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Oxidation of Flavone, 5-Hydroxyflavone, and 5,7-Dihydroxyflavone to Mono-, Di-, and Tri-Hydroxyflavones by Human Cytochrome P450 Enzymes

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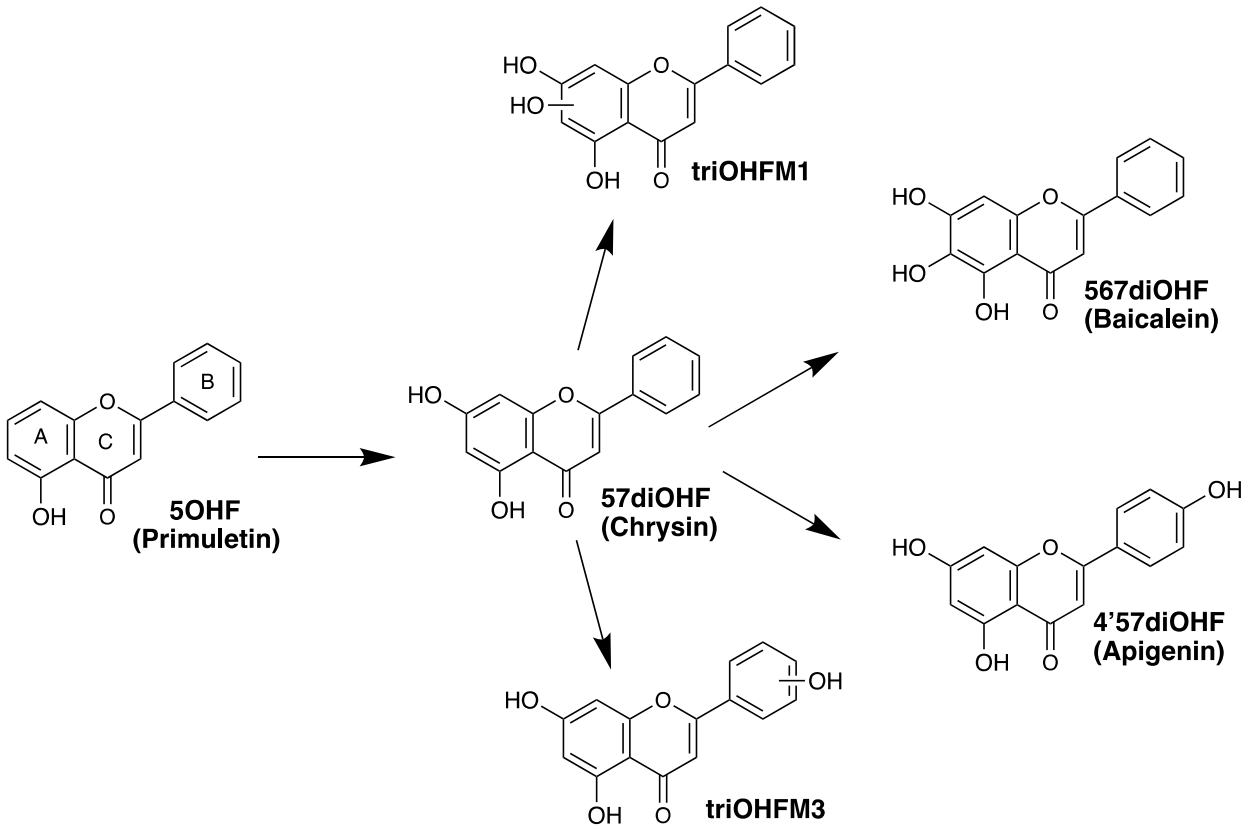

ABSTRACT: Biologically active plant flavonoids, including 5,7-dihydroxyflavone (57diOHF, chrysin), 4′,5,7-trihydroxyflavone (4′57triOHF, apigenin), and 5,6,7-trihydroxyflavone (567triOHF, baicalein), have important pharmacological and toxicological significance, e.g. anti-allergic, anti-inflammatory, anti-oxidative, anti-microbial, and anti-tumorgenic properties. In order to better understand the metabolism of these flavonoids in humans, we examined the oxidation of flavone, 5-hydroxyflavone (5OHF), and 57diOHF to various products by human cytochrome P450 (P450 or CYP) and liver microsomal enzymes. Individual human P450s and liver microsomes oxidized flavone to 6-hydroxyflavone, small amounts of 5OHF, and 11 other mono-hydroxylated products at different rates and also produced several di-hydroxylated products (including 57diOHF and 7,8-dihydroxyflavone) from flavone. We also found that 5OHF was oxidized by several P450 enzymes and human liver microsomes to 57diOHF and further to 567triOHF, but the turnover rates in these reactions were low. Interestingly, both CYP1B1.1 and 1B1.3 converted 57diOHF to 567triOHF at turnover rates (on basis of P450 contents) of >3.0 min^{-1}, and CYP1A1 and 1A2 produced 567triOHF at rates of 0.51 and 0.72 min^{-1}, respectively. CYP2A13 and 2A6 catalyzed the oxidation of 57diOHF to 4′57triOHF at rates of 0.7 and 0.1 min^{-1}, respectively. Our present results show that different P450s have individual roles in oxidizing these phytochemical flavonoids and that these reactions may cause changes in their biological and toxicological properties in mammals.

INTRODUCTION

Flavonoids constitute a set of over 5,000 structurally different natural chemicals in the environment, and many of these flavonoids have been shown to cause various biological benefits in preventing a number of diseases including cancer, heart disorders, and bone loss in humans.[1-5] The biological properties of flavonoids also include toxicities *per se*. A search of the literature (PubMed, 1 April 2019) showed 10,590 items in a search "toxicity of flavonoids." Further, the search "toxicity of quercetin" yielded 1,789 entries, and much remains to be known about the toxicity of many flavonoids and the role of metabolism in changing the biological properties. The flavonoids 5,7-dihydroxyflavone (57diOHF, chrysin), 4´5,7-trihydroxyflavone (4´57triOHF, apigenin), and 5,6,7-trihydroxyflavone (567triOHF, baicalein) have been reported to show significant anti-allergic, anti-inflammatory, anti-oxidative, anti-microbial, anti-tumorgenic, and anti-mutagenic properties in mammals, including humans.[6-22] Collectively, these three flavonoids yielded 593 hits in the PubMed searches ("toxicity of [each flavonoid]," 1 April 2019) and are the subject of the present investigation.

Recent studies have shown that cytochrome P450 (P450 or CYP) Family 1 enzymes in human breast cancer MDA-MB-468 cell lines are involved in the activation of flavonoids, eupatorine, diosmetin, and nobiletin, to more active metabolites, e.g. cirisiliol and luteolin,[23-29] which have been reported to show various antitumor activities in ovarian cancer[30,31] and gastric

cancer cells,[32] and to show suppression of metastasis in breast cancer[33] and malignant melanoma.[34] Such activation of these flavonoids was not seen in normal mammary MCF-10A cells, which do not express CYP1A1 and 1B1, indicating that these P450s have critical roles in the activation of these flavonoids.[27,29,23,35]

We recently reported that flavone is oxidized by different forms of human P450 enzymes to several mono-hydroxy (mono-OH) and further to dihydroxy (di-OH) products[36] and that CYP2A6 has specific roles in oxidizing flavone to yet unidentified mono-OH product 6 (OHFM6) and OHFM9 and in oxidizing flavanone to 2′-hydroxyflavanone.[37] During course of these studies, we noted that flavone is oxidized to 5-hydroxyflavone (5OHF) and 5,7-dihydroxyflavone (57diOHF) by different human P450s and liver microsomes, although the rates of formation of two products by these enzymes were not very high.[36,37]

In this study, we further examined roles of human P450s and liver microsomes to oxidize flavone, 5OHF, and 57diOHF to form respective mono-OH, di-OH, and tri-OH products by analyzing product formation using LC-MS/MS. Purified human P450 enzymes used included CYP1A1, 1A2, 1B1.1, 1B1.3, 2A6, 2C9, and 3A4, and we also used recombinant CYP2B6 expressed in microsomes of *Trichoplusia ni* cells in which human NADPH-P450 reductase was co-expressed. Among liver microsomes prepared from human samples HH2, HH47, and HH54, the

HH2 sample was defective in coumarin 7-hydroxylation activity, thus categorized to be a poor metabolizer of CYP2A6.[37] Molecular docking analysis of the interaction of 57diOHF with different forms of human P450s, particularly CYP1B1.1 and 1B1.3, were studied to examine the basis of catalytic differences found in these P450s in oxidizing these flavonoids.

EXPERIMENTAL PROCEDURES

Chemicals. Flavone, 5-, 6-, and 7-hydroxyflavone (5OHF, 6OHF, and 7OHF), and 5,7- and 7,8-dihydroxyflavone (57diOHF and 78diOHF, respectively) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Wako Pure Chemicals (Osaka, Japan). 4′,5,7-Trihydroxyflavone (apigenin; 4′57triOHF) and 5,6,7-trihydroxyflavone (baicalein; 567triOHF) were also purchased from Sigma-Aldrich. Other chemicals and reagents used in this study were obtained from sources described previously or were of the highest quality commercially available. [36-38]

Enzymes. Purified preparations of wild type of human CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2C9, and CYP3A4 expressed *in Escherichia coli* were obtained by methods described previously.[38,39,40,41] In this study, we were also used variant form CYP1B1.3 and compared it with (wild-type) CYP1B1.1 for their catalytic differences. NADPH-P450 reductase and cytochrome b_5 (b_5) were also purified from membranes of recombinant *E. coli* as described

elsewhere.[39,40] Recombinant CYP2B6 expressed in microsomes of *T. ni* cells, infected with baculovirus containing CYP2B6 and NADPH-P450 reductase cDNA inserts, was obtained from Corning-GENTEST (Woburn, MA).

Liver microsomes prepared from human samples HH2 (Cat No. 452002), HH47 (Cat No. 452047), HH54 (Cat No. 452054), and HG95 (Cat No. 452095) were obtained from Corning-GENTEST. The data sheets provided by the manufacturer indicated that these microsomes contained 0.19, 0.26, 0.35, and 0.25 nmol P450/mg protein, respectively. The oxidation activities towards typical P450 substrates such as (*S*)-mephenytoin, paclitaxel, diclofenac, bufuralol, chlorzoxazone, and testosterone were reported in these liver microsomes, except that HH2 had no detectable coumarin 7-hydroxylation activity and the data sheets indicated that this individual was classified a poor metabolizer (PM) for CYP2A6 activity.[37]

Oxidation of Flavone, 5OHF, and 57diOHF by P450 Enzymes by Human Liver Microsomes. Oxidation of flavone, 5-hydroxyflavone (5OHF), and 5,7-dihydroxyflavone (57diOHF) by P450 enzymes was determined by the methods described previously.[36,37] Briefly, liver microsomes (50 pmol of P450), baculosome-derived CYP2B6 (20 pmol of P450), or reconstituted monooxygenase systems consisting of each purified P450 (50 pmol), NADPH-P450 reductase (100 pmol), b_5 (100 pmol, in the cases of CYP2A6, 2A13, 2C9, and 3A4 enzymes), and L-α-1,2 dilaouryl-

sn-glycero-3-phosphocholine (DLPC) (50 μg) were incubated (0.25 mL of total volume) with 60 μM flavone, 5OHF, or 57diOHF at 37 °C for 20 min, following a pre-incubation of 1 min before adding an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 0.5 unit of yeast glucose 6-phosphate dehydrogenase/mL). Each reaction was terminated by the addition of 0.5 mL of ice-cold CH_3CN. The mixture was mixed vigorously (with a vortex device) and centrifuged at 10,000 × *g* for 5 min, and an aliquot of the upper CH_3CN layer was injected and analyzed with LC-MS/MS as described.[36,37]

LC-MS/MS analyses were performed using an HPLC system (ACQUITY UPLC I-Class system; Waters, Milford, MA) coupled to a tandem quadruple mass spectrometer (XevoTQ-S; Waters) by the methods as described previously.[36,37] MS/MS analysis was performed in the positive electrospray ionization mode with a capillary voltage of 3000 V and cone voltage of 30 V as described previously.[36,37]

Kinetic parameters were determined by non-linear regression analysis (mean ± standard error) employing the Michaelis-Menten equation, $v = V_{max} \times [S]/(K_m + [S])$ or the allosteric Hill equation model, $v = V_{max} \times [S]^n/(S^n_{50} + [S]^n)$ using the program Kaleida-Graph (Synergy Software, Reading, PA, USA) or GraphPad Prism (GraphPad, La Jolla, CA, USA).

Other Assays. P450 and protein contents were determined by the methods described previously.[42,43]

Docking Simulations of Flavone, 5OHF, and 57diOHF into P450 Enzymes. Crystal structures of CYP1A1 (Protein Data Bank 418V), 1A2 (2H14), 1B1.1 (3PMO), 2A6 (2FDV), 2A13 (3T3S), 2B6 (3QOA), 2C9 (1R9O), and 3A4 (5TE8) have been reported and were used in this study.[44-52] CYP1B1.3 variant (L432V) model was established by incorporating the mutation into CYP1B1.1 (3PMO). Chemical structures of 5OHF and 57diOHF were taken from PubChem (an open chemistry database at the Untied States National Institutes of Health) and were optimized in MOE software (ver. 2018.0101, Computing Group, Montreal, Canada). Simulations were carried out in the MOE by the methods as described previously.[36,37] Ligand-interaction energies (*U* values) were obtained by use of the program ASEdock in MOE.

RESULTS

Different Roles of Human P450 Enzymes in the Oxidation of Flavone. Oxidations of flavone to mono-OH flavones (Figure 1A-1G) and further to di-OH flavones (Figure 1H-1N) by purified P450 enzymes (Figure 1A-1E and 1H-1L) and by human liver microsomes (Figure 1F, 1G, 1M, and 1N) were examined. As we have reported earlier,[36,37] CYP2A6 had important roles in the oxidation of flavone to form unidentified mono-OH flavones OHM6 and OHFM9 in liver microsomes (Figure 1E) because these products were produced slowly by liver microsomes from sample HH2, a poor metabolizer of CYP2A6,[38] as compared with those catalyzed by HH47, an extensive metabolizer of CYP2A6 (Figures 1F and 1G). Our present studies also showed that OHFM10 was produced by CYP2A6 in liver microsomes, because formation of this product was slowly catalyzed by microsomes from liver sample HH2 (Figures 1M and 1N).

CYP1B1.3 was shown to be unique in oxidizing flavone to form mono-OH flavones OHFM1, M2. M3, and M4; these products were not produced by other human P450s and liver microsomes (Figure 1D). It was also found that OHFM11 was not formed by CYP1B1.3 but that formation was catalyzed by CYP1B1.1 and other human P450s and liver microsomes.

CYP1A2 was more active than CYP2A6 in catalyzing the oxidation of flavone to 6OHF

and both liver microsomal HH2 and HH47 samples were found to catalyze formation of 6OHF at higher rates (on a P450 basis) than CYP2A6 did, indicating that CYP2A6 does not have a major role in this reaction (Figure 1). We also found that 5OHF (t_R 11.2 min) was produced from flavone by CYP2A6 and human liver microsomes, but turnover rates were low (< 0.01 min^{-1}).

Human P450 enzymes and liver microsomes were able to form di-OH flavones, as well as mono-OH flavones, when flavone was used as a substrate (Figure 1H-1N). Major di-OH products were diOHFM3, diOHFM4, and diOHFM4 with CYP1A2 and CYP2A6 and liver microsomes (Figure 1I, 1L, 1M, and 1N). CYP1A1 and 1B1.1 produced diOHFM5 and formed small amounts of diOHFM3 and diOHFM4. CYP1B1.3 did not produce di-OH flavones at significant levels (Figure 1K). An interesting finding was that CYP2A6 and human liver microsomes produced 57diOHF (t_R 7.9 min) and that the latter enzymes formed 78diOHF (t_R 4.1 min); these di-OH products formed were characterized by co-chromatography with standards (*vide infra*).

LC-MS/MS chromatograms of mono-OH products produced following incubation of flavone with HH2 and HH54 microsomes showed that 5OHF (t_R 11.2 min) (as well as 6OHF) was detected when examined using the transition *m/z* 239>137 (but not *m/z* 239>121) (Figure 2C and 2D). The product ion spectra of 5OHF and 6OHF produced from liver microsomes of sample HH54 (Figure 2F) and of 6OHF from the HH2 microsomes (Figure 2H) were similar to those of standard

5OHF (Figure 2E) and 6OHF (Figure 2G). We did not detect formation of 7OHF following incubation of flavone with any P450s or human liver microsomal samples.

Chemical Structures of Unidentified Mono- and Di-OH Flavones. The product ion spectra of unidentified products of OHFM1-OHFM12, as well as standards 5OHF, 6OHF, and 7OHF, were examined with LC-MS/MS (Figure 3). By analyzing the product ion spectra of 11 unidentified mono-OH flavones (OHFM1-OHFM12, except for OHFM8 which was a very minor product), we were able to tentatively classify these mono-OH flavones into two groups (Figure 3). The first group included OHFM4, OHFM5, OHFM7, and OHFM12, which showed a characteristic *m/z* 128.9 ion; of the products detected, OHFM4 and M7 were also found to contain *m/z* 102.9 and 136.9 ions as well as an *m/z* 128.9 ion, indicative of similarity to the spectra of the standard 5OHF, 6OHF, and 7OHF products (Figure 3). OHFM5 contained *m/z* 102.9 and 128.9 fragments, but not 136.9, while OHFM12 had characteristic *m/z* 128.9 and 136.9 fragments but not one at *m/z* 102.9. Among the products determined, OHM1, OHFM6, OHFM9, and OHFM10 contained a characteristic *m/z* 144.8 ion and OHFM11 was found to contain an *m/z* 139.0 ion as well as an *m/z* 120.9 ion (Figure 3).

We also determined the product ion spectra of unidentified di-OH flavones following incubation of flavone with these P450s and liver enzymes (Figure 4). Standard spectra of flavone, 57diOHF, and 78diOHF were included for comparison (Figure 4A, 4B, and 4D, respectively). A

product of 57diOHF produced from HH54 showed a similar spectrum to standard 57diOHF, having *m/z* 128.9 and 152.9 ions, but dissimilar in the presence of an *m/z* 135.0 ion and the absence of *m/z* 146.9 (Figure 4C). The product 78diOHF, formed from HH54 microsomes, was also similar to the standard, except that the former had a different ion, *m/z* 141.0, in the spectrum (Figure 4E). The unidentified diOHFM1 and OHFM4 products, having an *m/z* 120.9 ion, were found to constitute one group, and the other included diOHFM3 and diOHFM5, which contained an *m/z* 128.9 ion (Figure 4). The diOHFM6 spectrum could be indicative of another group, having *m/z* 125.0 as well as *m/z* 102.9 ions (Figure 4J).

Oxidation of 5OHF by Human P450s and Liver Microsomes. Human CYP1A2, 2A6, and 2A13 and liver microsomes from sample HH54 converted 5OHF into 57diOHF and further to 567triOHF (Figure 5). Sample HH54 was highly active in oxidizing 5OHF to 57diOHF, followed by CYP1A2, 2A6, and 2A13, although the oxidation activities for this reaction were not very high among these human enzymes examined (*vide infra*). CYP2A13 produced diOHFM6 from flavone at low rates (Figure 5C). These P450s and microsomal sample HH54 catalyzed formation of 567diOHF (and triOHFM1 in CYP1A2 and 2A13) from flavone, at low rates (*vide infra*).

Oxidation of 57diOHF by Human P450s and Liver Microsomes. CYP1A1, 1A2, and 1B1.1 (and also 1B1.3, results not shown) were found to be active in converting 57dOHF into

567triOHF; the turnover rates (on P450 basis) were higher with CYP1B1.1 and 1B1.3 than CYP1A1 and 1A2 (*vide infra*) when the concentrations of P450 and 57diOHF used were 0.2 μM and 60 μM, respectively (Figure 6). CYP1A1, but not CYP1A2, produced small amounts of 4´57triOHF and triOHFM3 (Figure 6A).

In contrast to the CYP1 enzymes, CYP2A6 and 2A13 were more active in forming 4´57triOHF than 567triOHF from 57diOHF and the opposite was the case for liver microsomes from human sample HH54; it should be mentioned that the peak areas obtained with the samples from CYP2A6 and 2A13 and human liver microsomes (Figure 6D-6F) were 10-fold lower than those obtained with CYP1 enzymes at the same concentrations of P450 and 57diOHF (0.2 and 60 μM, respectively) used for the assays (Figure 6A-6C). CYP2A6 and 2A13 and HH54 microsomes produced triOHFM3 in small amounts. Product ion spectra were determined with these metabolites obtained on incubation of 57diOHF with human P450 enzymes (Figure 7). The reference peak *m/z* 122.8 in the spectrum of 567triOHF observed from the incubation with CYP1B1.3 was similar to that of the authentic standard (Figure 7A and 7B).

The 4´57triOHF product isolated from CYP2A13 (Figure 7E) had a similar spectrum to the standard (Figure 7D), except that the latter was missing *m/z* 168.9. The unidentified triOHFM1 (from CYP1A2) and triOHFM3 products (from CYP1A1) were also characterized from their

spectra; the former was similar to 567triOHF and the latter was to 4′57triOHF, based on their spectra.

Rates of Oxidation of 5OHF and 57diOHF by Human P450s and Liver Microsomes. Oxidations of 5OHF (Figure 8A and 8B) and 57diOHF (Figure 8C and 8D) by human P450s and liver microsomes were determined under standard reaction condition (0.2 μM P450 and 60 μM 5OHF or 57diOHF), and rates of product formation was calculated (nmol product formed/min/nmol P450).

The rates of oxidation of 5OHF to form 57diOHF and 567triOHF by human P450s were low, < 0.1 min^{-1}, although the rates measured with human liver microsomes were higher than with purified P450 enzymes (Figure 8A and 8B). In contrast, the oxidation of 57diOHF to 4′57triOHF (Figure 8C) and 567triOHF (Figure 8D) was more evident with the P450 enzymes than with human liver microsomes. CYP2A13 was most active in converting 57diOHF to 4′57triOHF, at rates of 0.7 min^{-1}, followed by CYP2A6 (0.13 min^{-1}) (Figure 8C). Both CYP1B1.1 and 1B1.3 were most active in oxidizing 57diOHF to 567triOHF at rates of >3.0 min^{-1}, followed by CYP1A1 and 1A2 at rates of 0.5 and 0.7 min^{-1}, respectively (Figure 8D).

Kinetic Analysis of Oxidation of 57diOHF to 4′57triOHF and 567diOHF by Human P450s. The K_m values for the formation of 4′57triOHF from 57diOHF by CYP1A1 and 1A2 were

85 and 97 μM and K_m values for the formation of 567triOHF by these P450s were 70 and 26 μM, respectively, with higher k_{cat} values in the latter reactions (Figure 9A, 9B, 9E, and 9F). CYP2A13 showed higher activity in oxidizing 57diOHF to 4′57triOHF than CYP2A6, having a K_m value of 14 μM and a k_{cat} value of 1.0 min^{-1}. CYP2A13 also had a low K_m value for the formation of 567triOHF, compared with CYP2A6 (K_m 200 μM) (Figure 9).

One interesting finding was that both CYP1B1 enzymes caused increases in the formation of 567triOHF from 57diOHF with sigmoidal character at lower substrate concentrations (Figure 9x and 9y). Homotropic cooperativity of wild-type CYP1B1.1 and variant CYP1B1.3 was seen in the formation of 567triOHF from substrate 57diOHF (1-80 μM), with Hill coefficients of 1.3 ± 0.4 and 1.2 ± 0.2 (means ± SEM) respectively (Figure 9X and 9Y). Moreover, the 95% confidential intervals of Hill coefficients for CYP1B1.1 (1.9 ± 0.2) and CYP1B1.3 (5.3 ± 1.1) were 1.5-2.6 and 1.8-8.8, respectively, in the substrate ranges of 1-16 μM.

Molecular Docking Analysis of Interaction of Flavonoids with Human P450s. In order to gain information of molecular interaction of these P450 enzymes with flavonoids examined in this study, we performed molecular docking analysis as described in Experimental Procedures. Because homotropic cooperativity of CYP1B1.1 and CYP1B1.3 was evident for the oxidation of 57diOHF (*vide supra*, Figure 9), docking simulation was first performed with the substrate 57diOHF

using the CYP1B1.1 structure and a CYP1B1.3 model. Both wild type CYP1B1.1 and variant CYP1B1.3 accommodated two 57diOHF molecules in the substrate-binding pocket (Figure 10). The first substrate molecule was stabilized at a site far from the heme of CYP1B1.1 (*U* energy of -52.2 kcal mol^{-1}, Fig. 10A) by trapping the phenyl B ring and A ring of 57diOHF in interactions with Leu-191 and His-216, respectively. Similarly, the first substrate molecule was also stabilized at a site far from the heme of CYP1B1.3 (*U* energy of -46.5 kcal mol^{-1}, Figure 10B) by trapping the phenyl B ring and A ring of 57diOHF in interactions with Arg-194 and Glu-229, respectively. The second substrate molecule of 57diOHF was able to dock with both of the heme of CYP1B1.1 and CYP1B1.3 with *U* energy values of -76.8 and -18.7 kcal mol^{-1}, respectively.

Flavone oxidation differed in CYP1B1.1 and 1B1.3, with the latter enzyme showing characteristic patterns in forming OHFM1-OHFM4. CYP1B1.1 did not form these products but was highly active in producing OHFM6-M12 (Figure 1). Docking analysis of interaction of flavone with these P450 enzymes suggested that there was a different orientation in these cases, one in which the active site of CY1B1.1 faced the C4′ position at the B-ring, while CY1B1.3 showed interaction with C5 of A-ring of flavone in its active site (Supporting information Figure S1). Such differences in the two P450 enzymes were not seen in the interactions with 5OHF and 57diOHF (Supporting Information Figure S1).

Molecular docking analysis was done for the interaction of CYP1A1, 1A2, 2A6, and 2A13 with 5OHF (Supporting Information Figure S2A-S2D) and 57diOHF (Supporting Information Figure S2E-S2H). The results showed that each P450 enzyme had similarities in interaction with 5OHF and 57diOHF. The A-ring of 5OHF and 57diOHF was close to the active sites of CYP1A1 and CYP2A13, but CYP1A2 and 2A6 interacted with the B-ring of 5OHF and 57diOHF in their molecular docking analysis (Figure S2).

The interaction of the active sites of CYP2B6, 2C9, and 3A4 with 5OHF and 57diOHF was also studied in molecular docking analysis (Supporting Information Figure S3).

DISCUSSION

Our present results can be summarized as follows. (i) Flavone was oxidized by human P450 and liver microsomal enzymes to several mono-OH and di-OH products, and CYP1B1.3 was found to be unique in forming mono-OHFM1, OHFM2, OHFM3, and OHFM4 but not di-OH products, compared with other P450s and liver microsomes. (ii) CYP2A6 was shown to catalyze the site-specific oxidation of flavone to mono-OHFM6 and OHFM9, as described previously,[37] and we also found that OHFM10 and diOHFM4 were produced by CYP2A6. (iii) Human liver microsomes were able to oxidize flavone to 5OHF, 57diOHF, and 78diOHF, as well as other mono-OH and di-OH flavones described above. (iv) 5OHF was converted by human P450 and liver microsomal enzymes to 57diOHF and further to 567diOHF, but the rates of formation were low. (v) CYP1B1.1 and 1B1.3 were highly active in oxidizing 57diOHF to 567triOHF at rates (on a P450 basis) of >3.0 min^{-1}, followed by CYP1A1 and 1A2. CYP1B1.1 and 1B1.3 showed sigmoidal patterns in the formation of 567triOHF from 57diOHF at lower substrate concentrations, indicative of homotropic cooperativity in the reaction. (vi) Finally, CYP2A6 and 2A13 were not very active in catalyzing the oxidation of 57diOHF to 567triOHF but were active in producing 4´57triOHF at rates of 0.7 and 0.1 min^{-1}, respectively.

As described above, CYP1B1.1 and 1B1.3 were found to be active in catalyzing oxidation

of 57diOHF to 567triOHF, followed by CYP1A1 and 1A2. Williams et al.[53] have also reported that CYP1A1 is able to catalyze the oxidation of 57diOHF to 567triOHF in yeast cells expressing human CYP1A1. Although both 57diOHF (chrysin) and 567triOHF (baicalein) have been shown to have several biological benefits (e.g. anti-proliferative, anti-microbial, anti-allergic, anti-inflammatory, and anti-oxidative properties, [6-10,14-16,22] it is not known which flavonoid is more biologically active. It is interesting in this connection to note recent studies showing that P450 Family 1 enzymes are involved in the activation of flavonoids eupatorine, diosmetin, and nobiletin to more active metabolites, e.g. cirisiliol and luteolin.[23,26,28,29] Such activation could not be observed in normal mammary MCF-10A cells which do not express CYP1A1 and 1B1,[23,27,29] indicating that CYP1 family enzymes have significant roles of in the activation of flavonoids in such human cancer cells.

CYP2A13 and 2A6 catalyzed oxidation of 57diOHF to form 4´57triOF at turnover rates of 0.7 and 0.1 min^{-1}, respectively; these P450s showed formation of 567triOHF at the relatively slow rates of 0.013 and 0.019 min^{-1}, respectively. Apigenin (4´57triOHF) has also been reported to be highly active in several biological effects described above. [6,9,14,19]

We obtained evidence for homotropic cooperativity in the oxidation of 57diOHF to 567triOHF catalyzed by purified CYP1B1.1 and 1B1.3. A number of studies have shown the presence of both homotropic and heterotropic oxidation of substrates by several P450 enzymes,

including human and rabbit CYP3A enzymes and several other P450s.[54-58] The mechanisms by which CYP1B1 enzymes display homotropic (and also heterotropic) cooperativity in the oxidation of 57diOHF are not known; however, our molecular docking studies showed that there were two ligand binding sites in the interaction of 57diOHF with CYP1B1.1 and 1B1.3 active sites and it was suggested that binding of the first ligand might affect the activation of the second substrate binding to cause increases in the oxidation of 57diOHF by these P450 enzymes. Molecular docking results also showed differences in the orientation of flavone in the active sites of CYP1B1.1 and 1B1.3 (Supporting Information Figure S1). These P450s showed different patterns of oxidation of flavone to form mono-OH and di-OH products; the former P450 catalyzed oxidation of flavone essentially similar patterns to form these oxidative metabolites while CYP1B1.3 produced unique products such as OHFM1-M4. Such products were not detected in other P450s and liver microsomes (Figure 1). More studies will be needed to determine if these molecular differences in the CYP1B1 variants affect their catalytic differences in flavone oxidation activities.

Some assignments of mono-, di-, and tri-OH flavone structures to the products obtained in the present study could be made based on the literature on mass spectrometry of flavones (the amounts of products formed were too low for NMR analysis). All assignments were made on the basis of positive ion mass spectra.[59-61] The preferred fragmentation is through the C ring, both with

flavones and 3-hydroxyflavones (Figure 10), and accordingly hydroxylations on the A and B rings can be distinguished from each other.[60]

We detected 11 unidentified mono-OF products on incubation of flavone with human P450 and liver microsomal enzymes (Figure 1). On the basis of the mass spectral literature,[60] OHFM11 was assigned as 3-OH flavone. The *m/z* 121 and 93 fragments are indicative of a non-A-ring hydroxylation (i.e., leaving M1, M2, M3, M6, 9, M10, and M11), and M11 has no *m/z* 145 ion (ruling at M1, M6, M9, and M10), but does have a strong *m/z* 115 ion, as expected for 3-OH flavone.[60] Mono-OH flavones M4, M5, M7, and M12 resemble standard 5-OH-, 6-OH-, and 7-OH flavones in showing characteristic *m/z* 137, 129, and 103 peaks (Figure 3A-3G). These patterns are indicative of hydroxylations on the A ring.[60] The products M1, M2, M3, M6, M9, and M10 showed strong *m/z* 121, 93, and 91 peaks, indicative of B ring hydroxylation.[60] However, we were unable to define specific sites of hydroxylation other than with 5-, 6-, and 7-OH flavone (for which standards were available). The enzymes formed 5- and 6-OH flavone, but not 7-OH flavone.[36,37] Thus, we have at least six A-ring hydroxy products (one of which could be 8-OH flavone) and six B-ring hydroxy products. The numbers of products raise a dilemma in that each ring only has three possibilities (because 7-hydroxylation on the A ring was not observed). Flavone and its mono-hydroxylated products are not chiral, so the explanation cannot be stereochemistry.

We detected 57diOHF and 78diOHF and five unidentified di-OH flavones on incubation of flavone with human P450 and liver microsomal enzymes (Figure 1). The 57diOHF and diOHFM6 were also detected on incubation of 5OHF with these P450 enzymes and the minor product diOHFM6 could be derived from 5OHF (Figure 4). The mass spectrum of diOHFM6 was very similar to that of 57diOHF in terms of the fragments (Figure 4) and only the A ring should be hydroxylated, suggesting that this might be either 5,6- or 5,8-dihydroxyflavone (known as rimetin). The other four unidentified di-OH flavones were assigned based on comparison of their mass spectral fragmentation patterns with those in the literature. Hydroxyl groups in the A ring show strong *m/z* 129 and 137 fragments (Figures 2, 3, *vide infra*) and hydroxyl groups in the B ring show strong *m/z* 103 and 121 ions. On the basis of comparisons, we assigned di-OHFM1 as 3′,4′-diOH flavone (NIST 1207822), di-OHFM3 as 4′,5-diOH flavone (NIST 1207763), di-OHFM4 as 3,4′-di-OH flavone[59] and di-OHFM5 as 6,4′-di-OH flavone (PubChem NP_C9_15_p5_E04_iTree_POS_05).

Two of the tri-OH products (Figure 6) were readily identified by their co-chromatography and the similarity of their fragmentation with commercial standards, i.e. 567triOHF (baicalein) and 4′57triOHF (apigenin). TriOHFM1 (formed with CYP1A2) had a mass spectrum similar to 567triOHF (Figure 7), dominated by *m/z* 123, apparently the remnant of the tri-hydroxy A ring

(Figure 6). This was formed from 57diOHF, so it can only be 5, 7, 8-tri-OH flavone. The other product (triOHFM3) fragmented in a very similar manner to 4′57triOHF (Figure 7), dominated by *m/z* 153, presumably MH^+-118. The pattern indicates that the hydroxylation is on the B ring. It cannot be at the 4′ position, because that compound is known and separates (Figure 6). The 2′ and 3′ sites are possible. The spectrum resembles that of 5,7,2′-triOH flavone (NIST 1210448), dominated by *m/z* 153. We prefer this assignment but cannot rule out the possibility of 5,7,3′-tri-OH flavone, in that a mass spectrum of this has not been published nor is otherwise available.

An explanation is provided in that there is the potential for P450s to induce regiochemical changes during the oxidation process. A precedent already exists in the isomerization of flavonone to isoflavone, catalyzed by both plant[62-65] and rat and human P450s.[66] A mechanism for the P450-catalyzed stereo isomerization of the drug (4-hydroxy) tamoxifen has already been proposed [67] and a similar mechanism, based on that outlined by Kagawa et al. [66] is shown in Fig. 11.

We are currently investigating to verify this hypothesis that human P450s could convert flavone to isoflavone as shown in Figure 12. We propose that the mono-OH flavone products (Figure 1D-1M) are mixtures of flavones and isoflavones, which have very similar UV and mass spectra. We cannot discern, with the data available, whether isomerization occurred before or after each hydroxylation.

In conclusion, the present studies showed that flavone, 5OHF, and 57diOHF were converted to mono-OH, di-OH, and tri-OH flavones, respectively, by human P450 and liver microsomal enzymes and that individual human P450s had different roles in oxidizing these flavonoids. Human P450 and liver microsomal enzymes produced various oxidized flavones, including 6OHF and 5OHF and at least 11 unidentified products whose chemical structures are proposed in this study by the analysis of product ion spectra (mass fragmentation) and comparison with previously reported data. Formation of 5OHF from flavone by P450s and liver microsomes was not very rapid, but the turnover rates of formation of 567triOHF and 4'57triOHF from 57diOHF were relatively high with CYP Family 1 enzymes and CYP2A13 and 2A6, respectively, and these phenomena may be relevant in the metabolic activation of numerous flavonoids into biologically active products with beneficial properties in humans, e.g. prevention of cancer due to anti-proliferative, anti-oxidant, and anti-inflammatory effects.

ASSOCIATED CONTENT

SUPPORTING INFORMATION

Molecular docking analysis of interaction of flavone, 5OHF, and 57diOHF with CYP1B1.1, CYP1B1.3, CYP1A1, CYP1A2, CYP2A6, CYP2A13, CYP2B6, CYP2C9, and CYP3A4.

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Notes

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ABBREVIATIONS: P450 or CYP, cytochrome P450; b_5, cytochrome b_5; DLPC, L-α-1,2 dilaouryl-*sn*-glycero-3-phosphocholine; mono-OH, di-OH, and tri-OH flavone, general term for mono-, di-, and tri-hydroxy flavone, respectively; 5OHF, 6OHF, 7OHF, 57diOHF, 78diOHF, 4'57triOHF, and 567triOHF, individual form for 5-hydroxyflavone (primuletin), 6-hydroxyflavone, 7-hydroxyflavone, 5,7-dihydroxyflavone (chrysin), 7,8-dihydroxyflavone, 4′,5,7-trihydroxyflavone (apigenin), and 5,6,7-trihydroxyflavone (baicalein), respectively. OHFM1-OHFM12, diOHFM1-diOHFM6, triOHFM1 and triOHF M3, individual terms for unidentified products of mono-, di-, and triOH flavones.

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Legends to figures

Figure 1. LC-MS/MS analysis of oxidation of flavone by human CYP1A1 (A and H), CYP1A2 (B and I), CYP1B1.1 (C and J), CYP1B1.3 (D and K), and CYP2A6 (E and L) and by liver microsomes derived from human samples HH2 (F and M) and HH47 (G and N) to form mono-OH flavones (A-G) and di-OH flavones (H-N). The mono-OH flavones and di-OH flavones were detected by analysis with *m/z* 239>121 and 239>137 and with *m/z* 255>129 and 255>153, respectively.

Figure 2. Oxidation of flavone by human liver microsomes of samples HH2 (A) and HH54 (B). Formation of mon-OH flavones was analyzed using *m/z* 239>121 and 239>137. The product ion spectra of standard 5OHF (C) and 6OHF (E) and that of 5OHF produced by HH2 microsomes (D) and of 6OHF produced by HH54 microsomes (F) are shown.

Figure 3. Product ion spectra of standard 5OHF (A), 6OHF (B), and 7OHF (C) and unidentified products of mono-OH flavones that were formed through metabolism of flavone with CYP1B1.3 (D, H, I, and J), CYP1A2 (E), CYP2A6 (F, G, K, L, and N), and HH54 (M) as indicated in the figures.

Figure 4. Product ion spectra of standard flavone (A), standard 57diOHF (B), 57diOHF produced by

HH54 (C), standard 78diOHF (D), 78diOHF produced by HH54 (E), diOHFM1, M3, M4, and M5 produced by CYP2A6 (F, G, H, and I, respectively), and diOHFM6 produced by HH54 (J).

Figure 5. Oxidation of 5OHF by CYP1A2 (A), CYP2A6 (B), CYP2A13 (C), and HH54 (C and F) to form di-OH flavones and tri-OH flavones. Product formation was determined using *m/z* 255>153 for dihydroxyflavones (colored in red) and *m/z* 271>123 for tri-OH flavones (colored in blue). The substrate 5OHF (shown in black) was analyzed with *m/z* 239>137.

Figure 6. LC-MS/MS analysis of oxidation of 57diOHF by reconstituted monooxygenase systems containing CYP1A1 (A), CYP1A2 (B), CYP1B1.1 (C), CYP2A6 (D), and CYP2A13 (E) and by HH54 liver microsomes (F). Detection was by analysis of *m/z* 255>153 for the substrate 5,7diOHF, *m/z* 271>119 for 457triOHF, *m/z* 271>123 for 567triOHF, and triOHFM1 and M3. The scale of the upper three panels was 10-fold higher than in the lower three panels; the assays were carried out with the same levels of P450 contents in these cases.

Figure 7. Product ion spectra of standard baicalein (A, 567triOHF) and apigenin (D, 4′57triOHF) and those of products of oxidation of 57diOHF with CYP1B1.3 (B) and CYP2A13 (E). Uncharacterized

triOHFM1 (C) and triOHFM3 (F) products, which were formed by oxidation of 57diOHF with CYP1A2 and CYP1A1, respectively, are also shown.

Figure 8. Oxidation of 5OHF to 57diOHF (A) and 567triOHF (B) by human P450 enzymes including CYP1A1, 1A2, 1B1.1, 1B1.3, 2A6, 2A13, 2B6, 2C9, and 3A4 and microsomes prepared from human liver samples HH2, HH47, and HH54. These P450 enzymes and liver microsomes were also used for the analysis of oxidation of 57diOHF to form 4´57triOHF (C) and 567triOHF (D). The data presented are means ± SD from 3-5 experiments. Note that the scales of the vertical axis for the oxidation of 5OHF (A and B) were 25-fold lower than those for the oxidation of oxidation of 57diOHF (C and D).

Figure 9. Kinetic analysis of oxidation of 57diOHF by human P450 enzymes. Different concentrations of 57diOHF were incubated with reconstituted systems containing CYP1A1 (A and E), CYP1A2 (B and F), CYP2A6 (C and G), and CYP2A13 (D and H) and the products of 4´57triOHF (A-D) and 567triOHF (E-H) formed were analyzed with LC-MS/MS. The K_m values for these P450 enzymes were estimated by nonlinear regression analysis of hyperbolic plots using Graphpad Prism (GraphPad, La Jolla, CA) and are shown in the figures. Formation of 567triOHF in incubations with different concentrations of 57diOHF with CYP1B1.1 (X and x) and CYP1B1.3 (Y and y) was also

determined; these small x and y show the data obtained using low concentrations of 57diOHF.

Figure 10. Docking analysis of interaction of 57diOHF with CYP1B1.1 (A) and CYP1B1.3 B). In the figures, two different ligand bindings with CYP1B1.1 and 1B1.3 are shown as the first and second ligand.

Figure 11. Preferred fragmentation sites for flavones.[59-61]

Figure 12. Proposed mechanism for rearrangement of flavone to isoflavone and oxidation to 3-OH flavone.[62,66-69]

Figure 1

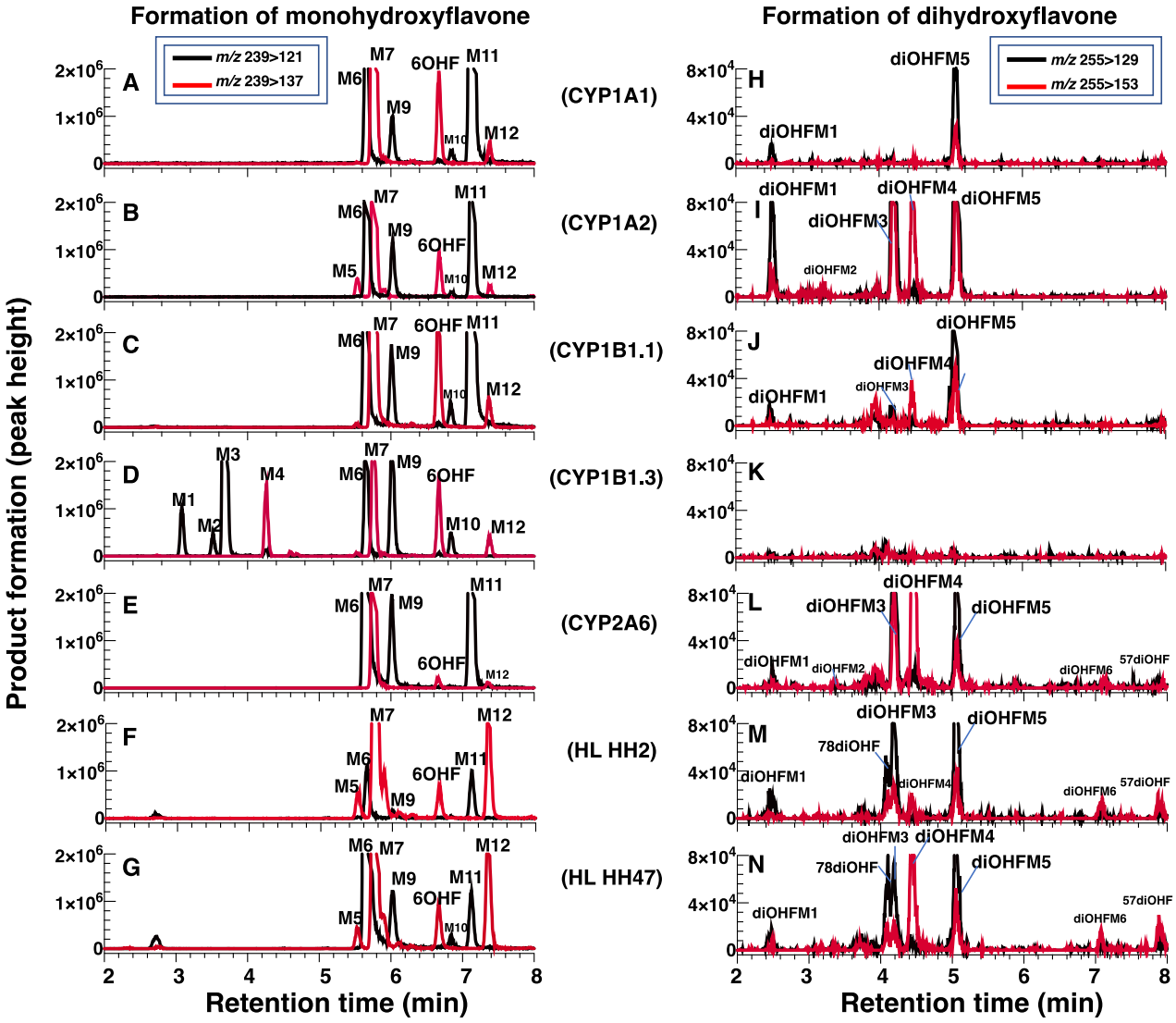

Formation of monohydroxyflavone
Formation of dihydroxyflavone
Product formation (peak height)
Retention time (min)
m/z 239>121
m/z 239>137
m/z 255>129
m/z 255>153
(CYP1A1)
(CYP1A2)
(CYP1B1.1)
(CYP1B1.3)
(CYP2A6)
(HL HH2)
(HL HH47)
M1
M2
M3
M4
M5
M6
M7
M9
M10
M11
M12
6OHF
diOHFM1
diOHFM2
diOHFM3
diOHFM4
diOHFM5
diOHFM6
78diOHF
57diOHF

Figure 2

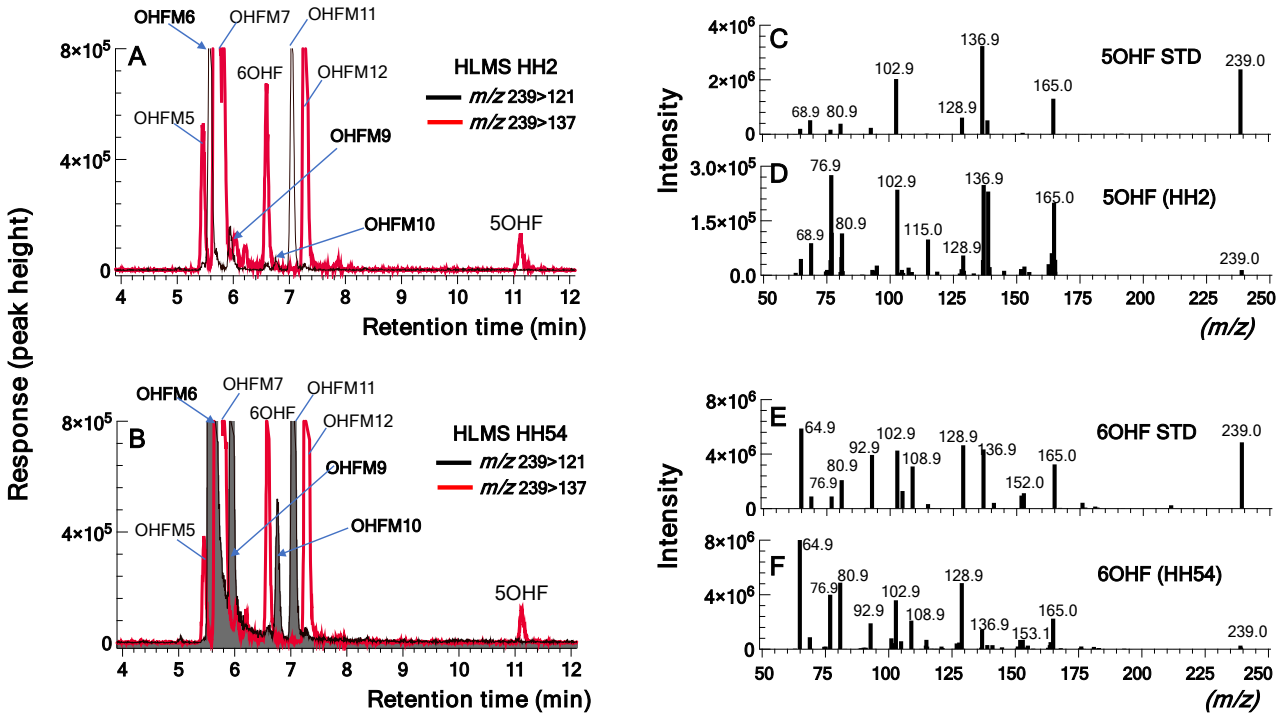

A
B
C
D
E
F
OHFM6
OHFM7
OHFM11
6OHF
OHFM12
OHFM5
OHFM9
OHFM10
5OHF
HLMS HH2
HLMS HH54
m/z 239>121
m/z 239>137
Response (peak height)
Retention time (min)
Intensity
(m/z)
5OHF STD
5OHF (HH2)
6OHF STD
6OHF (HH54)

Figure 3

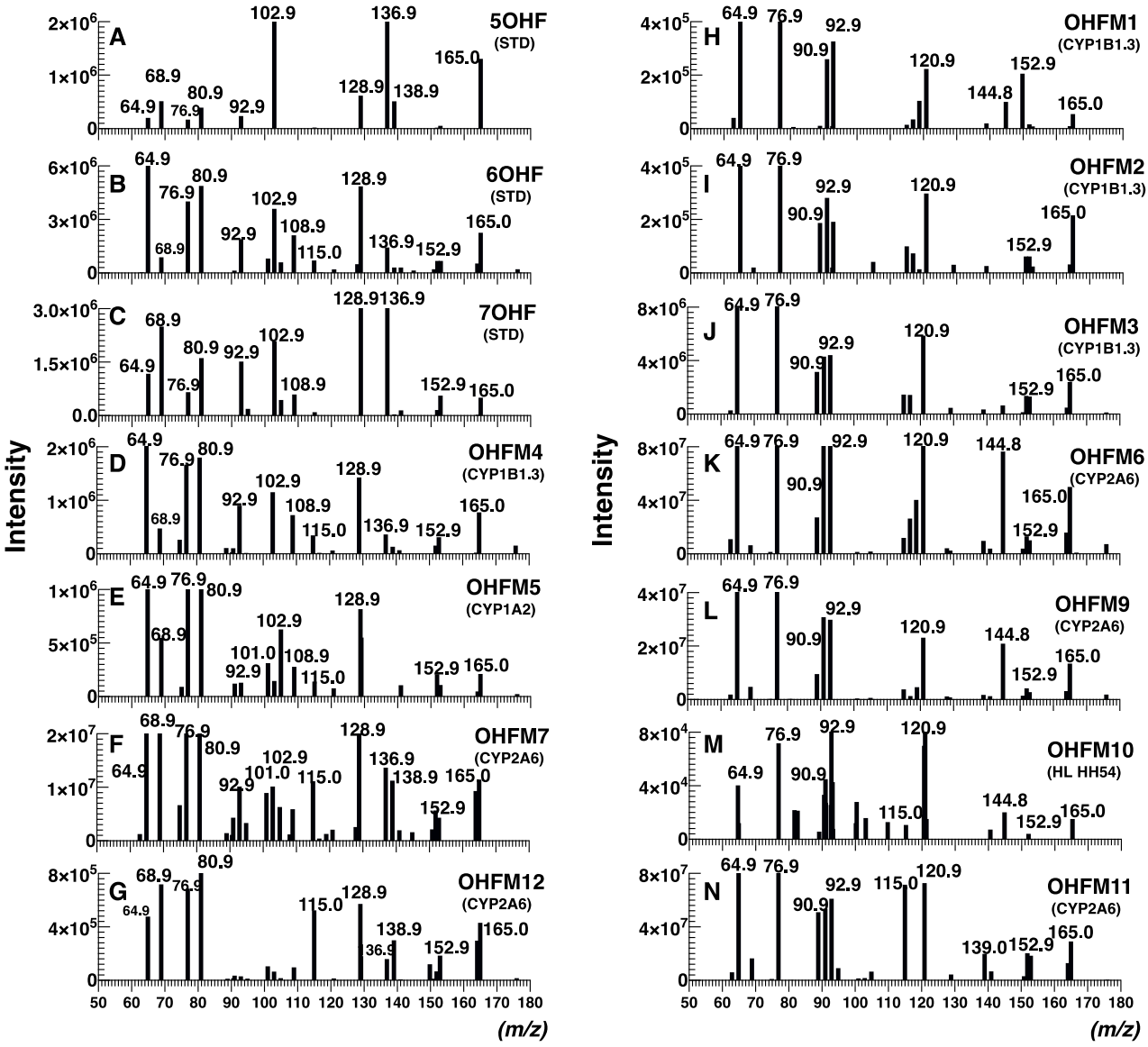

A
5OHF
(STD)
B
6OHF
(STD)
C
7OHF
(STD)
D
OHFM4
(CYP1B1.3)
E
OHFM5
(CYP1A2)
F
OHFM7
(CYP2A6)
G
OHFM12
(CYP2A6)
H
OHFM1
(CYP1B1.3)
I
OHFM2
(CYP1B1.3)
J
OHFM3
(CYP1B1.3)
K
OHFM6
(CYP2A6)
L
OHFM9
(CYP2A6)
M
OHFM10
(HL HH54)
N
OHFM11
(CYP2A6)
Intensity
Intensity
(m/z)
(m/z)

Figure 4

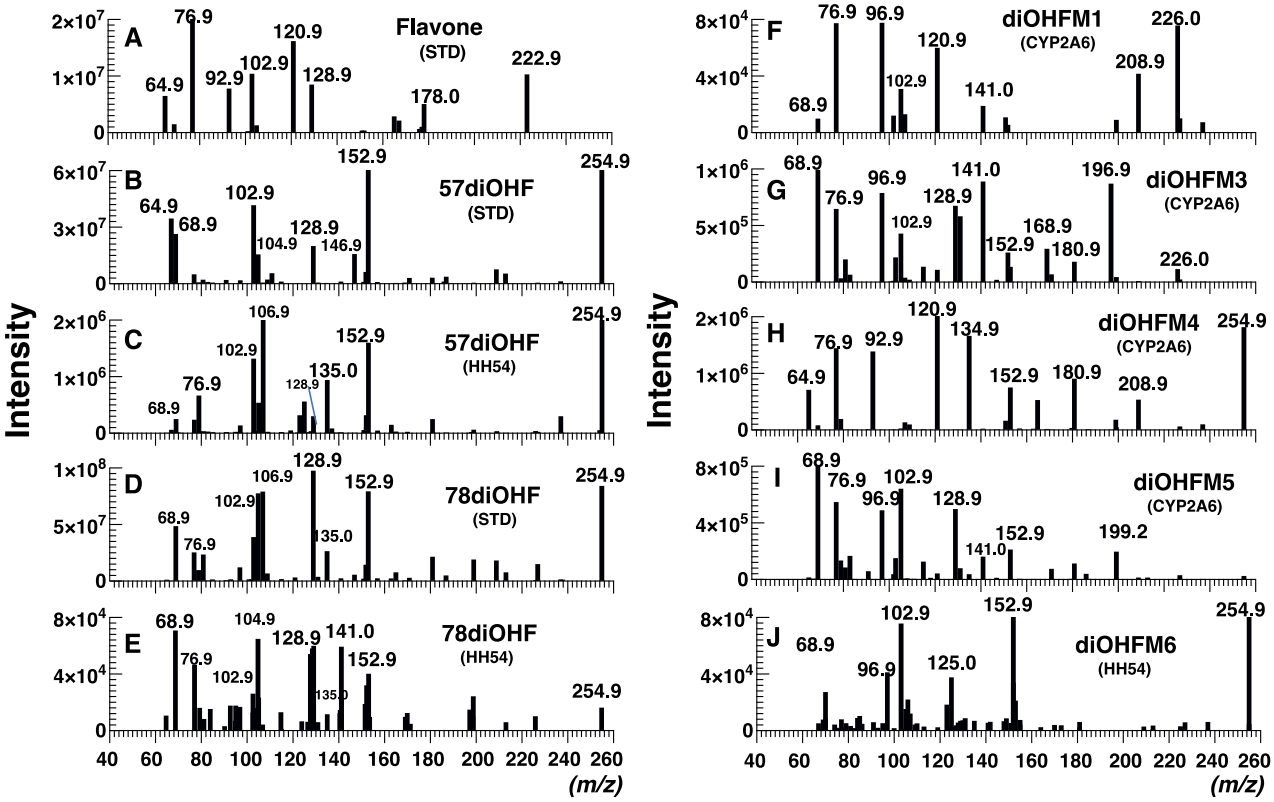

Intensity
A
Flavone
(STD)
B
57diOHF
(STD)
C
57diOHF
(HH54)
D
78diOHF
(STD)
E
78diOHF
(HH54)
F
diOHFM1
(CYP2A6)
G
diOHFM3
(CYP2A6)
H
diOHFM4
(CYP2A6)
I
diOHFM5
(CYP2A6)
J
diOHFM6
(HH54)
(m/z)

Figure 5

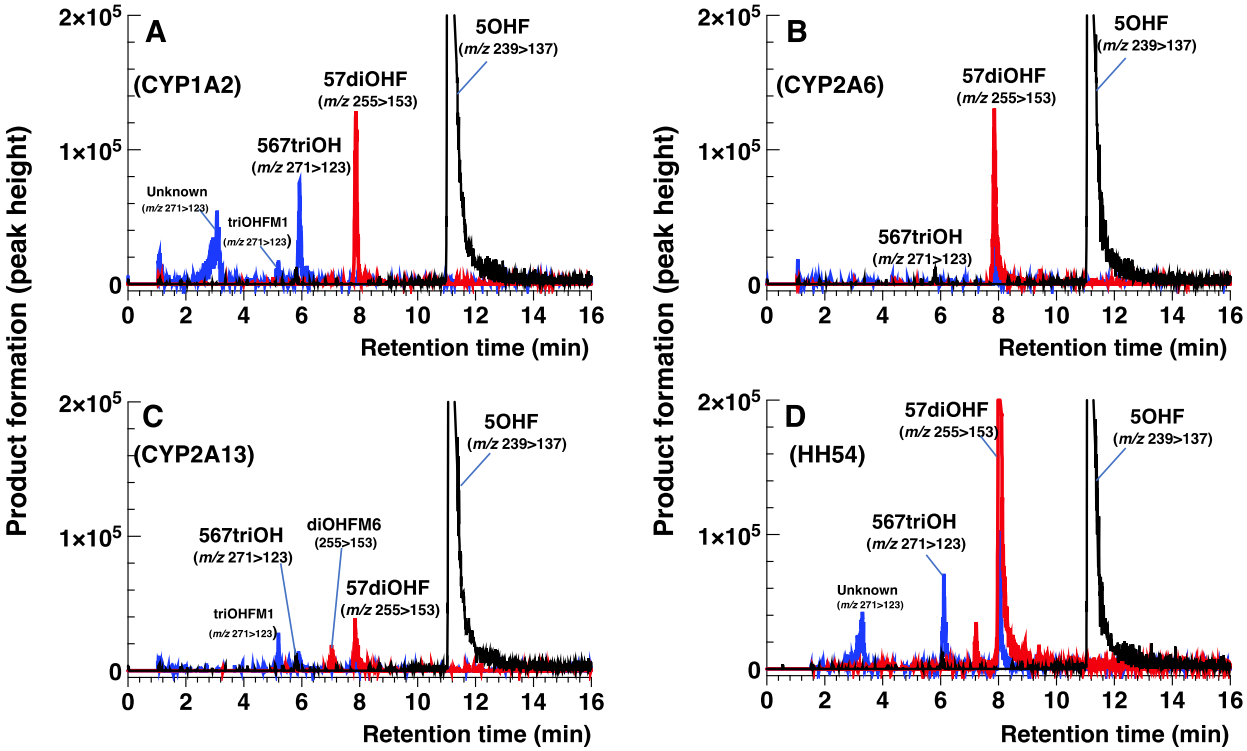

A
(CYP1A2)
Unknown
(m/z 271>123)
triOHFM1
(m/z 271>123)
567triOH
(m/z 271>123)
57diOHF
(m/z 255>153)
5OHF
(m/z 239>137)
B
(CYP2A6)
567triOH
(m/z 271>123)
57diOHF
(m/z 255>153)
5OHF
(m/z 239>137)
C
(CYP2A13)
triOHFM1
(m/z 271>123)
567triOH
(m/z 271>123)
diOHFM6
(255>153)
57diOHF
(m/z 255>153)
5OHF
(m/z 239>137)
D
(HH54)
Unknown
(m/z 271>123)
567triOH
(m/z 271>123)
57diOHF
(m/z 255>153)
5OHF
(m/z 239>137)
Product formation (peak height)
Retention time (min)
2×10^5
1×10^5
0
0 2 4 6 8 10 12 14 16

Figure 6

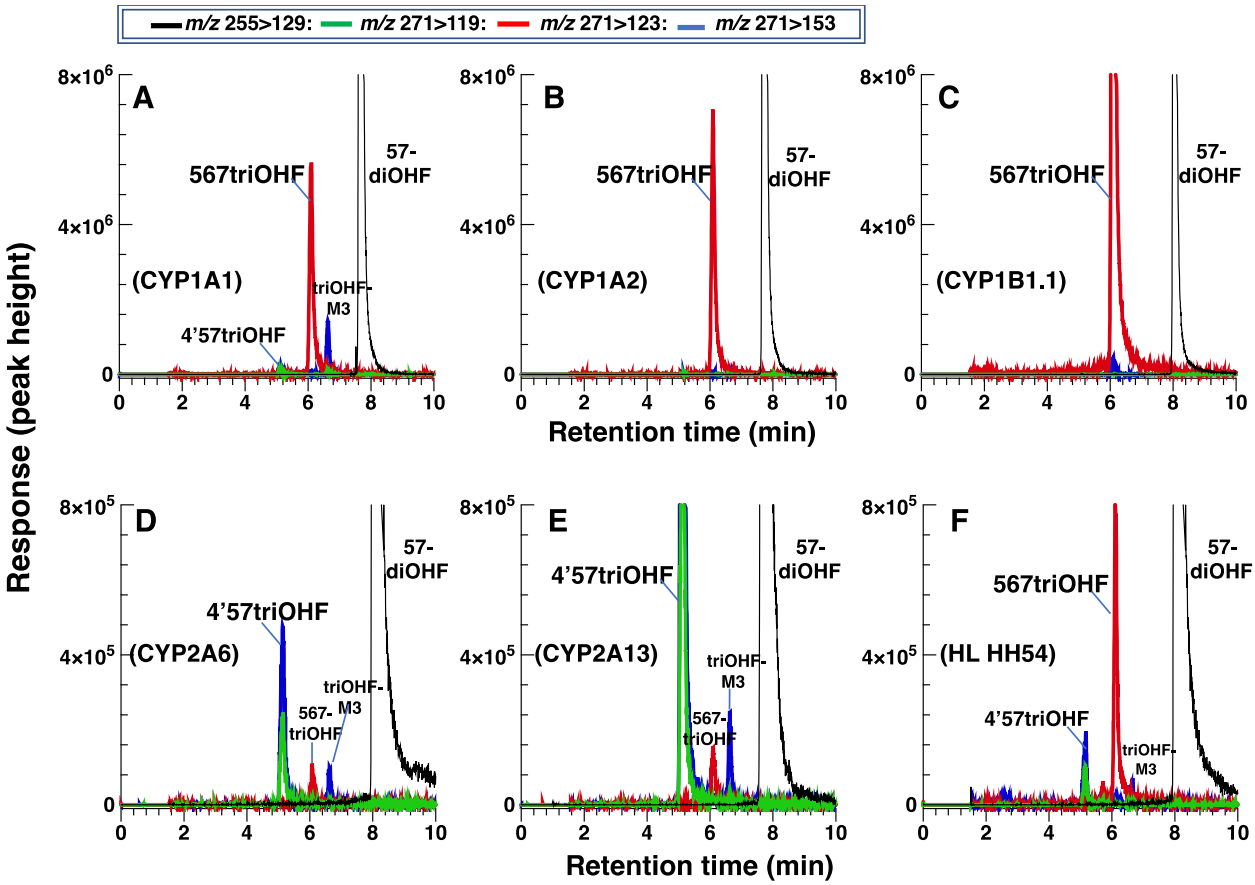

m/z 255>129:
m/z 271>119:
m/z 271>123:
m/z 271>153
Response (peak height)
Retention time (min)
A
B
C
D
E
F
(CYP1A1)
(CYP1A2)
(CYP1B1.1)
(CYP2A6)
(CYP2A13)
(HL HH54)
567triOHF
4'57triOHF
57-diOHF
triOHF-M3
567-triOHF
8x10^6
4x10^6
8x10^5
4x10^5
0
2
4
6
8
10

Figure 7

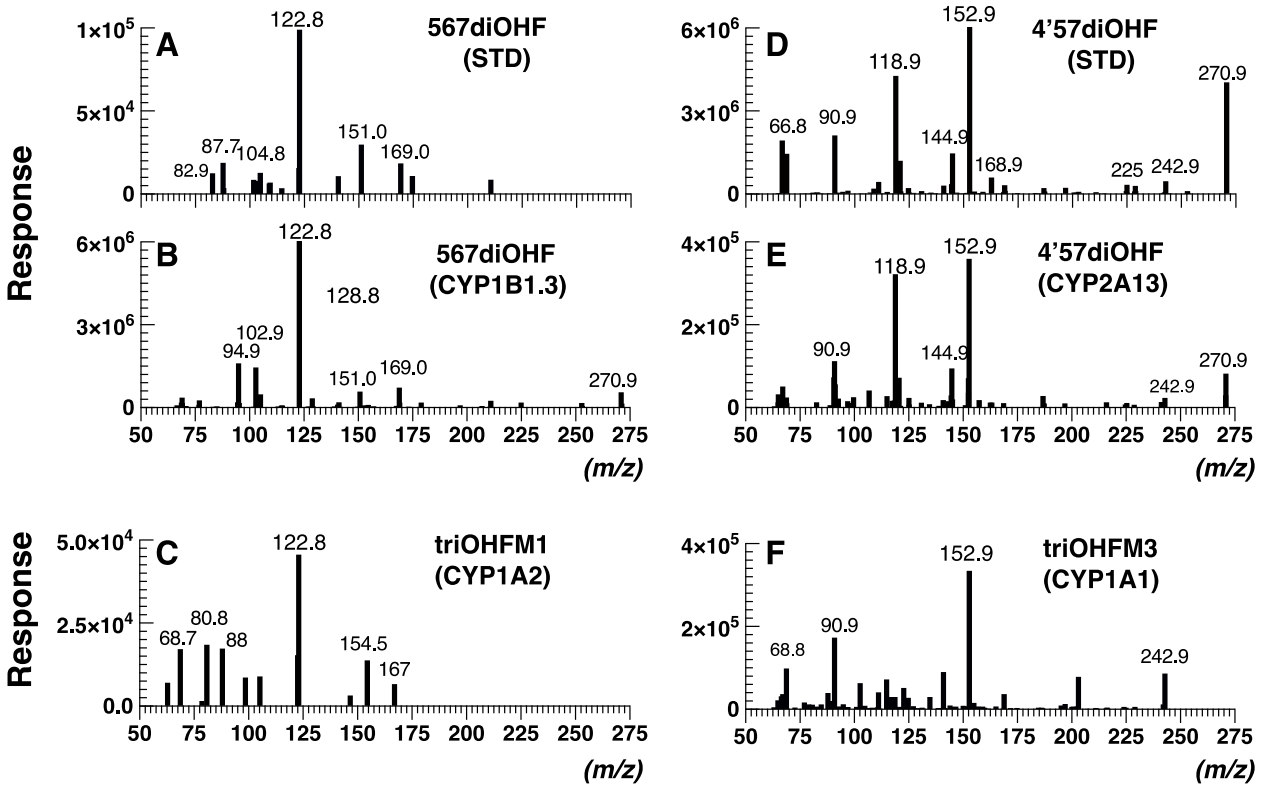

A
567diOHF
(STD)
B
567diOHF
(CYP1B1.3)
C
triOHFM1
(CYP1A2)
D
4'57diOHF
(STD)
E
4'57diOHF
(CYP2A13)
F
triOHFM3
(CYP1A1)
Response
(m/z)

Figure 8

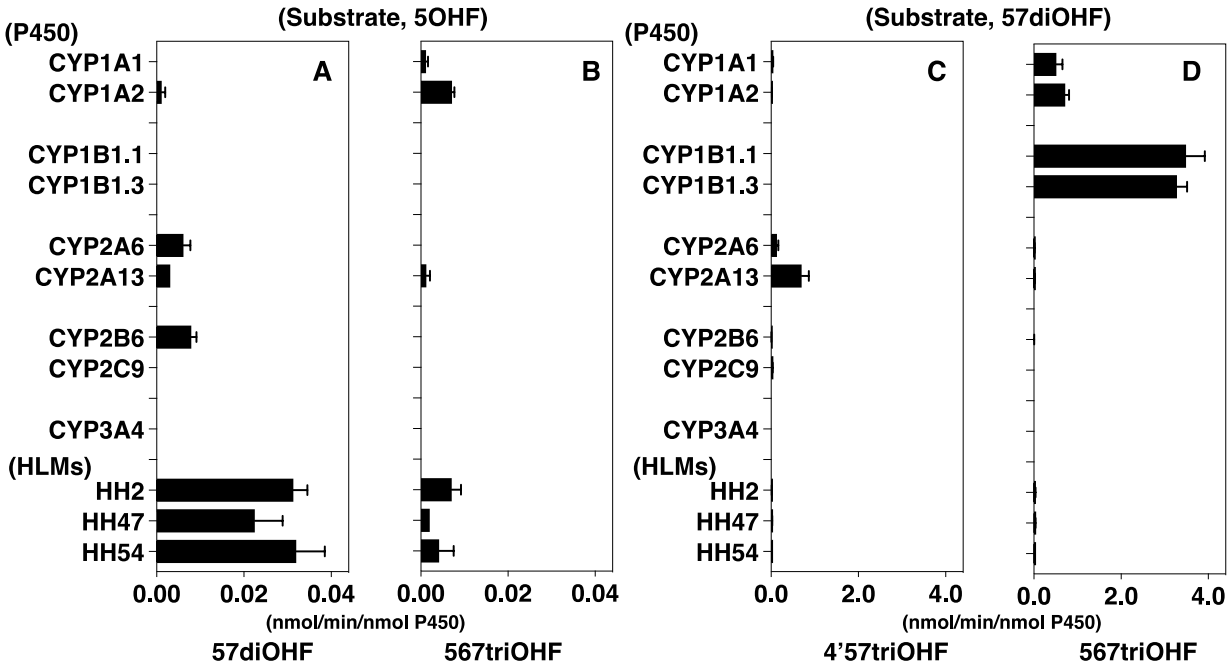

(Substrate, 5OHF)
(Substrate, 57diOHF)
(P450)
CYP1A1
CYP1A2
CYP1B1.1
CYP1B1.3
CYP2A6
CYP2A13
CYP2B6
CYP2C9
CYP3A4
(HLMs)
HH2
HH47
HH54
A
B
C
D
0.00
0.02
0.04
0.0
2.0
4.0
(nmol/min/nmol P450)
57diOHF
567triOHF
4'57triOHF
567triOHF

Figure 9

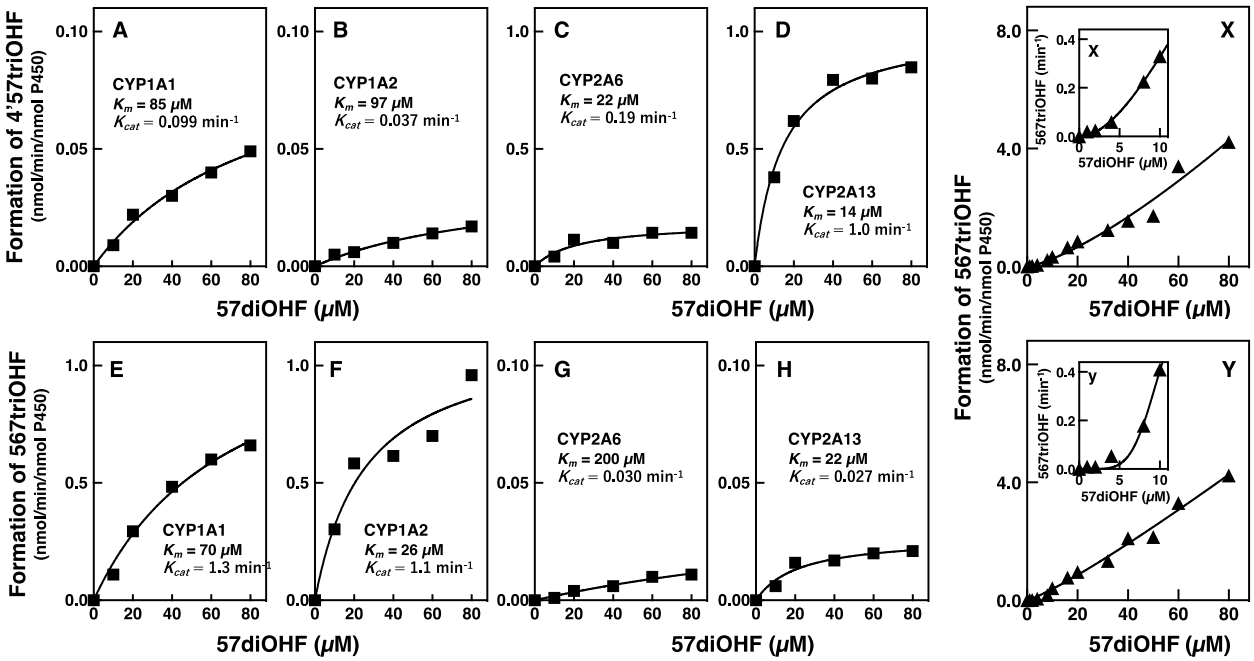

A
CYP1A1
K_m = 85 μM
K_{cat} = 0.099 min^{-1}
B
CYP1A2
K_m = 97 μM
K_{cat} = 0.037 min^{-1}
C
CYP2A6
K_m = 22 μM
K_{cat} = 0.19 min^{-1}
D
CYP2A13
K_m = 14 μM
K_{cat} = 1.0 min^{-1}
E
CYP1A1
K_m = 70 μM
K_{cat} = 1.3 min^{-1}
F
CYP1A2
K_m = 26 μM
K_{cat} = 1.1 min^{-1}
G
CYP2A6
K_m = 200 μM
K_{cat} = 0.030 min^{-1}
H
CYP2A13
K_m = 22 μM
K_{cat} = 0.027 min^{-1}
Formation of 4'57triOHF (nmol/min/nmol P450)
Formation of 567triOHF (nmol/min/nmol P450)
57diOHF (μM)
X
Y
567triOHF (min^{-1})

Figure 10

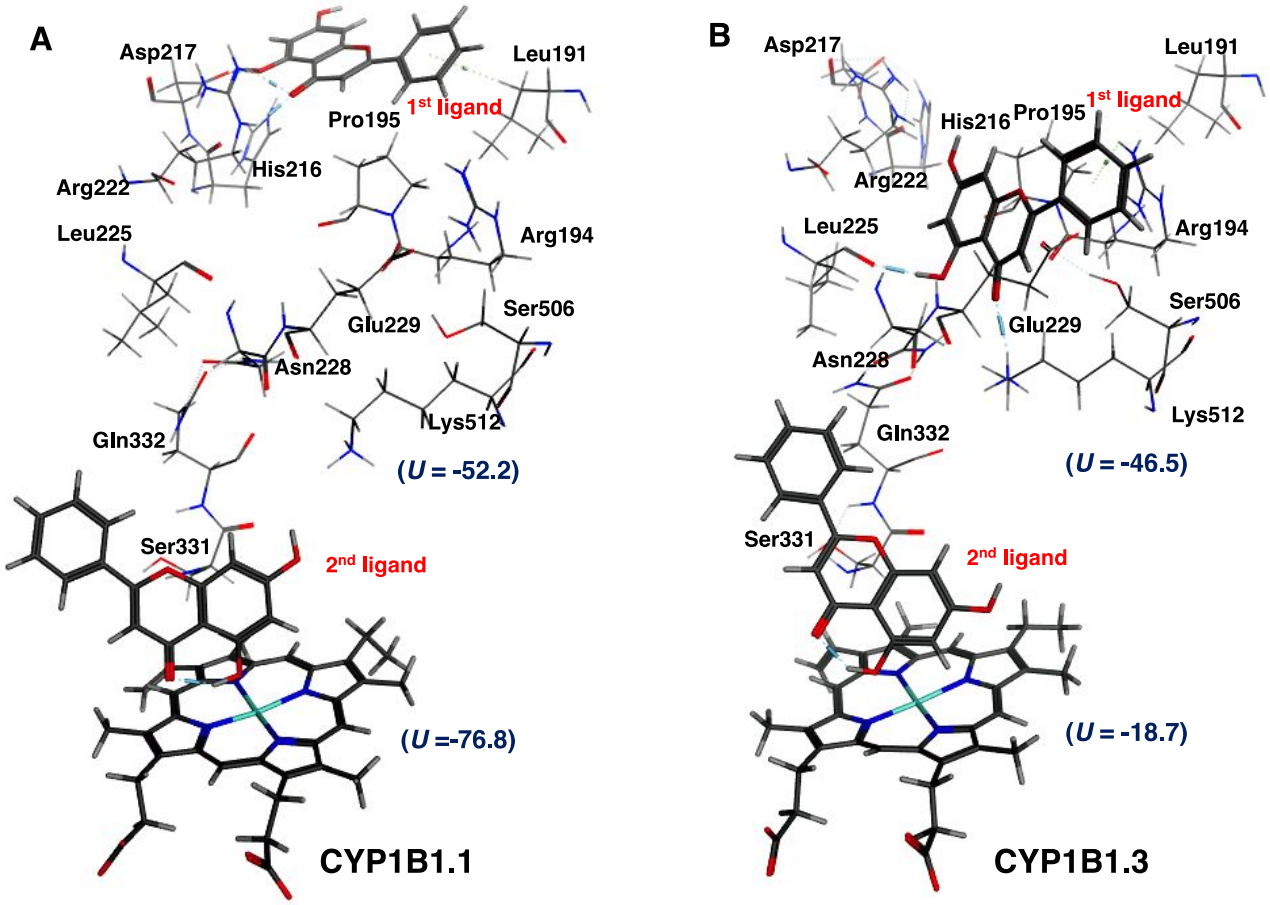

A
Asp217
Leu191
Pro195
1st ligand
His216
Arg222
Leu225
Arg194
Glu229
Ser506
Asn228
Lys512
Gln332
(U = -52.2)
Ser331
2nd ligand
(U =-76.8)
CYP1B1.1
B
Asp217
Leu191
His216
Pro195
1st ligand
Arg222
Leu225
Arg194
Ser506
Glu229
Asn228
Lys512
Gln332
(U = -46.5)
Ser331
2nd ligand
(U = -18.7)
CYP1B1.3

Figure 11

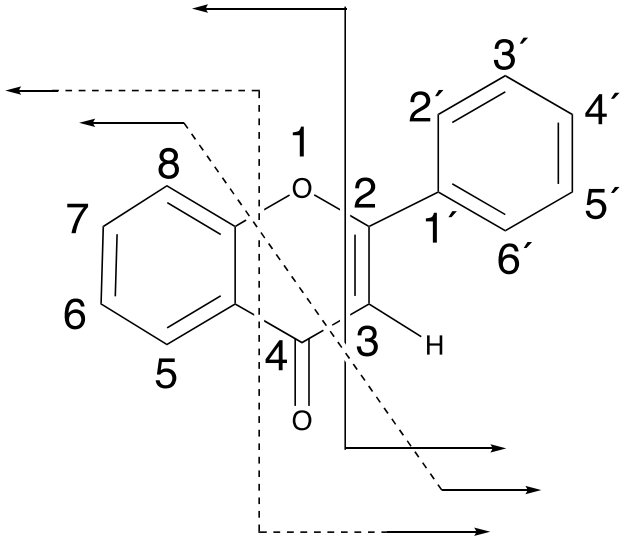
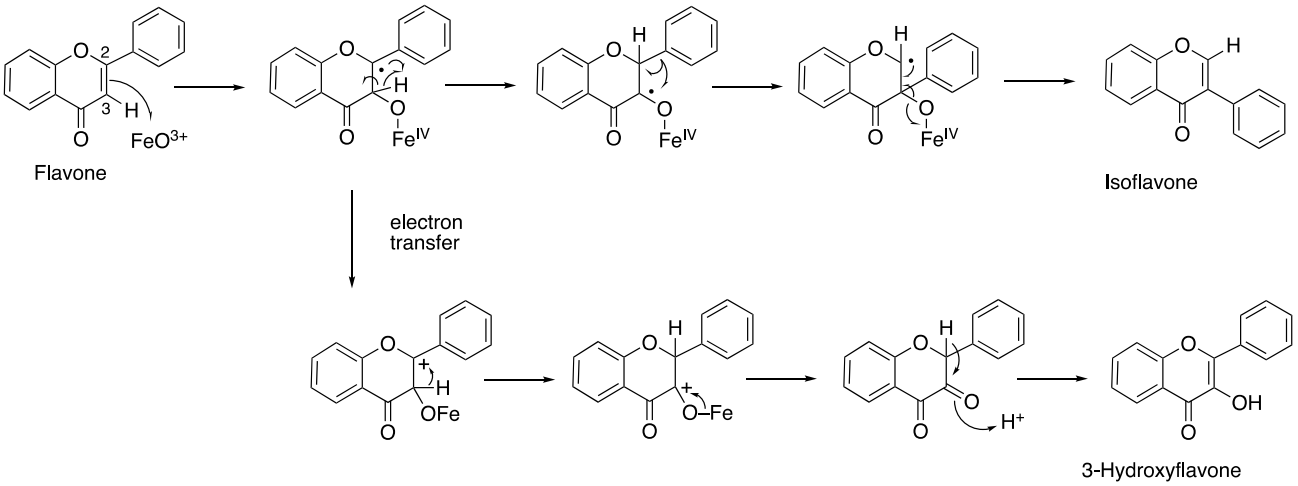


Figure 12


Flavone
FeO3+
Isoflavone
electron
transfer
3-Hydroxyflavone