation (8.4 ± 0.6 min^{-1}). The enzyme specificity constants k_0^{app}/K_m for CYP1B1-catalyzed demethylation reactions show that daidzein formation (13 ± 4 min^{-1} μM^{-1}) is favored over genistein formation (3.8 ± 0.4 min^{-1} μM^{-1}). Notably, the K_m value for 4′-*O*-demethylation of formononetin by CYP1B1 (0.27 ± 0.06 μM) is 22-fold lower than that for the same reaction catalyzed by CYP1A1.

Comparison of enzyme specificity constants in **Table 2** shows that CYP1A1 catalyzes hydroxylation at the C3′ position of formononetin (0.21 ± 0.08 min^{-1} μM^{-1}) less efficiently than 4′-*O*-demethylation (2.3 ± 0.7 min^{-1} μM^{-1}) or hydroxylation

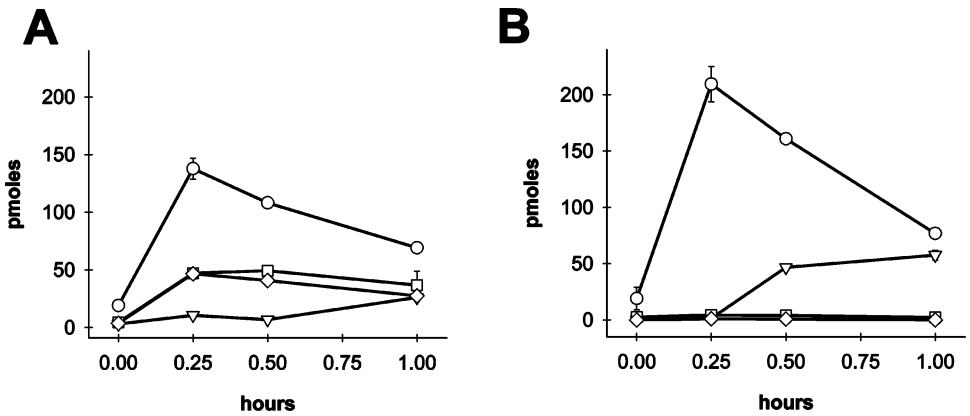


Figure 3. Time course for metabolism of biochanin A by human cytochromes P450 1A1 and 1B1: (**A**) time course for formation of primary metabolites of biochanin A by CYP1A1; (**B**) time course for formation of primary metabolites of biochanin A by CYP1B1; genistein (○); 3′-hydroxybiochanin A (▽); 8-hydroxybiochanin A (□); 6-hydroxybiochanin A (◇). Reactions (0.5 mL) consisted of human cytochrome P450 (10 pmol) with 0.5 nmol of biochanin A, initiated by adding the NADPH regeneration system, and quenched as described under Materials and Methods.

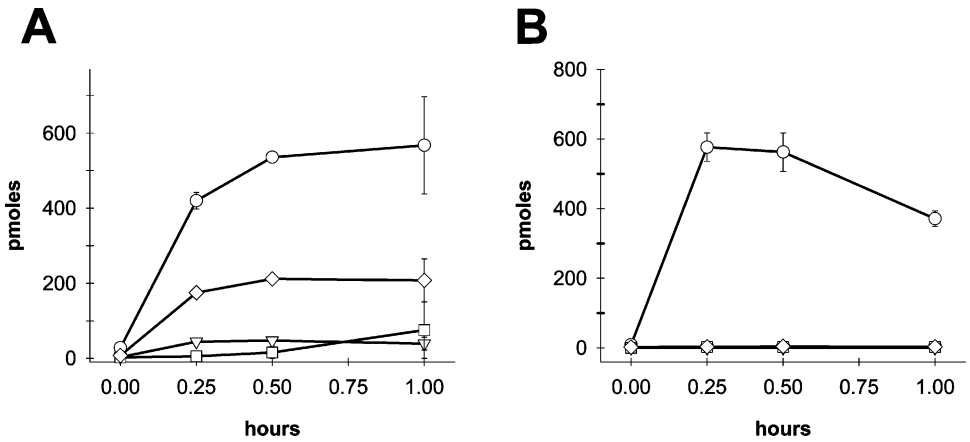


Figure 4. Time course for metabolism of formononetin by human cytochromes P450 1A1 and 1B1: (**A**) time course for formation of primary metabolites of formononetin by CYP1A1; (**B**) time course for formation of primary metabolites of formononetin by CYP1B1; daidzein (○); 3′-hydroxyformononetin (▽); 8-hydroxyformononetin (□); 6-hydroxyformononetin (◇). Reactions (0.5 mL) consisted of human cytochrome P450 (10 pmol) with 0.5 nmol of formononetin, initiated by adding the NADPH regeneration system, and quenched as described under Materials and Methods.

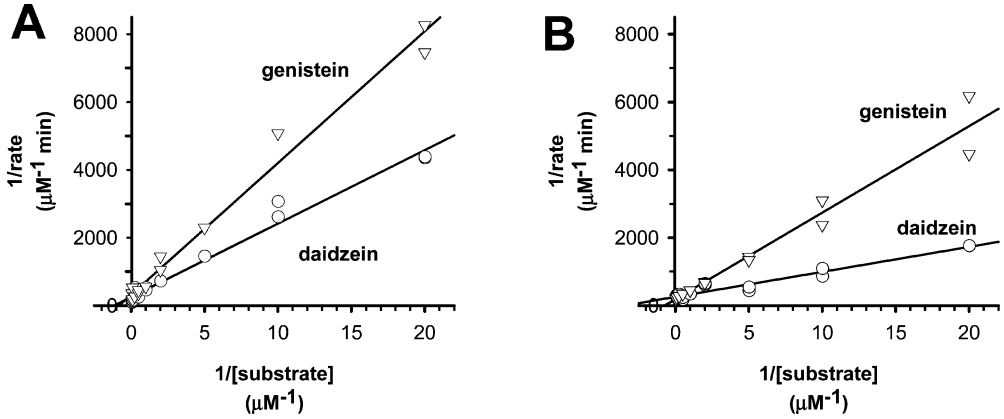


Figure 5. Lineweaver–Burk plots for isoflavone 4′-*O*-demethylation reactions catalyzed by human cytochromes P450 1A1 (**A**) and 1B1 (**B**): 4′-*O*-demethylation of biochanin A (▽) or of formononetin (○). Reactions (0.5 mL) consisted of 0–20 μM isoflavones with CYP1A1 or CYP1B1 (1 pmol) initiated by adding the NADPH regeneration system and incubated for 1 h at 37 °C. Extracts were dissolved in methanol and analyzed by HPLC-EC.

at either C6 (1.6 ± 0.4 min^{-1} μM^{-1}) or C8 (1.2 ± 0.3 min^{-1} μM^{-1}), but much more efficiently than CYP1B1. Although all known monohydroxylated formononetin and biochanin A metabolites were detectable in reactions containing CYP1B1, K_m and k_0^{app} values could not be determined reliably for hydroxylation at C6, C8, or C3′ of formononetin or for hydroxylation of biochanin A at C6. Hydroxylation at C3′ of biochanin A was catalyzed by CYP1B1 with a K_m value similar to that for 4′-*O*-demethylation, albeit at a 49-fold lower rate.

Inhibition of Cytochrome P450 1B1 7-Ethoxyresorufin Deethylase Activity by Genistein and Daidzein. Microsomal incubations containing CYP1A1 or CYP1B1 and 0.2−200 μM genistein or daidzein were also conducted as described for the biochanin A and formononetin incubations. As noted above,

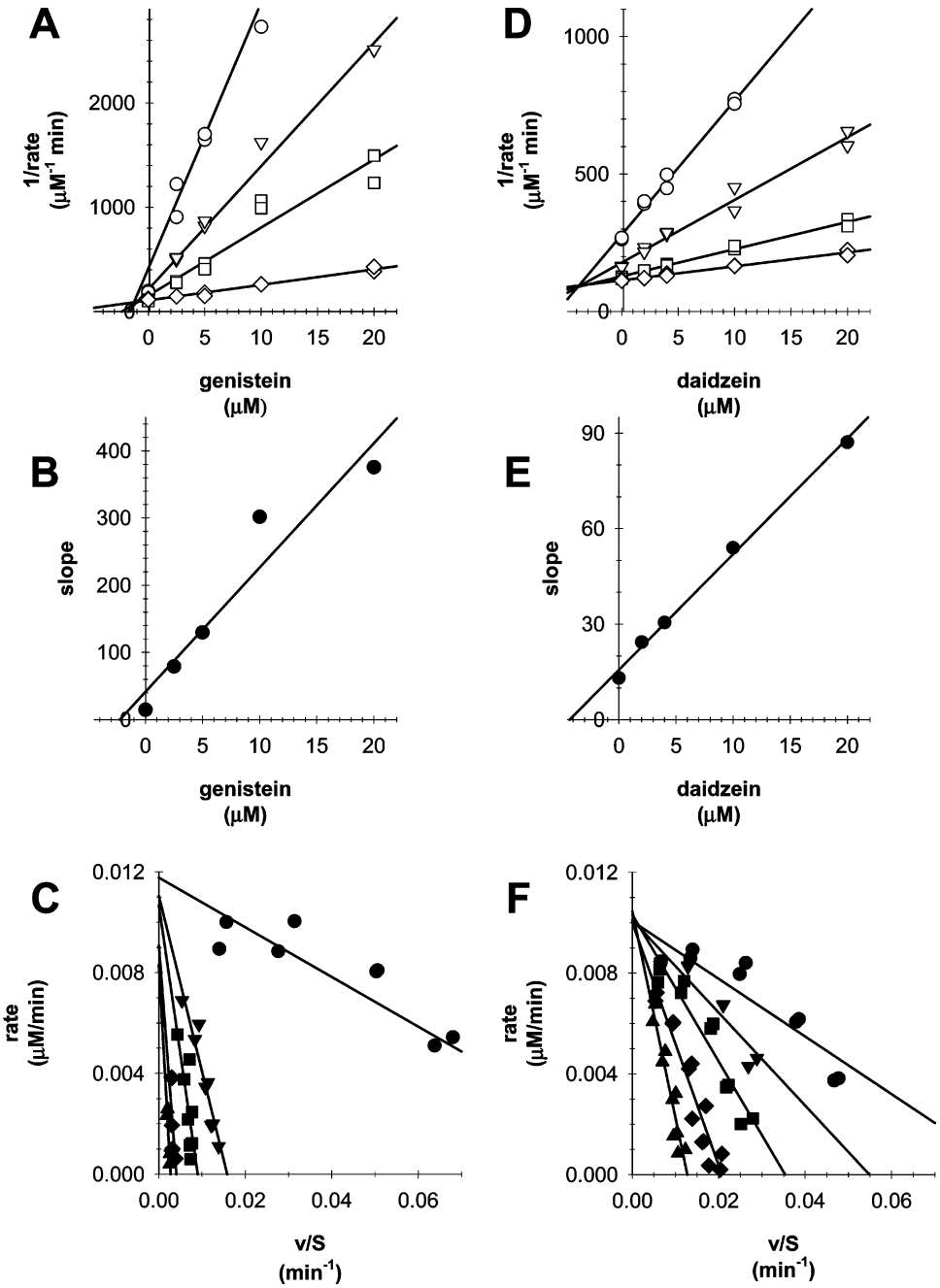


Figure 6. Inhibition of human cytochrome P450 1B1-dependent 7-ethoxyresorufin deethylase activity by genistein and daidzein: (**A**–**C**) inhibition of CYP1B1 activity by genistein; (**D**–**F**) inhibition of CYP1B1 activity by daidzein; (**A**, **D**) Dixon plots [(○) 80 nM, (▽) 160 nM, (□) 320 nM, (◇) 640 nM 7-ethoxyresorufin vs isoflavone inhibitor concentrations]; (**B**, **E**) slopes (●) from Lineweaver–Burk analyses vs concentration of isoflavone inhibitor; (**C**, **F**) Eadie–Hofstee plots [(●) 0.0, (▼) 2.5, (■) 5.0, (◆) 10, and (▲) 20 μM genistein; or (●) 0.0, (▼) 2.0, (■) 4.0, (◆) 10, or (▲) 20 μM daidzein]. Reaction progress was monitored in real time using a 96-well fluorescent plate reader (550 nm excitation, 590 nm emmission).

CYP1B1 exhibited greater selectivity for isoflavone demethylation than CYP1A1. This observation led us to investigate the ability of the demethylated isoflavones genistein and daidzein to inhibit CYP1B1 EROD activity. The data are summarized in **Figure 6**. We found evidence for inhibition of CYP1B1 EROD activity by both genistein ($K_i = 1.9 \pm 0.2$ μM; **Figure 6A**−**C**) and daidzein ($K_i = 3.7 \pm 0.4$ μM; **Figure 6D**−**F**). Enzyme inhibition constants, K_i, were determined by replotting slopes from Lineweaver−Burk analyses versus inhibitor concentrations (**Figure 6B**,**E**). Dixon and Eadie−Hofstee plots (**Figure 6D**,**F**, respectively) indicated competitive inhibition of CYP1B1 EROD activity by daidzein. The evidence for simple competitive inhibition of CYP1B1 EROD activity by genistein (**Figure 6A**,**C**) was not as strong as for daidzein and was more consistent with mixed-type inhibition. These results reveal a potential negative feedback loop in which the end products of 4′-*O*-demethylation of biochanin A or formononetin act as potent CYP1B1 inhibitors.

DISCUSSION

Although it is generally acknowledged that isoflavones have only a fraction of the estrogenic activity ($\sim 1/1000$) of endogeneous estrogens, individuals consuming diets or nutritional supplements rich in isoflavones may have circulating levels of isoflavones that are orders of magnitude higher than the levels

of endogeneous estrogens (*21*, *23*). This is particularly evident in postmenopausal women.

Approximately 90% of the isoflavones in red clover extract are formononetin and biochanin A, and the remaining isoflavones are the corresponding 4′-*O*-demethylated isoflavones daidzein and genistein. If 4′-*O*-demethylation by gut enterocytes or the liver occurs during absorption and enterohepatic circulation, then the isoflavones reaching peripheral blood and organs are predominantly genistein and daidzein. However, if the mechanisms that would normally 4′-*O*-demethylate formononetin and biochanin A are overwhelmed or inhibited, the local metabolism of the parent 4′-methoxyisoflavones may produce an altered constellation of effects associated with the local production of hydroxylated metabolites of biochanin A and formononetin. Alternatively, local 4′-*O*-demethylation products of formononetin and biochanin A, daidzein and genistein, could compete with endogeneous estrogens for local CYP1B1, potentially reducing the formation of estrogen 3,4-catechols.

Genistein and daidzein are the principal metabolites from red clover isoflavone extracts produced by human CYP450 isoforms. Among the human hepatic CYP450 isoforms, CYP1A2 is clearly the most efficient catalyst for the formation of genistein from biochanin A (*9*, *18*). Hu and co-workers found that human CYP1A2 exhibited V_{max} = 903 pmol/min/mg of protein for biochanin A demethylation (*18*), 9-fold higher than that for pooled human liver microsomes (*9*). In the present study, we found that human CYP1A1 catalyzes isoflavone 4′-*O*-demethylation and hydroxylations with similar efficiency, whereas CYP1B1 strongly favors isoflavone 4′-*O*-demethylation over hydroxylation (**Table 2**). Furthermore, CYP1B1 exhibits K_m values for the 4′-*O*-demethylation of biochanin A and formononetin that are ~11-fold lower than those determined for similar reactions catalyzed by pooled human liver microsomes (*9*). Notably, V_{max} values (derived from k_0^{app} determinations) for the synthesis of genistein from biochanin A catalyzed by CYP1A1 and CYP1B1 are 150- and 90-fold greater, respectively, than those measured for CYP1A2. Thus, these extrahepatic CYP450 isoforms represent higher efficiency catalysts, exhibiting lower K_m and higher k_0^{app} for the 4′-*O*-demethylation reactions than related hepatic enzymes.

The data (**Figure 6**) also suggest that isoflavone 4′-*O*-demethylation may create negative feedback loops modulating CYP1A1 and CYP1B1 activity. This conclusion is substantiated by our observation that CYP1B1 EROD activity was inhibited by genistein (K_i = 1.9 ± 0.2 μM) and daidzein (K_i = 3.7 ± 0.4 μM). A similar effect was deduced for human CYP1A2, the phenacetin *O*-demethylation activity of which was inhibited by genistein (IC_{50} = 16 μM) (*18*). Recent studies have shown that genistein, but not daidzein, added to cultured MCF-7 human breast carcinoma cells treated with 7,12-dimethylbenz[*a*]anthracene (DMBA) prevented the formation of DMBA−DNA adducts (*24*), and this protective effect was reproduced in similar cultures treated with biochanin A (*25*). The same authors found that genistein and biochanin A are competitive inhibitors of recombinant human CYP1A1 (K_i = 15 and 4 μM, respectively) and CYP1B1 (K_i = 0.68 and 0.59 μM, respectively) EROD activity using assay conditions comparable to those used in this study (*24*, *25*).

Interestingly, human CYP1B1 EROD activity appears to be more sensitive to inhibition by daidzein or genistein than other CYP450 isoforms. For instance, both daidzein and genistein have been found to inhibit mouse CYP1A1-dependent hydroxylation of benzo[*a*]pyrene noncompetitively, but the K_i values (325 and 140 μM, respectively) (*26*) were 2 orders of magnitude higher that those determined for the inhibition of CYP1B1 EROD activity in the present study. Similarly, equol (IC_{50} = 1.7 ± 0.8 mM) and genistein (IC_{50} = 5.6 ± 1.8 mM) noncompetitively inhibit mouse liver microsomal CYP1A and human CYP1A2 EROD activities (*27*). Noncompetitive inhibition of CYP2E1-dependent *p*-nitrophenol hydroxylation by equol (IC_{50} = 560 μM) and genistein (IC_{50} = 10 mM) has also been established (*27*).

In addition to their estrogenic activity, ingested isoflavones or their metabolites may modulate hormonal carcinogenesis by influencing the metabolism and local availability of endogeneous hormones. Humans express at least seven isoforms of 17β-hydroxysteroid dehydrogenase (17β-HSD), which are key enzymes in the formation of both androgens and estrogens (*28*). Inactivation of 17β-HSD type 3 is considered to be critical for male pseudohermaphroditism. Biochanin A (10.3 μM) and baicalein (9.3 μM) are potent inhibitors of recombinant human 17β-HSD type 3 (*29*). Human 17β-HSD type 5 is inhibited effectively by quercetin (3,5,7,3′,4′-pentahydroxyflavone) and biochanin A (*30*). The isoflavones genistein, biochanin A, and equol inhibit human steroid 5α reductase isoforms 1 and 2, which convert testosterone to dihydrotestosterone, a more potent androgen (*31*). 3β-Hydroxysteroid dehydrogenase (3β-HSD, progesterone reductase) functions as a dehydrogenase and a steroid 4-ene/5-ene isomerase. Both 3β-HSD activities are strongly inhibited by genistein, daidzein, biochanin A, and formononetin (IC_{50} values of 0.4−11 μM) (*32*).

In humans, isoflavones have been shown to affect the metabolism of endogeneous estrogens and, specifically, the formation of mutagenic estrogen quinone metabolites (*33*). The current study provides mechanistic insight into this important observation. Biochanin A and formononetin and their metabolites have the potential not only to inhibit CYP1B1 or to act as alternative substrates for CYP1B1, affecting the production of mutagenic estrogen 3,4-catechols, but also to compete with catechol-*O*-methyltransferase (*34*, *35*) which normally detoxifies estrogen catechols. The catechol and pyrogallol metabolites of biochanin A and formononetin need to be further considered, not only in the context of potential health benefits but also in the context of their potential toxicity. These metabolites are candidates for further metabolism to quinones and associated redox cycling (*36*, *37*), and their potential to form carcinogenic depurinating DNA adducts (*38*, *39*) should also be considered.

ABBREVIATIONS USED

k_0^{app}, apparent enzyme-catalyzed rate constant; COSY, correlation spectroscopy; CYP450, cytochrome P450; K_i, enzyme inhibition constant; k_0^{app}/K_m, enzyme selectivity constant; EROD, 7-ethoxyresorufin deethylase; HPLC-EC, high-pressure liquid chromatography with electrochemical detection; HPLC-MS, high-pressure liquid chromatography with mass spectrometric detection; IC_{50}, concentration of an agent in vitro producing 50% inhibition of the biological effect; K_m, Michaelis−Menten constant; V_{max}, maximal enzyme-catalyzed reaction velocity.

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