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Roles of cytochrome P450 2A6 in the oxidation of flavone, 4′-hydroxyflavone, and 4′-, 3′-, and 2′-methoxyflavones by human liver microsomes

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Abstract

1. Nine forms of recombinant cytochrome P450 (P450 or CYP) enzymes were used to study roles of individual P450 enzymes in the oxidation of flavone and some other flavonoids, 4′-hydroxyflavone and 4′-, 3′-, and 2′-methoxyflavones, by human liver microsomes using LC-MS/MS analysis.

2. As has been reported previously (Nagayoshi et al., Xenobiotica **50**, 1158-1169, 2020), 4′-, 3′-, and 2′-methoxyflavones were preferentially *O*-demethylated by human liver P450 enzymes to form 4′-, 3′-, and 2′-hydroxylated flavones and also 3′,4′-dihydroxyflavone from the former two substrates.

3. In comparisons of product formation by oxidation of these methoxylated flavones, CYP2A6 was found to be a major enzyme catalyzing flavone 4′- and 3′-hydroxylations by human liver microsomes but did not play significant roles in 2′-hydroxylation of flavone, *O*-demethylations of three methoxylated flavones, and the oxidation of 4′-hydroxyflavone to 3′,4′-dihydroxyflavone.

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Declaration of interest Statement

The authors declare no conflict of interest associated with this manuscript.

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4. The effects of anti-CYP2A6 IgG and chemical P450 inhibitors suggested that different P450 enzymes, as well as CYP2A6, catalyzed oxidation of these flavonoids at different positions by liver microsomes.

5. These studies suggest that CYP2A6 catalyzes flavone 4′- and 3′-hydroxylations in human liver microsomes and that other P450 enzymes have different roles in oxidizing these flavonoids.

Keywords

Flavone; 3′- and 4′-Hydroxylation; Flavonoids; CYP2A6; Human liver; LC-MS/MS

Introduction

A variety of plant flavonoids are found in nature and many of these natural products have been reported to have various biological activities in *in vitro* experiments and animal studies, e.g. anti-allergic, anti-inflammatory, anti-oxidative stress, anti-microbial, and anti-tumor activities (Arct and Pytowska, 2008; Kale et al., 2008; Martens and Mithöfer, 2005; Tanaka et al., 2010; Tanaka and Brugliera, 2013; Zhang et al., 2005). These biological activities depend on the chemical structures, including the substitution positions on the parent chemicals and their hydroxyl and/or methoxy groups in the flavonoid molecules (Breinholt et al., 2002; Hodek et al., 2002; Walle and Walle, 2007; Zhang et al., 2005). Studies have been done to examine biotransformation of these flavonoids to biologically active and/or inert compounds by cellular enzymes, particularly by P450s, in plants and mammals (Akashi et al., 1998; 1999; Du et al., 2010; Fliegmann et al., 2010; Hodek et al., 2002; Kagawa et al., 2004; Kakimoto et al., 2019; Nikolic and van Breemen, 2004; Tanaka et al., 2010; Tanaka and Brugliera, 2013; Uno et al., 2013; 2015; Zhang et al., 2005).

Our previous studies have suggested that human cytochrome P450 (P450 or CYP) enzymes play important roles in the oxidation of various flavonoids, including flavone, flavanone, 5-hydroxyflavone (5OHF), 5,7-dihydroxyflavone (chrysin, 57diOHF), 2′-, 3′-, and 4′-MeF, and 2′-, 3′-, 4′-, and 6-hydroxylated flavanones (Kakimoto et al., 2019; Nagayoshi et al., 2019a; 2019b; Nagayoshi et al., 2020; Shimada et al., 2021). During course of these studies, we found that CYP2A6 catalyzes flavanone 2′-hydroxylation and flavone to two chemically uncharacterized products, OHFM6 and OHFM9, in human liver microsomes. Formation of these products was low in microsomes of a human sample HH2, which was defective in CYP2A6-dependent coumarin 7-hydroxylation activity. The activity was strongly inhibited by coumarin and anti-CYP2A6 IgG in human liver samples that had significant coumarin 7-hydroxylation activity (Nagayoshi et al., 2019a).

In this study, LC-MS/MS analysis was performed to study roles of human P450 enzymes in the oxidation of flavone, 4′-hydroxyflavone (4′OHF), and 4′-, 3′-, and 2′-methoxyflavones (4′MeF, 3′MeF, and 2′MeF, respectively) in human liver microsomes. Nine forms of recombinant human P450 enzymes, including CYP1A1, 1A2, 1B1.1, 1B1.3, 2A6, 2A13, 2B6, 2C9, and 3A4, and three human liver samples (HH2, HH47, and HH54) were used

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in this study (Nagayoshi et al., 2019a). The effects of anti-CYP2A6 and chemical P450 inhibitors and molecular docking analysis were also examined.

Materials and methods

Chemicals

Flavone, 4´OHF, 3´4´diOHF, and 57diOHF (Figure 1) were purchased from Tokyo Kasei Co. (Tokyo), Sigma-Aldrich (St. Louis, MO, USA) and Wako Pure Chemicals (Osaka, Japan). 2´MeF, 3´MeF, and 4´MeF (Figure 1) were kindly donated by Dr. Maryam K. Foroozesh (Xavier University of Louisiana, New Orleans, LA, USA). Other chemicals and reagents were obtained from sources described previously or were of the highest quality commercially available (Kakimoto et al, 2019; Nagayoshi et al., 2019a; 2019b; 2020).

Enzymes and anti-CYP2A6 IgG

Purified preparations of human CYP1A1, CYP1A2, CYP1B1.1, CYP1B1.3, CYP2A6, CYP2A13, CYP2C9, and CYP3A4 enzymes expressed *in Escherichia coli* were obtained by the methods described previously (Han et al., 2012; Kim et al., 2018; Parikh 1997; Sandhu et al., 1993; 1994; Shimada et al., 2018). Recombinant *E. coli* membranes expressing both CYP2B6 and NADPH-P450 reductase were prepared as described previously (Han et al., 2012; Kim et al., 2018). NADPH-P450 reductase and cytochrome b_5 (b_5) were purified from membranes of recombinant *E. coli* by methods described elsewhere (Guengerich 2014; Shen et al., 1989).

Liver microsomes prepared from human samples HH2 (Cat No., 452002), HH47 (Cat No., 452047), and HH54 (Cat No., 452054) were obtained from GENTEST-Corning (Woburn, MA). The data sheets provided by the manufacturer indicated that these microsomes contained 0.19, 0.26, and 0.35 nmol P450/mg protein, respectively. HH2 reportedly had no detectable coumarin 7-hydroxylation activity and thus this individual is considered to be a poor metabolizer for CYP2A6 activity, while the other two samples had considerable coumarin 7-hydroxylation activity.

Anti-CYP2A6 IgG was prepared as described previously (Yun et al., 1991).

Oxidation of flavonoids by recombinant human P450 enzymes and liver microsomes

The oxidation of flavone, 4´OHF, 2´MeF, 3´MeF, and 4´MeF by P450 enzymes and liver microsomes was determined by methods described previously (Kakimoto et al, 2019; Nagayoshi et al., 2019a; 2019b). 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 dilauoryl-*sn*-glycero-3-phosphocholine (DLPC) (50 μg) were incubated (0.25 mL of total volume) with 60 μM flavonoids 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). CYP2B6 membranes (50 pmol of P450) in *E. coli* that express both CYP2B6 and NADPH-P450 reductase were also incubated with these flavonoids. Human liver microsomes (50 pmol of P450) were also used for the assays.

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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 (Kakimoto et al, 2019; Nagayoshi et al., 2019a; 2019b; Shimada et al., 2021).

LC-MS/MS analyses were performed using an HPLC system (ACQUITY UPLC I-Class PLUS system; Waters, Milford, MA) coupled to a tandem quadruple mass spectrometer (XevoTQ-XS; Waters) by the methods as described previously (Kakimoto et al, 2019; Nagayoshi et al., 2019a; 2019b). Chromatographic separation was performed on CORTECS C_{18}, 100 mm × 2.1 mm i.d., 1.6 μm column (Waters, Milford, MA) at 45 °C. The gradient elution was done using a mixture of solvents A (10 mM ammonium acetate containing 0.1% formic acid) and B (5% methanol in acetonitrile containing 0.1% formic acid) with gradient in B from 20% (v/v) to 80% (v/v) over 16 min, at a flow rate of 0.2 ml/min. MS/MS analysis was performed and the positive electrospray ionization mode was employed with a capillary voltage of 3000 V and cone voltage of 30 V as described previously (Kakimoto et al, 2019; Nagayoshi et al., 2019a; 2019b).

Other assays

P450 contents were determined by the method of Omura and Sato (1964). Protein contents were determined by the method of Brown et al. (1989).

Docking simulations of flavone with CYP2A6

The one available crystal structure of CYP2A6 bound to 4,4′-dipyridyl disulfide (Protein Data Bank 2FDY) was used in this study (Yano et al., 2006; DeVore et al., 2012a; 2012b). The chemical structure of flavone was from PubChem (an open chemistry database at the National Institutes of Health) and optimized in MOE software (ver. 2020.0900, Computing Group, Montreal, Canada). Simulations were carried out in MOE by the methods described previously (Nagayoshi et al., 2019a; Shimada et al., 2021). Ligand-interaction energies (*U* values) were obtained by use of the program ASEdock in MOE.

Results

Oxidation of 4′MeF, 3′MeF, and 2′MeF to hydroxylated products by P450s and comparison of these products with those of oxidation of flavone by human liver microsomes

4′MeF, 3′MeF, and 2′MeF were first incubated with CYP1B1.1, 1A2, and 1B1.1, respectively, and the resultant *O*-demethylated products, 4′OHF, 3′OHF, and 2′OHF, respectively, were analyzed using LC-MS/MS (Figure 2). Although only standard 4′OHF was available (but not 3′OHF and 2′OHF), our previous studies indicated that the major products of 3′- and 2′-MeF are the *O*-demethylated products, 3′OHF and 2′OHF, respectively, by analysis with LC-MS/MS (Nagayoshi et al., 2020). In this study, we assigned these peaks (M2, M3, and M4 in Figure 2A, 2B, and 2C) to be 4′OHF (retention time, 5.6 min), 3′OHF (5.9 min), and 2′OHF (6.7 min), respectively; we analyzed the formation of 4′OHF (and also 3′4′diOHF) by determination with comparison to authentic standards on LC-MS/MS. The formation of 3′4′diOHF (M1 in Figure 2A and 2B; retention time 4.4 min) as detected by *m/z* 255>121 on LC/MS/MS was confirmed by analyzing

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an authentic standard. In addition, we analyzed fragment (product) ion spectra from LC-MS/MS and the results suggested that M1, M2, M3, and M4 corresponded to 3′4′diOHF, 4′OHF, 3′OHF, and 2′OHF, respectively (Figures 2G–2J).

Subsequently, we analyzed oxidation of flavone by liver microsomes of human samples HH54 and HH2 (extensive and poor metabolizers, respectively, for CYP2A6 activities) (Figure 2D and 2E) (Nagayoshi et al., 2019a). The effects of anti-CYP2A6 IgG on the oxidation of flavone catalyzed by HH54 microsomes were also studied (Figure 2E). The chromatograms of products obtained on incubation of flavone with these liver microsomes were compared with those of M1 (3′4′diOHF), M2 (4′OHF), M3 (3′OHF), and M4 (2′OHF), as mentioned above (Figure 2A–2C and 2D–2F). As reported previously (Nagayoshi et al., 2019a), HH54 microsomes produced mono-hydroxylated products, OHFM6, OHF9, OHFM10, and OHFM11 (2D-2F), with the former three products showing similar chromatographic patterns of M2, M3, and M4, respectively, when determined at *m/z* 239>121 (2A-2B). The fragment ion spectra (2K-2L) of these three products were very similar to the spectra of M2, M3, and M4 (Figure 2H–2J). OHFM11 (retention time 7.05 min), on the other hand, was not detected on incubation of three MeFs with P450 enzymes and the fragment ion spectrum of this product (Figure 2N) was different from those of M2, M3, and M4. Small amounts of formation of diOHFM4, suggested to be M1 (3′4′diOHF) by analyzing *m/z* 255>121, were detected on incubation of flavone with human liver microsomes. Sample HH2 liver microsomes were found to produce very little OHFM6, OHFM9, and diOHFM4, and this was also in the case for sample HH54 liver microsomes in the presence of anti-CYP2A6 IgG (2E).

Formation of mono-hydroxylated products of flavone by nine human P450s

Nine forms of human P450 (eight P450s, two variants of CYP1B1) were examined to catalyze flavone to mono-oxygenated products on analysis at *m/z* 239>121 (A-I) and *m/z* 239>129 (J-R) (Figure 3). Detection at *m/z* 239>121 was sensitive for products hydroxylated on the B-ring, while the *m/z* 239>129 showed preference for products oxidized on the A-ring (*vide infra*).

The results showed that CYP2A6 was highly active in oxidizing flavone to produce OHFM6 (4′OHF) and OHFM9 (3′OHF) but not in forming OHFM10 (2′OHF) (Figure 3E). Formation of OHFM11 was catalyzed by P450s, including CYP1A1, 1A2 1B1.1, 1B1.3, 2A6, and 2A13 (Figure 3A, C, D, and E). CYP2B6, 2C9, and 3A4 were not very active in oxidizing flavone. By analyzing the *m/z* 239>129 traces, we found that OHFM7 formation (suggested to result from oxidation of the A-ring) was catalyzed by CYP1A1, 1B1.1, 1B1.3, and 2A6 (Figure 3J, 3L, 3M, and 3N). Relatively minor products (OHFM5, 6OHF, and OHFM12) were also detected in these P450 enzymes studied.

Eight mono-hydroxylated products were obtained in P450 enzyme and liver microsomal incubations, as judged by analysis with *m/z* 239>121 and 239>129 (Figure 3). We characterized fragment ion spectra of these products (Figure 4). OHFM5, M7, and M12 (as well as 6OHF)—suggested to be products oxidized on the A-ring—typically had *m/z* 129 and *m/z* 137 fragments in their spectra (the proposed structures of these fragments were described in Figure 4A). On the other hand, other products of OHFM6 (4′OHF), M9

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(3′OHF), M10 (2′OHF), and M11 contained *m/z* 119 and *m/z* 121 fragments, suggestive of products oxidized on the B-ring (proposed structures of these fragments are described in Figure 4B).

Formation of di-hydroxylated products of flavone by human P450s and liver microsomes

Three different di-hydroxylated products (diOHFM3, diOHFM4, and diOHFM5) were determined by analyzing the *m/z* 255>129 and *m/z* 255>153 fragments by LC-MS/MS (Figure 5). (The results with CYP2A13, 2B6, 2C9, and 3A4 are not presented because of low activities with these enzymes.) Fragment ion spectra of 3′4′diOHF (Figure 5I) and 57diOHF (5M) standards are shown for comparison. The major product diOHFM5 was found in these P450s and liver microsomes, and fragment ion spectra (Figure 5L) were similar to that of 57diOHF (Figure 5M). The product diOHFM4 (which was found in CYP1A2, CYP2A6, and HH54 microsomal incubations at significant levels and in CYP1A1, HH54 (in the presence of anti-2A6 IgG), and HH2 at lower levels) had a retention time of 4.4 min on LC-MS/MS. The fragment ion spectra were similar to 3′4′diOHF (Figure 5), as in the cases of oxidation of 4′MeF, 3′MeF, and flavone described above (Figure 2). Other product, diOHFM3, was found to be high in CYP1A2 incubations, and its fragment ion spectra differed from diOHFM4 and diOHFM5 (Figure 5J).

Comparison of catalytic activities of oxidation of flavone by P450s and liver microsomes.

We have three standard hydroxylated flavones, 4′OHF, 6OHF, and 3′4′diOHF, available in this study and were able to compare turnover numbers (nmol products formed/min/nmol P450) with human P450s and liver microsomes (Table 1). Among the nine forms of P450 enzymes examined, CYP2A6 had the highest activity in catalyzing flavone to form 4′OHF. In addition, liver microsomes from sample HH2 showed 14-fold lower activity for formation of 4′OHF than did HH54 microsomes (Table 1). Formation of 6OHF was catalyzed by CYP2A6, 1A1, CYP1B1.1, 1B1.3, 1A2, and 2A13 and liver microsomes, but the activities were <0.07 nmol/min/nmol P450 with these enzymes. CYP1A2 and 2A6 catalyzed formation of 3′4′diOHF at rates of 0.033 and 0.020 nmole/min/nmol P450, respectively, which were higher than those catalyzed by HH54 and HH2.

Effects of anti-CYP2A6 IgG and ANF and coumarin on oxidation of flavone by human liver microsomes.

The above results collectively suggested that CYP2A6 is the major enzyme catalyzing oxidation of flavone to form OHFM6 (4′OHF), OHFM9 (3′OHF), and diOHFM4 (3′4′diOHF) in human liver microsomes. The effects of anti-CYP2A6 IgG on oxidation of flavone by human liver microsomes were examined (Figure 6). The formation of OHFM6 and OHFM9 was markedly inhibited by anti-CYP2A6 in HH54 liver microsomes, but formation of OHFM10 and M11 was not inhibited as much (Figure 6A). The formation of other mono-hydroxylated products (e.g., OHFM5, M7, M12, and 6OHF) was only weakly inhibited by anti-CYP2A6 (Figure 6B).

Anti-CYP2A6 also inhibited the formation of diOHFM4 (3′4′diOHF), but not diOHFM3 and diOHFM5 (Figure 6C); however, inhibition of diOHFM4 formation was not as

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much compared with that seen with OHFM6 and OHFM9, particularly at lower antibody concentrations (Figure 6A).

The above results suggested that CYP1A2, as well as CYP2A6, plays an important role in oxidation of flavone by human liver microsomes (Figures 3 and 4). In order to characterize these enzymes in the reactions, ANF and coumarin, known to be specific inhibitors for CYP1 and CYP2A6 enzymes, respectively (Shimada, 2006; 2017; Guengerich, 2015), were used to examine roles of these enzymes in the oxidation of flavone by sample HH47 liver microsomes (Figure 7). Formation of OHFM6 and OHFM9 was strongly inhibited by coumarin but not by ANF. In contrast, the formation of OHFM5, OHFM7, 6OHF, and OHFM12 was inhibited only by ANF (Figure 7). Coumarin and ANF inhibited formation of diOHFM4 (3′4′diOHF) by 75% and 65%, respectively, in human sample HH54 liver microsomes of (results not shown).

Oxidation of 4′OHF to 3′4′diOHF by P450 enzymes and human liver microsomes

Although these results suggested that CYP2A6 is a major enzyme in catalyzing the formation of OHFM6 (4′OHF) and OHFM9 (3′OHF) forom flavone, it was not clear which P450 enzymes are involved in the oxidation of 4’OHF to 3′4′diOHF in human liver microsomes. We examined the oxidation of 4′OHF by P450s and human liver microsomes, analyzing formation of 3′4′diOHF (Figure 8). The catalytic activity of CYP2A6 (0.06 nmol/min/nmol P450) in forming 3′4′diOHF was much lower than that observed with CYP1A2 (0.46 min^{-1}) and 2A13 (0.21 min^{-1}) and also by liver microsomes of both the HH54 (0.16 min^{-1}) and HH2 (0.16 min^{-1}) samples (Figure 8).

A minor role for CYP2A6 in the oxidation of 4′OHF was also supported by the experiments of oxidation of 4′MeF and 3′MeF by CYP2A6 and liver microsomes (Figure 9) (Nagayoshi et al., 2019). CYP2A6 catalyzed *O*-demethylation of 4′MeF to form M2 (4′OHF) and then M1 (3′4′diOHF) at rates of 0.029 min^{-1} and 0.0004 min^{1}, respectively, but these activities were much lower than those catalyzed by HH54 (0.76 and 0.06 min^{-1}, respectively) and HH2 (0.63 and 0.06 min^{-1}, respectively) liver microsomes (Figure 9A–9C). CYP2A6 was also found to have only very low activity in oxidizing 3′MeF to form 3′4′diOHF, at a rate of 0.0007 min^{-1}, as compared with by the HH54 (0.084 min^{-1}) and HH2 (0.12 min^{-1}) microsomal samples (Figure 9D–9F).

Molecular docking analysis of interaction of flavone with CYP2A6

The interaction of flavone with the active site of CYP2A6 was examined using molecular docking analysis. We simulated the interactions between flavone and the active site of CYP2A6 by determining ligand-interaction energies (U values) with the program ASEdock in MOE and obtained 143 cases with different U values (in the figure, only five cases are shown). (Figure 10). The results showed that the C4′- and C3′-moieties of flavone were interfaced to the active site of CYP2A6 with the lowest stabilizing energy ($U = -26.0$) and most significant interaction was obtained (Figure 10A). The distances between iron center of CYP2A6 and C4′ and C3′ were calculated to be 6.44 and 6.77 Å, respectively.

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Discussion

Plant P450 enzymes, e.g. CYP73, CYP84, and CYP98, catalyze reactions in phenylpropanoid pathways, leading to the formation of phenolic compounds, including UV protectants, antioxidants, and antimicrobials (Wei and Chen, 2018). Members of the CYP75A and CYP75B P450 Sub-families play roles in flavonoid biosynthetic pathways leading to flower colors in plants (Tanaka et al., 2010; Tanaka and Brugliera, 2013). Mammalian P450 enzymes are also reported to convert various flavonoids to biologically active compounds as well as chemically inert products (Breinholt et al., 2002; Hodek et al., 2002; Nagayoshi et al., 2019; Nikolic et al., 2004; Schlupper et al., 2006; Walle and Walle, 2007). Recent studies have established that sequential oxidation of flavonoids by P450s produces more biologically active products, such as 3′4′diOHF, 57diOHF (chrysin), 4′,5,7-trihydroxyflavone (apigenin), 5,6,7-trihydroxyflavone (baicalein), 3′,4′,5,7-tetrahydroxyflavone (luteolin), 3,3′,4′,5,7-pentahydroxyflavone (quercetin), and 3′,4′,5,6,7,8-hexahydroxyflavone (noblletin) (Schlupper et al., 2006; Kim et al., 2018; Samarghandian et al., 2017; Farkhondeh et al., 2019; Patel et al., , 2007; Nabavi et al., 2015; Madunić et al., 2018; Li et al., 2012; Liu et al., 2016; Surichan et al., 2018). It is of interest to know why such sequential oxidation of flavonoids by P450 enzymes produces more biologically active compounds in mammals as well as in plants.

By comparing products formed through oxidation (*O*-demethylation) of 4′-, 3′-, and 2′-MeF by P450 enzymes, we found that human liver microsomes catalyzed oxidation of flavone to form several mono- and di-hydroxylated products, including 4′-, 3′-, and 2′-OHF, and 3′4′diOHF on LC-MS/MS analysis (Figures 2–5). Among nine forms of human P450s examined, CYP2A6 was found to be a major enzyme in producing OHFM6 (4′OHF) and OHFM9 (3′OHF) in human liver microsomes. Formation of these products was very low in microsomes from human liver sample HH2, which was defective in CYP2A6-dependent coumarin 7-hydroxylation activity. The activity was strongly inhibited by the CYP2A6-inhibitor coumarin and by anti-CYP2A6 IgG in human samples HH47 and HH54, which had normal coumarin 7-hydroxylation activities. Formation of OHFM10 (2′OHF) was catalyzed by different P450s, including CYP1A1, 1B1.1, 1B1.3, 2A6, 2A13, and 2C9, but was suggested to be low when compared with other mono-hydroxylated products in their peak intensities (Figure 3). CYP2A6 also oxidized flavone to produce OHFM7, 6OHF, OHFM11, and OHFM12 but did not play significant roles, based on the effects of anti-2A6 IgG and coumarin (Figures 6 and 7).

Although the Family 1 CYP enzymes (CYP1A1, 1A2, 1B1.1, and 1B1.3) were found to catalyze the oxidation of flavone to several mono-hydroxylated products, the rates were lower than by CYP2A6, except that CYP1A2 was more active in forming OHFM5 than CYP2A6 (Figure 3). ANF, a known CYP1 enzyme inhibitor, suppressed the formation of OHFM5, OHFM7, 6OHF, and OHFM12 in human liver microsomes, indicating that CYP1 enzymes, as well as CYP2A6, play catalytic roles in these flavone oxidation reactions. Only traces of CYP 1A1 and 1B1 are present in liver (Guengerich 2015), and the Family 1 activity in liver microsomes is largely due to CYP1A2.

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Anti-CYP2A6 IgG and coumarin inhibited formation of diOHFM4 (3′4′diOHF) in human liver microsomes and these results indicate that CYP2A6 is also the major enzyme involved in this reaction. However, CYP2A6 did not have high activity in catalyzing the oxidation of 4′OHF to 3′4′diOHF, indicating that suppression of formation of diOHFM4 (3′4′diOHF) by anti-2A6 IgG and coumarin was due to inhibition of the first step, i.e. oxidation of flavone to form OHFM6 and OHFM9 (4′OHF and 3′OHF). The formation of diOHFM4 (3′4′diOHF) was catalyzed by CYP1A2 and CYP2A13 at much higher rates than CYP2A6 in reconstituted monooxygenase systems, and ANF inhibited the formation of diOHFM4 (3′4′diOHF) in human liver microsomes. These results indicate that flavone is first oxidized to 4′OHF and 3′OHF by CYP2A6 and that these products are oxidized to 3′4′diOHF by other P450 enzymes, particularly CYP1A2 and 2A13.

The ionization of flavonoid molecules in mass spectrometry produces various fragment ions through dehydration, losses of carbon monoxide, and fission at C-ring bonds, namely retro Diels-Alder cleavage, and these fragments can be used for the analysis of chemical structures of flavonoids and their metabolites (Heiden and Phillips, 1992; Nikolic and Breeman, 2004; Tsimogiannis et al., 2007; Sasaki et al., 1966). Our studies showed that eight mono-hydroxylated products of flavone determined thus far can be classified into (at least) two types, oxidation on the A- and B-rings. OHFM5, OHFM7, and OHFM12 (as well as 6OHF) had typical *m/z* 129 and *m/z* 137 fragments in their spectra and are suggested to be the products oxidized on the-ring. However, OHFM6 (4′OHF), M9 (3′OHF), M10 (2′OHF), and M11 (containing *m/z* 119 and *m/z* 121 fragments) were classified as products oxidized on the B-ring. In addition, fragment ion spectra showed that diOHFM4 can be characterized as 3′4′diOHF, by comparison of spectra with a standard material, and that diOHFM5 was similar in its spectra to standard 57diOHF (Figure 5).

Molecular docking analysis supports the preference of interaction of the C4′ and C3′ moieties of flavone with active site of CYP2A6, resulting in the initial formation of 4′OHF and 3′OHF, respectively. By analyzing 143 cases with different ligand-interaction energies (*U* values), we determined the possible interaction of flavone with the enzyme in the lowest stabilizing energy ($U = -26.0$) and found that the distance between C4′ and C3′ moieties of flavone with iron center of CYP2A6 were 6.44 and 6.77 Å, respectively (Figure 9A). It should be mentioned that we used the crystal structure of CYP2A6 (bound to 4,4′-dipyridyl disulfide) for molecular docking analysis, and our results might be affected when other crystal structures of CYP2A6 are used (Sheng et al., 2014).

Pathways for the formation of 3′4′diOHF formed in incubations of flavone, 4′MeF, and 3′MeF with human liver microsomes can be proposed (Figure 11). Flavone is first oxidized to 4′- and 3′-OHF mainly by CYP2A6 and these hydroxylated products are further oxidized to 3′4′diOHF by P450 enzymes, e.g. CYP1A2 and 2A13, in liver microsomes, although the level of CYP 2A13 in liver is rather low in comparison to CYP1A2 (Su et al. 2000;Guengerich 2015). After 4′MeF and 3′MeF are oxidized to demethylated products (4′OHF and 3′OHF, respectively), CYP1A2 and possibly 2A13 may also play significant roles to produce 3′4′diOHF in liver microsomes (Nagayoshi et al., 2019). As has been reported previously, CYP2A6 has low catalytic activity in the oxidation (*O*-demethylation) of 4′MeF and 3′MeF to 4′OHF and 3′OHF (Nagayoshi et al., 2020). Although 2′MeF

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was also oxidized (demethylated) by human P450s to form 2′OHF (Figure 2C and 2J), the formation of di-hydroxylated products was found to be low (Figure 2) (Nagayoshi et al., 2020).

Finally, it is of interest to note the results of Das and Griffiths (1966) who showed that oral administration of 30 mg of flavone to guinea pigs resulted in urinary excretion of 622, 309, 15, and 0 μg of 4′OHF on the first, second, third, and fourth days, respectively, following administration of flavone. They also showed that small amounts of 3′4′diOHF were found in urine after oral or intraperitoneal administration of flavone to rats. Excretion of hydroxylated flavones was not suppressed by high doses of antibiotics, indicating that microflora do not support the excretion of flavone (Das and Griffiths (1966). The biological significance of 3′4′diOHF in experimental animal models has been reported in several laboratories (Schlupper et al., 2006; Kim et al., 2018).

In conclusion, we studied the roles of CYP2A6 and other human P450s in the oxidation of flavone and other flavonoids in human liver microsomes by analyzing product formation with LC-MS/MS. By comparing the products formed through metabolism of these methoxylated flavones by P450 enzymes, CYP2A6 was found to be a major enzyme in catalyzing flavone 4′- and 3′-hydroxylations but it does not play a significant role in 2′-hydroxylation of flavone, the *O*-demethylations of three methoxylated flavones, and the oxidation of 4′OHF to 3′4′diOHF. The effects of anti-CYP2A6 IgG and chemical P450 inhibitors suggest that different P450 enzymes, as well as CYP2A6, play significant roles in the oxidation of flavone.

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Abbreviations used,

P450 or CYP
cytochrome P450

b **5**
cytochrome b_5

4′OHF
4′-hydroxyflavone

3′OHF
3′-hydroxyflavone

2′OHF
2′-hydroxyflavone

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6OHF
6-hydroxyflavone

3′4′diOHF
3′,4′-dihydroxyflavone

57diOHF
5,7-dihydroxyflavone

4′MeF
4′-methoxyflavone

3′MeF
3′-methoxyflavone

2′MeF
2′-methoxyflavone

ANF
α-naphthoflavone

DLPC
L-α-1,2-dilauoryl-*sn*-glycero-3-phosphocholine

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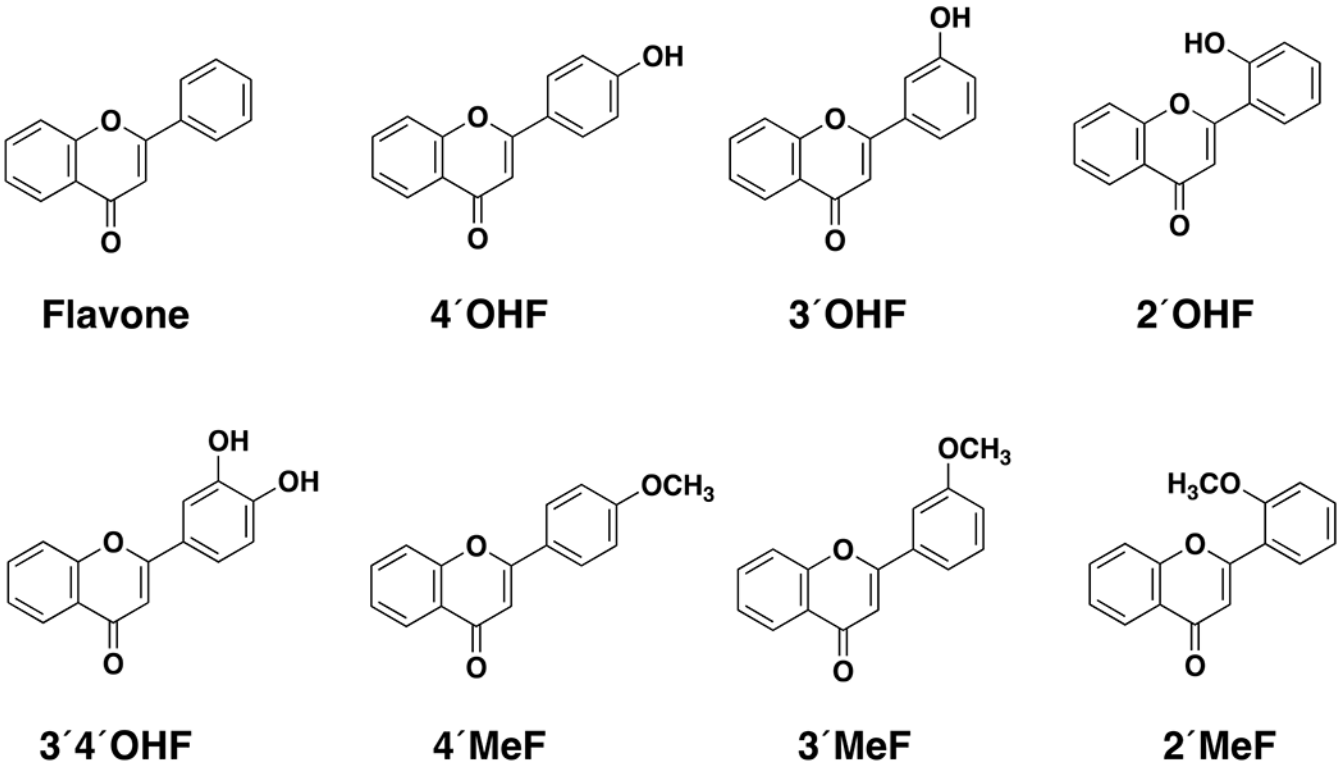

Figure 1.
Chemical structures of flavonoids studied in this study.

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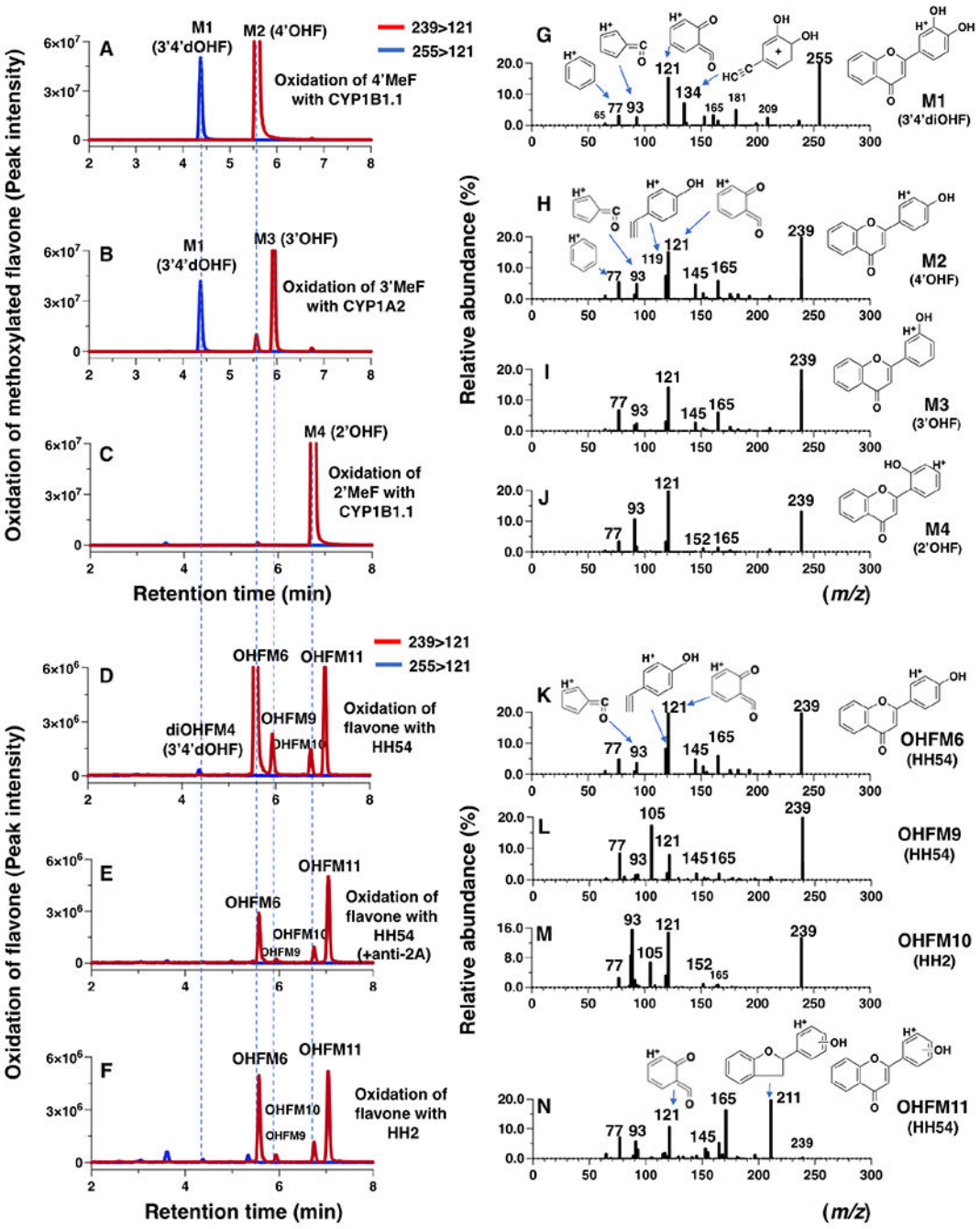

Figure 2.

LC-MS/MS analyses of oxidation of 4´MeF by CYP1B1.1 (A), 3´MeF by CYP1A2 (B), and 2´MeF (C) by CYP1B1.1 in reconstituted monooxygenase systems. The fragment (product) ion spectra of these products are shown in Figures 2G–2J. LC-MS/MS analysis of flavone metabolites was also examined with liver microsomes of human sample HH54 in the absence (D) and presence (E) of anti-CYP2A6 IgG and human sample HH2 liver microsomes (F). The fragment (product) ion spectra of OHFM6 (K), OHFM9 (L), OHFM10 (M), and OHFM11 (N) are shown in the figure.

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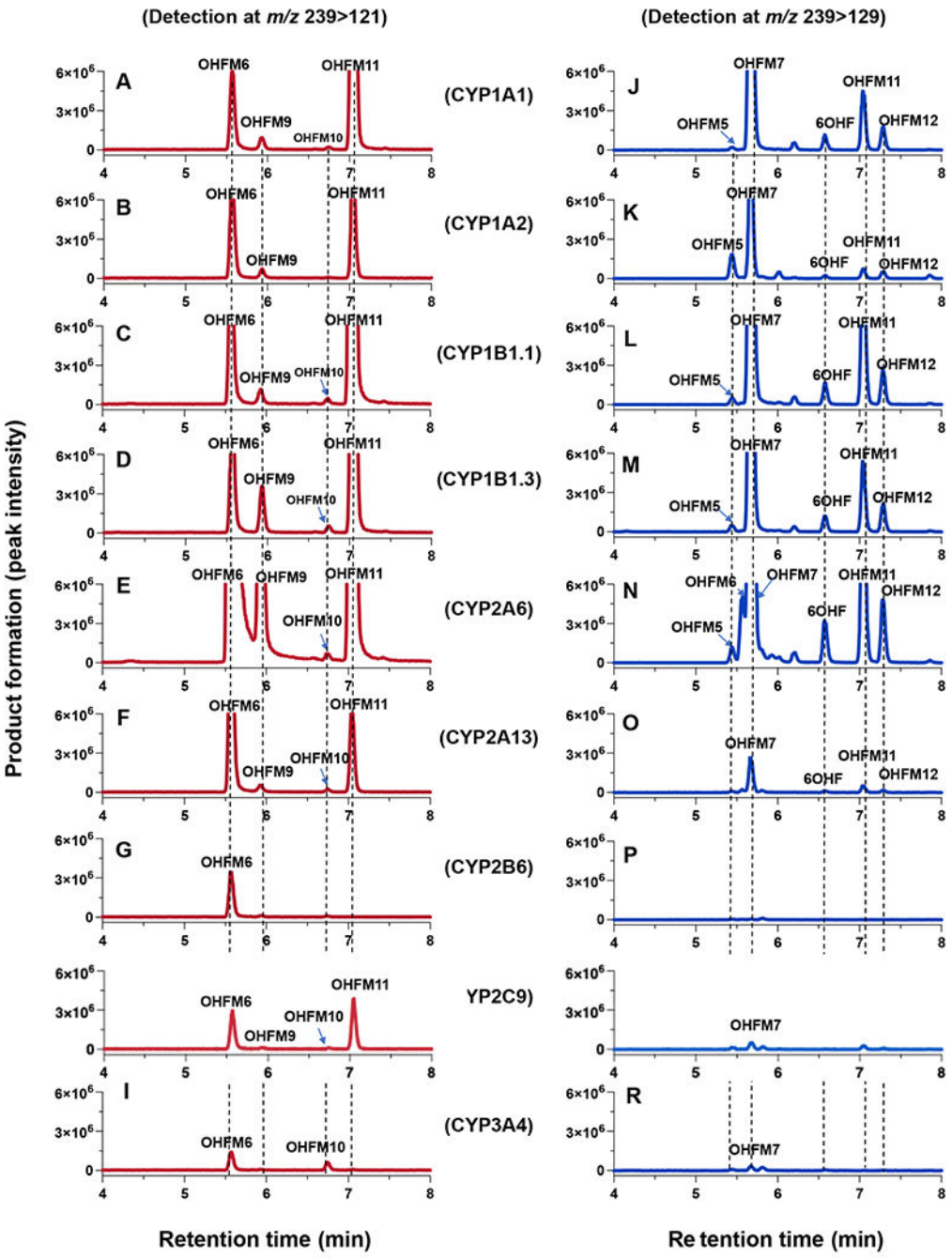

Figure 3.

Oxidation of flavone to form mono-hydroxylated products by CYP1A1 (A and J), CYP1A2 (B and K), CYP1B1.1 (C and L), CYP1B1.3 (D and M), CYP2A6 (E and N), CYP2A13 (F and O), CYP2B6 (G and P), CYP2C9 (H and Q), and CYP3A4 (I and R).

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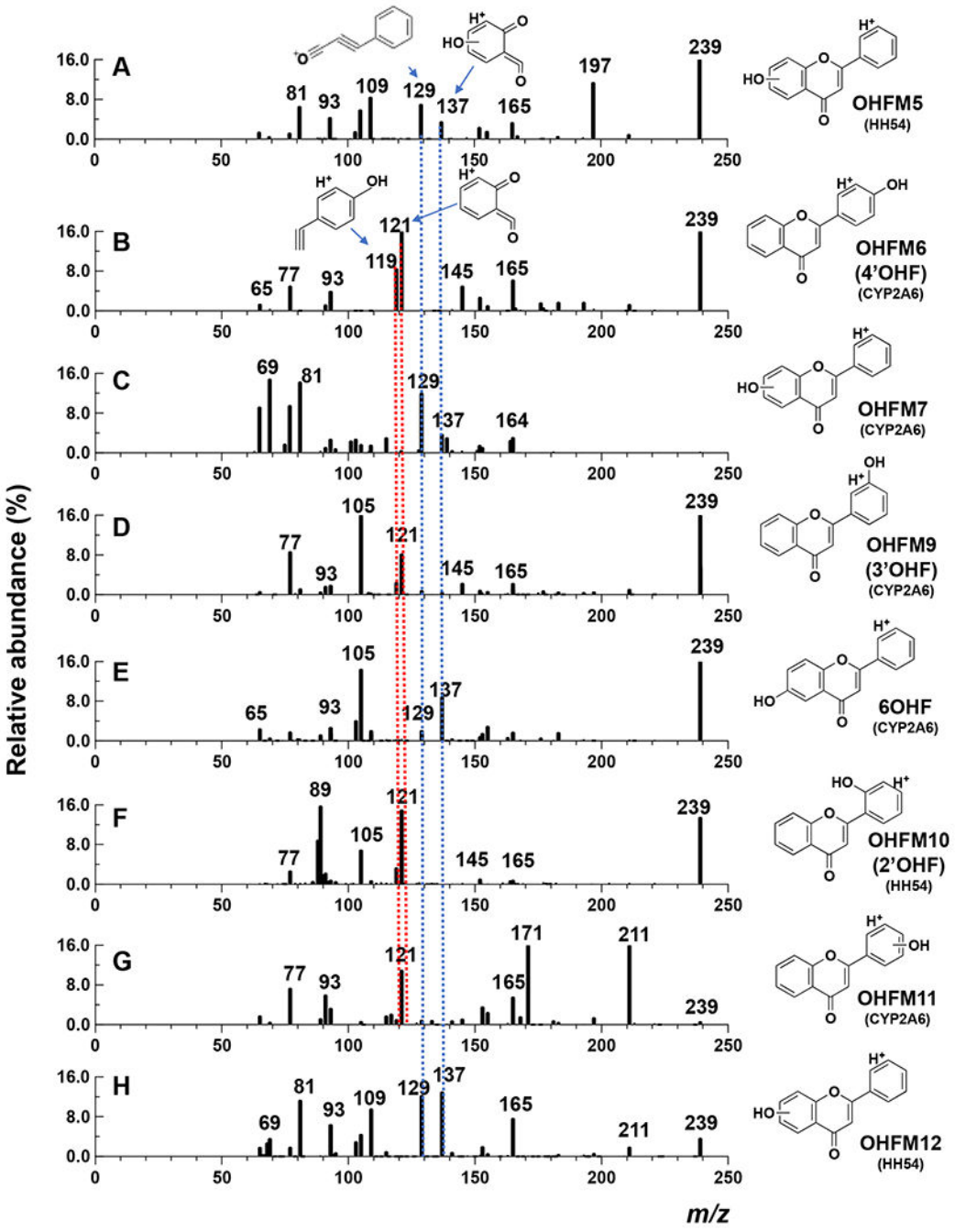

Figure 4.

Fragment ion spectra of mono-hydroxylated products of flavone formed by CYP2A6 and human sample HH54 liver microsomes.

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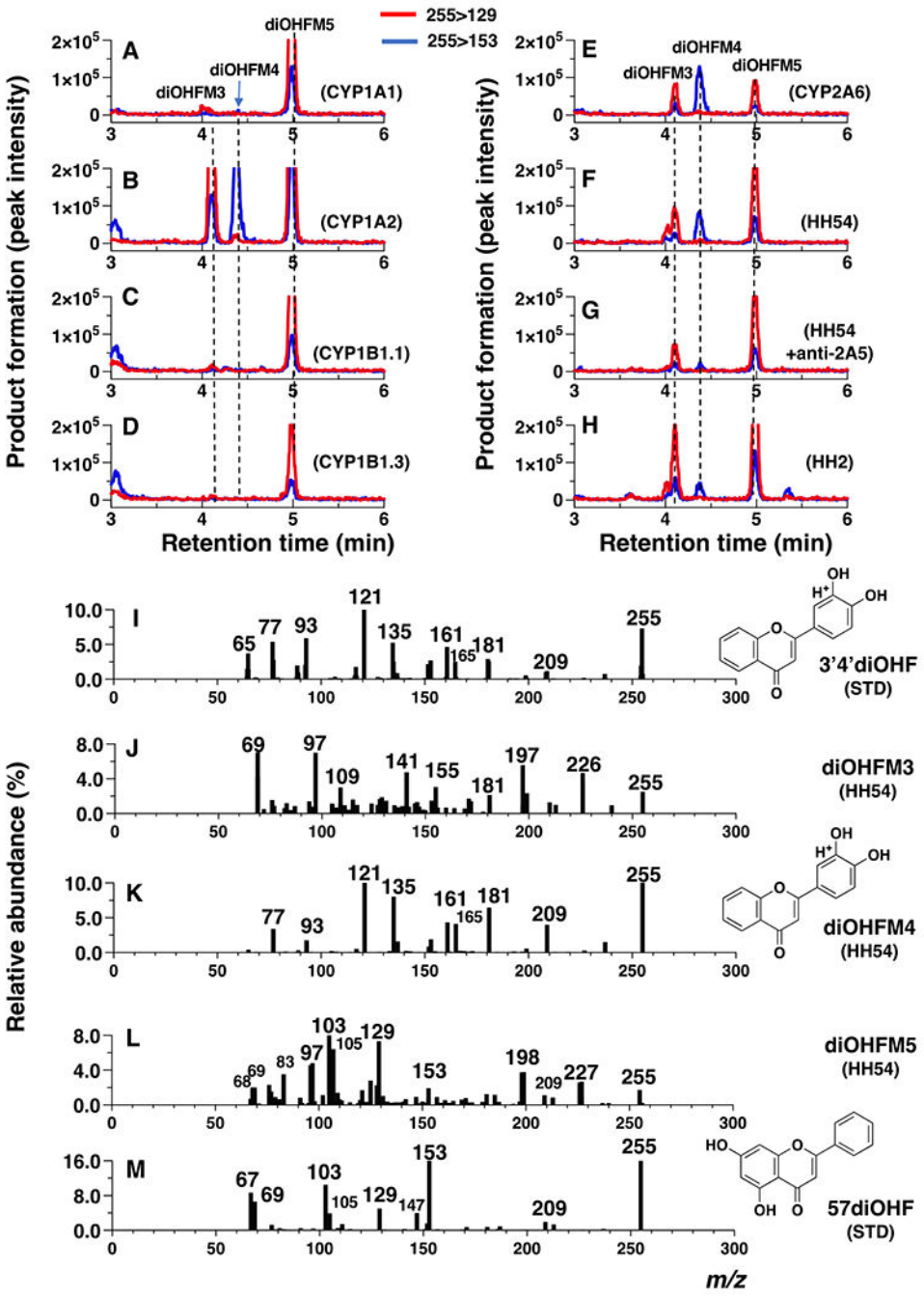

Figure 5.

Oxidation of flavone to form di-hydroxylated products by CYP1A1 (A), 1A2 (B), 1B1.1 (C), 1B1.3 (D), and 2A6 (E) and by liver microsomes from sample HH54 (F), HH54 with anti-CYP2A6 IgG (G), and HH2 (H). The products diOHFM3, M4, and M5 formed were analyzed with the *m/z* 255>129 and *m/z* 255>153 transitions. The fragment ion spectra of these products (J, K, and L) and the standards 3´4´diOHF (I) and 57diOHF (M) are shown in the figure.

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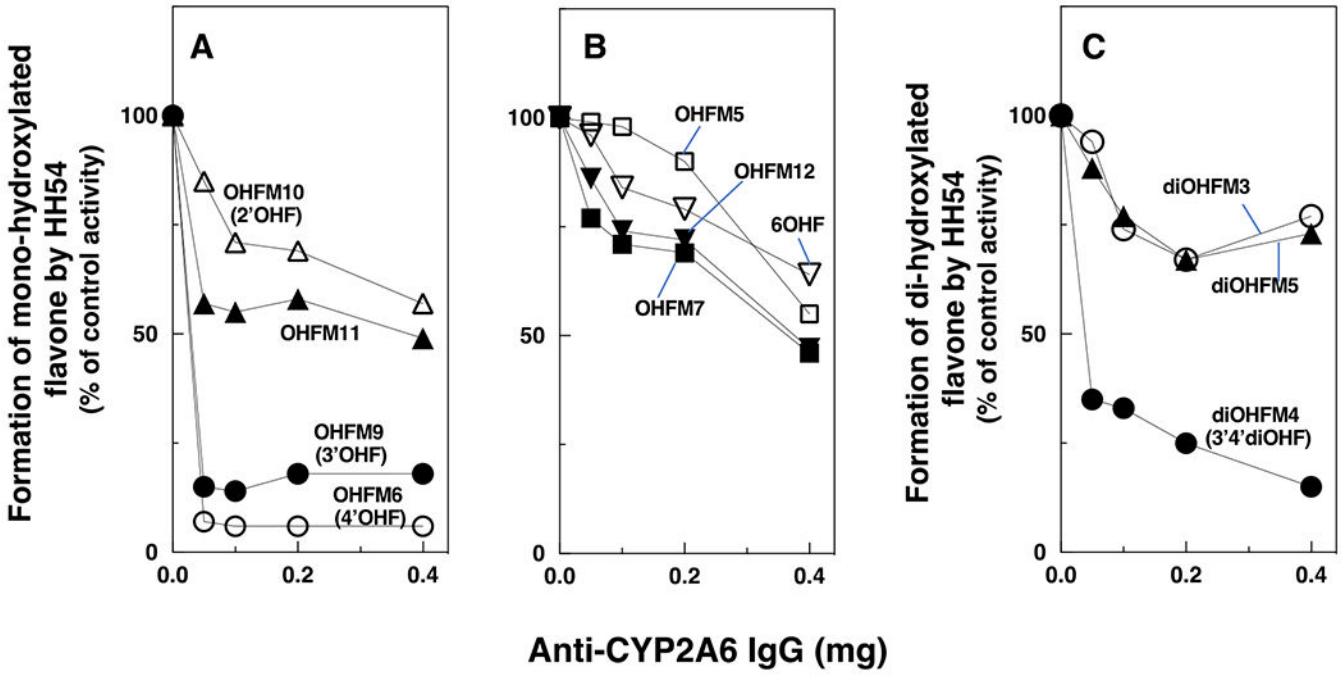

Figure 6.

Effect of anti-CYP2A6 IgG on oxidation of flavone to mono-hydroxylated products (A and B) and di-hydroxylated products (C) by liver microsomes of HH54. The mono-hydroxylated products were measured using the *m/z* 239>121 (A) or *m/z* 239>129 transition (B) and the di-hydroxylated products with *m/z* 255>129 (for diOHFM3 and M5) and *m/z* 239>153 transitions (for diOHFM4, 3´4´diOHF) (C).

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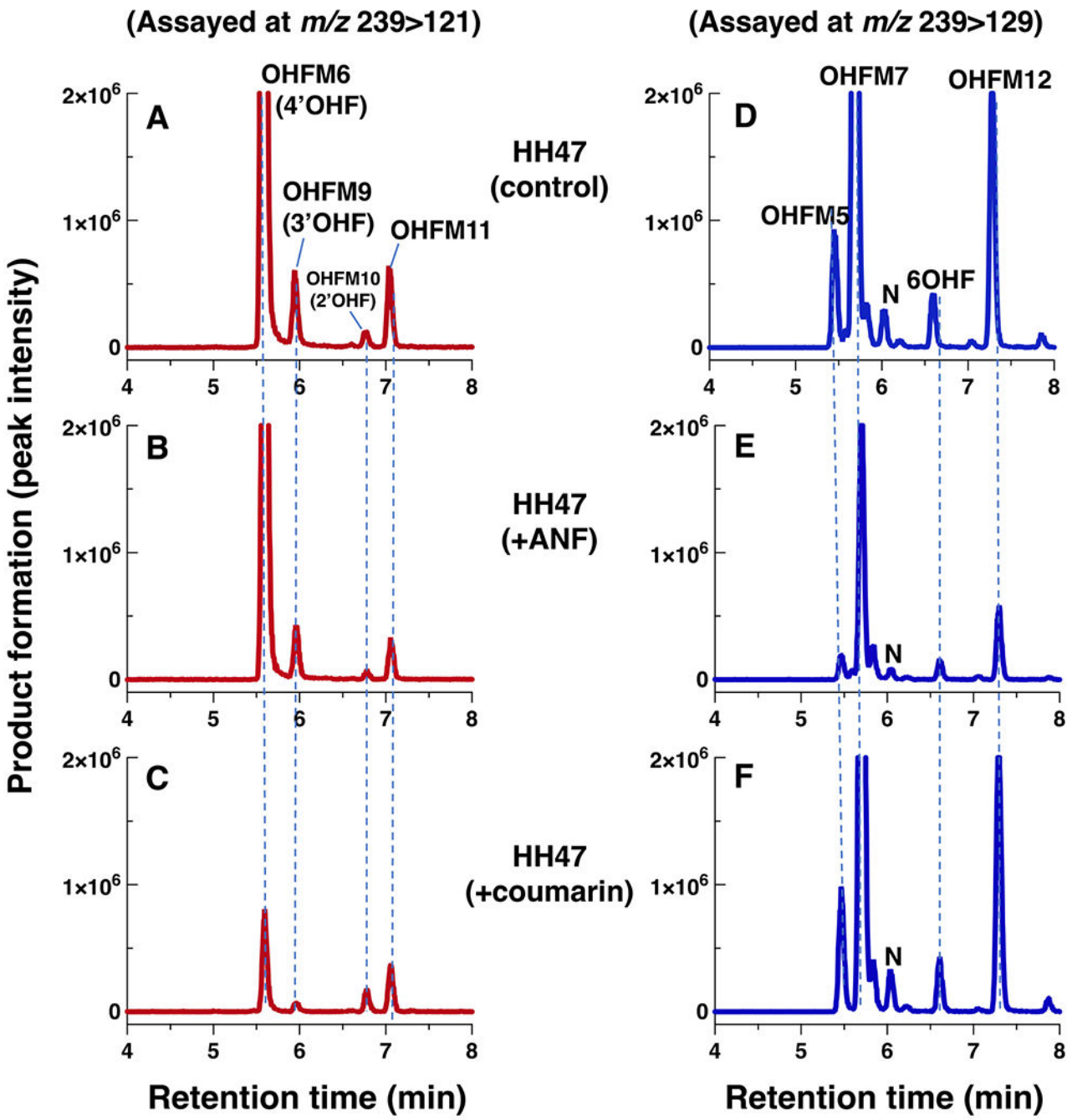

Figure 7.

Effects of ANF (B and E) and coumarin (C and F) on oxidation of flavone to form mono-hydroxylated products catalyzed by sample HH47 liver microsomes (control reaction without inhibitors, Figure 7A and 7D). The concentrations of inhibitors and the substrate flavone used were 10 μM and 60 μM, respectively, and product formation was determined at *m/z* 239>121 (A-C) and at *m/z* 239>129 (D-F). N, non-identified peak.

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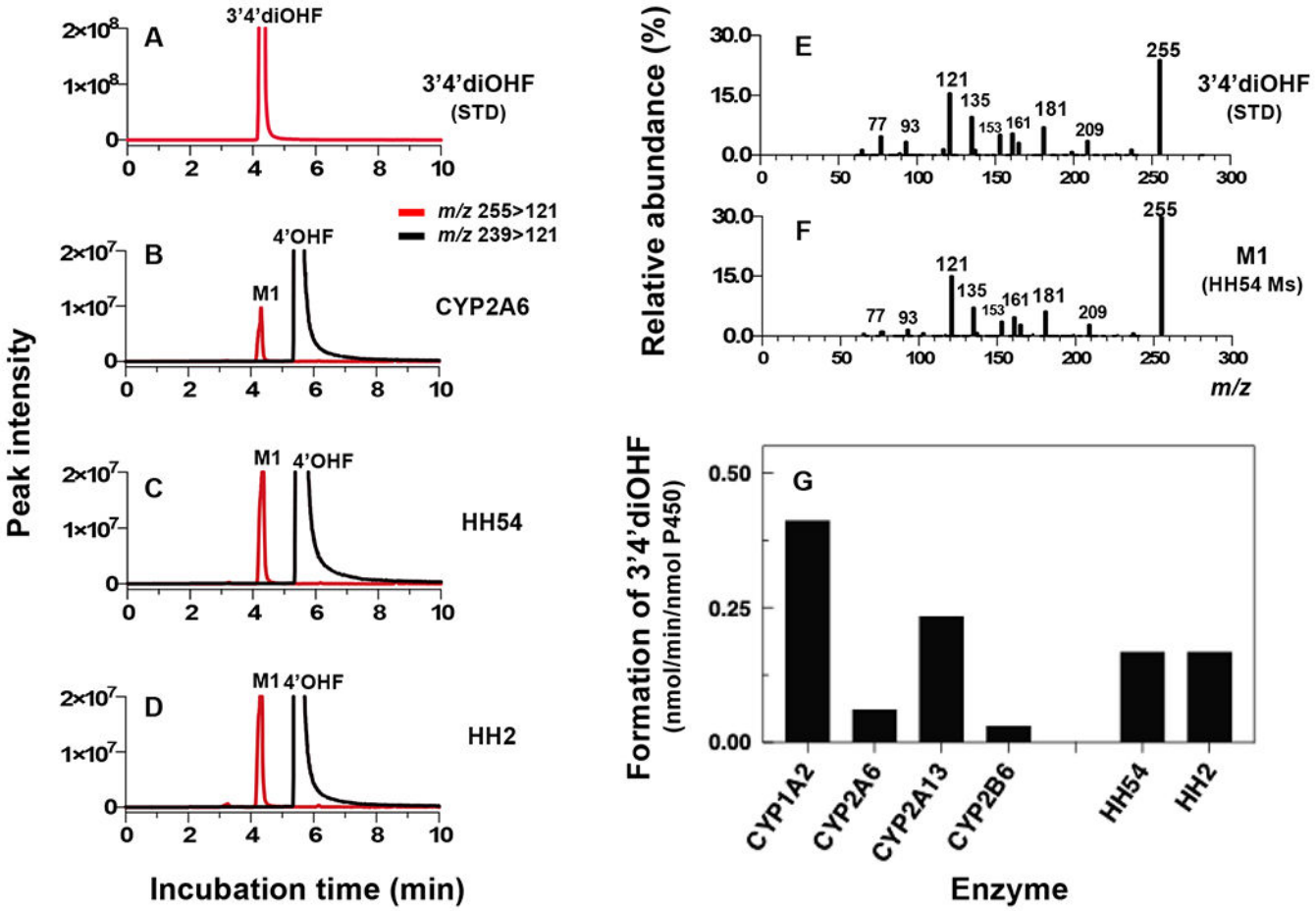

Figure 8.

LC-MS/MS analysis of oxidation of 4´OHF to form 3´4´diOHF by CYP2A6 (A) and by liver microsomes of human samples HH54 (C) and HH2 (D). The standard spectrum of 3´4´diOHF is shown in Figure 8A. The fragment ion spectra of a standard 3´4´diOHF and M1 (from HH54) are shown in Figures 8E and 8F, respectively. The turnover numbers (nmol 3´4´diOHF formed/min/nmol P450) by CYP1A2, 2A6, 2A13, 2B6, HH54, and HH2 are shown in Figure 8G. Results are means of duplicate determinations.

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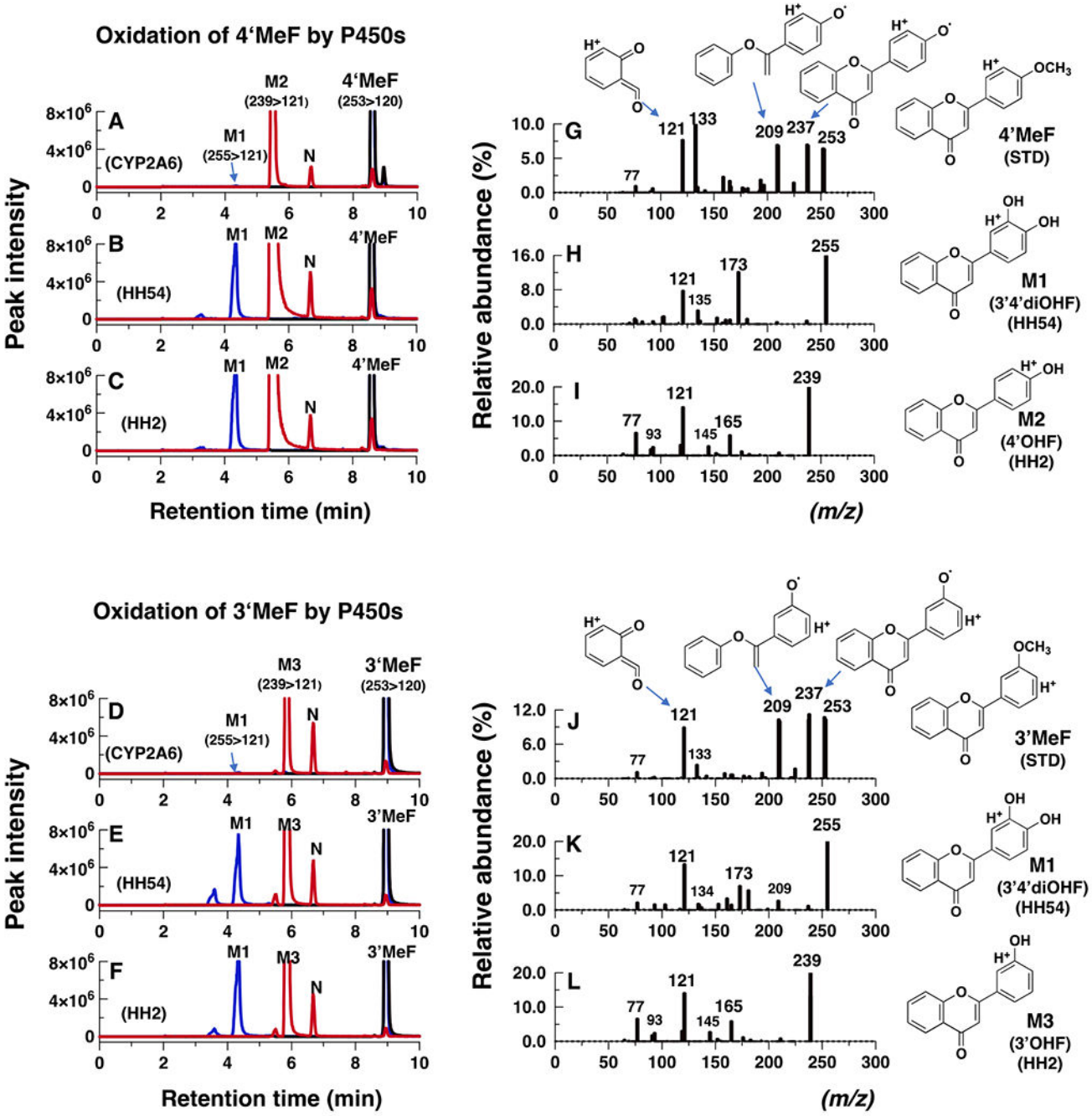

Figure 9.

Oxidation of 4´MeF (A-C, G-I) and 3´MeF (D-F, J-L) by CYP2A6 (A and D), HH54 liver microsomes (B and E), and HH2 liver microsomes (C and F). The products formed were M1 (3´4´diOHF) and M2 (4´OHF) in the case of 4´MeF and M1 (3´4´diOHF) and M3 (3´OHF) in the case of 3´MeF. Fragment ion spectra of 4´MeF (Figure 9G) and 3´MeF (9J) and their products (9H, 9I, 9K, and 9L) are shown in the figure. N, not characterized.

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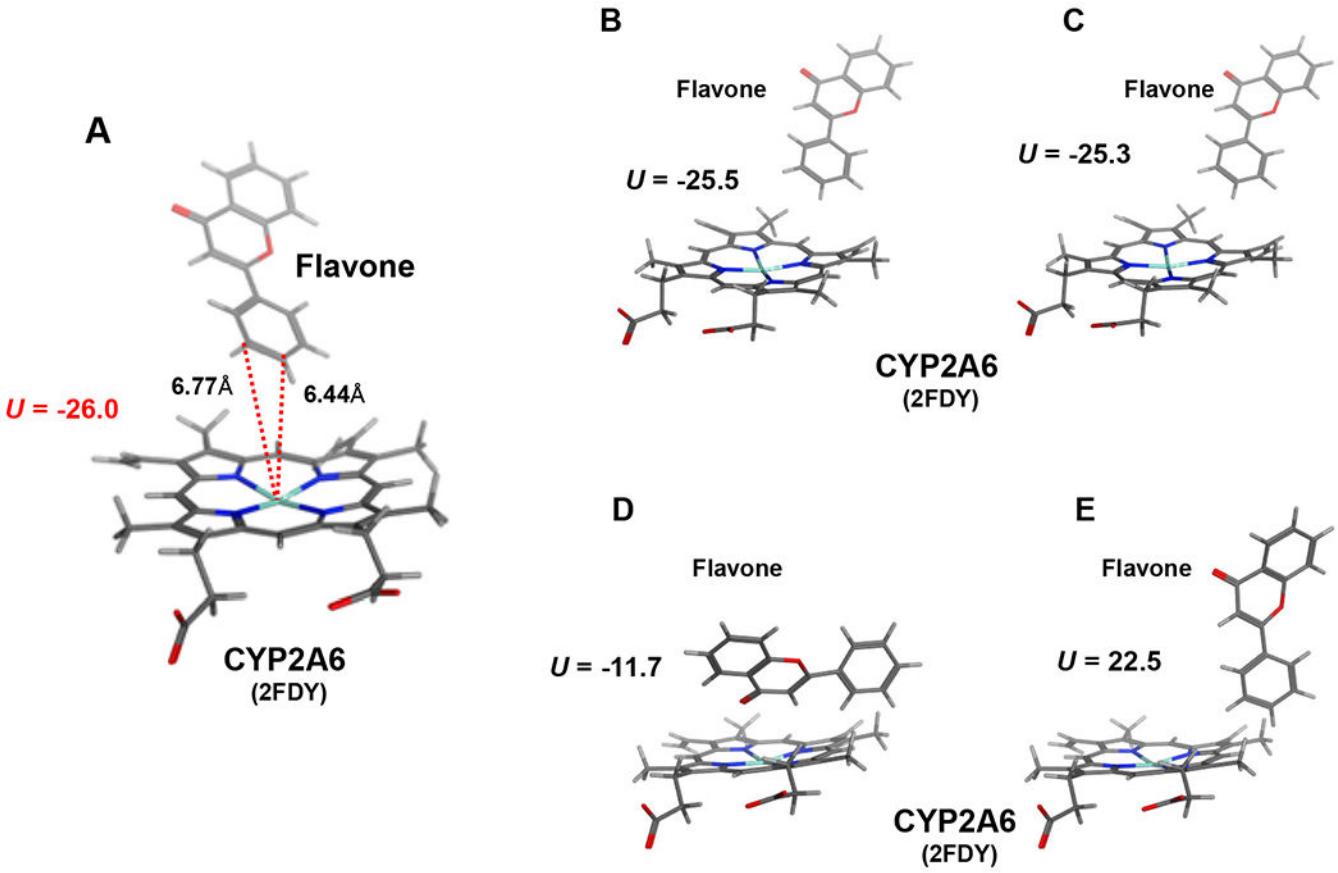

Figure 10.

Molecular docking analysis of interaction of flavone with active sites of CYP2A6. Five cases with different ligand-interaction energies (*U* values) are shown in the figure and most significant interaction was obtained in Figure 8A.

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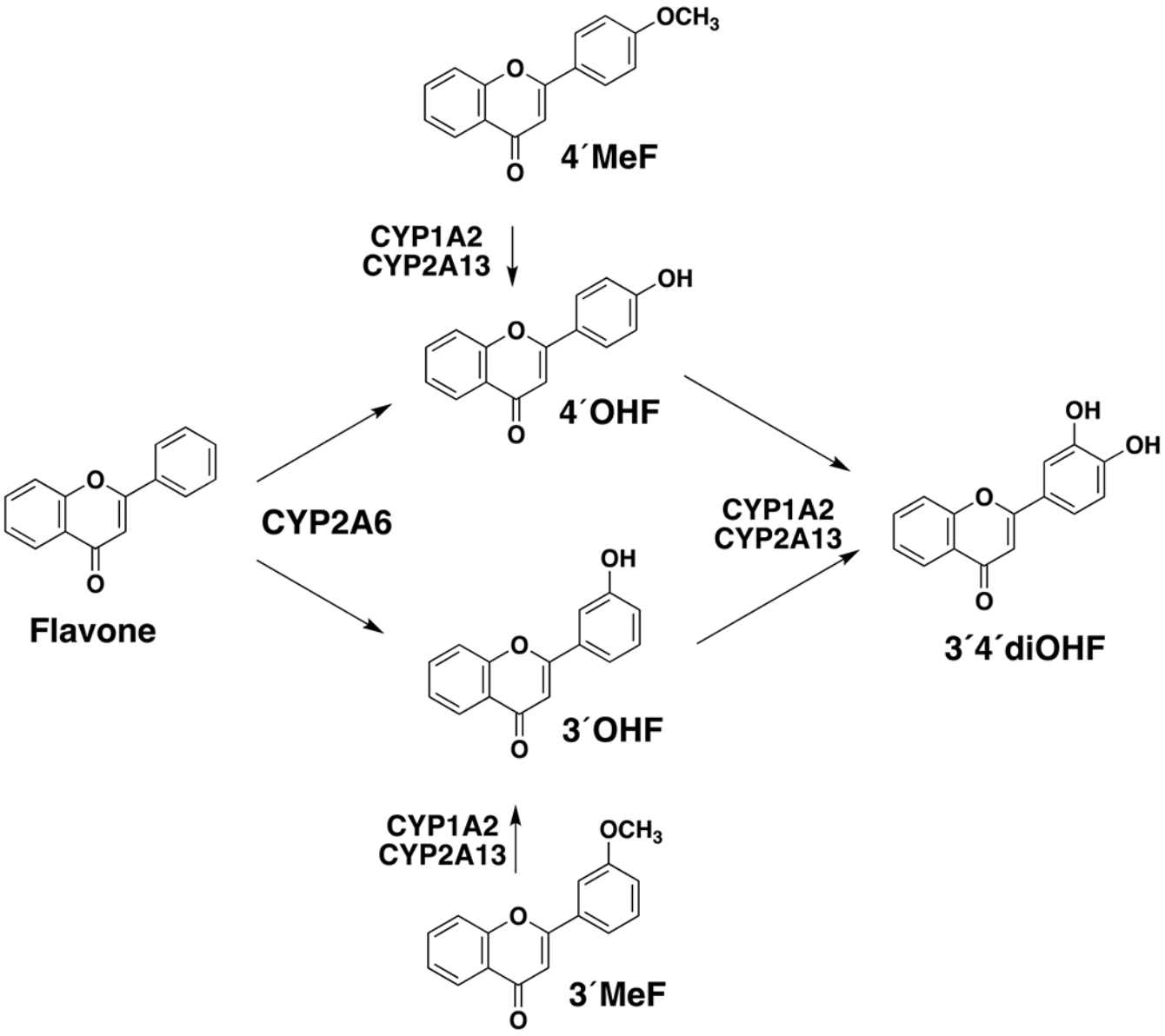

Figure 11.
Proposed pathway for oxidative metabolism of flavone, 4´MeF, and 3´MeF to form 3´4´diOHF by human P450 enzymes.

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Table 1.

Oxidation of flavone to form 6OHF, 4´OHF, and 3´4´diOHF by nine forms of human P450 enzymes and liver microsomes

Enzymes	Product formation (nmol products/min/nmol P450)		
	6OHF	4´OHF	3´4´diOHF
P450 enzymes			
CYP1A1	0.023	<0.0001	<0.0001
CYP1A2	0.006	0.020	0.033
CYP1B1.1	0.034	0.048	<0.0001
CYP1B1.3	0.026	0.026	<0.0001
CYP2A6	0.070	1.84	0.020
CYP2A13	0.003	0.053	<0.0001
CYP2B6	<0.0001	0.007	<0.0001
CYP2C9	<0.0001	0.005	<0.0001
CYP3A4	0.001	<0.0001	<0.0001
Liver microsomes			
HH54	0.031	0.136	0.009
HH2	0.022	0.010	0.004

Results presented are means and duplicate determinations and the standard errors in these values were found to be < 15% of the means.