

Mechanism of CYP2C9 Inhibition by Flavones and Flavonols

Dayong Si, Ying Wang, Yi-Han Zhou, Yingjie Guo, Juan Wang, Hui Zhou, Ze-Sheng Li, and J. Paul Fawcett

College of Life Science, Jilin University, Changchun, China (D.S., Y.W., Y.G., J.W., H.Z.); State Key Laboratory of Theoretical and Computational Chemistry, Institute of Theoretical Chemistry, Jilin University, Changchun, China (Y.-H.Z., Z.-S.L.); and School of Pharmacy, University of Otago, Dunedin, New Zealand (J.P.F.)

Received July 16, 2008; accepted December 11, 2008

ABSTRACT:

This article describes an *in vitro* investigation of the inhibition of cytochrome P450 (P450) 2C9 by a series of flavonoids made up of flavones (flavone, 6-hydroxyflavone, 7-hydroxyflavone, chrysin, baicalein, apigenin, luteolin, scutellarein, and wogonin) and flavonols (galangin, fisetin, kaempferol, morin, and quercetin). With the exception of flavone, all flavonoids were shown to inhibit CYP2C9-mediated diclofenac 4'-hydroxylation in the CYP2C9 RECO system, with K_i value $\leq 2.2 \mu\text{M}$. In terms of the mechanism of inhibition, 6-hydroxyflavone was found to be a noncompetitive inhibitor of CYP2C9, whereas the other flavonoids were competi-

tive inhibitors. Computer docking simulation and constructed mutants substituted at residue 100 of CYP2C9.1 indicate that the noncompetitive binding site of 6-hydroxyflavone lies beside Phe100, similar to the reported allosteric binding site of warfarin. The other flavonoids exert competitive inhibition through interaction with the substrate binding site of CYP2C9 accessed by flurbiprofen. These results suggest flavonoids can participate in interactions with drugs that act as substrates for CYP2C9 and provide a possible molecular basis for understanding cooperativity in human P450-mediated drug-drug interactions.

Flavonoids are polyphenolic secondary metabolites that are widely distributed in higher plants and ingested by humans in their regular food (Kuhnau, 1976; Bravo, 1998). Flavones and flavonols are two major classes of flavonoids (Table 1). Flavonols are present in a variety of fruits and vegetables, whereas flavones are mainly found in cereals and herbs (Hertog et al., 1993; Bravo, 1998; Peterson and Dwyer, 1998). In the West, the estimated daily intake of both flavonols and flavones is in the range 20 to 50 mg per day (Cermak and Wolfram, 2006). However, given the growing demand for food supplements or herbal remedies containing flavonoids, and given that in some countries flavonoids are commonly used as therapeutic agents (2008 State Food and Drug Administration RPC, <http://app1.sfd.gov.cn/datasearch/face3/dir.html>), it is likely that some individuals are exposed to relatively high levels of flavonoids. This points to a need for more information on the safety and potential toxicity of flavonoids.

In the early 1980s, several studies reported the effects of flavonoids on the activity of hepatic cytochrome P450 (P450) enzymes (Buening et al., 1981; Lasker et al., 1982). Since then, the ability of flavonoids to inhibit isoforms of CYP450, particularly CYP1A1 and CYP1A2, has been extensively confirmed (Cermak and Wolfram, 2006). Several clinical studies have reported that some flavonoids have the capacity to alter drug metabolism *in vivo* (Peng et al., 2003; Rajnarayana et al., 2003; Choi et al., 2004). However, for CYP2C9, which

ranks among the most important drug-metabolizing enzymes in humans and hydroxylates 10 to 20% of commonly prescribed drugs (Kirchheiner and Brockmüller, 2005), only two flavones, luteolin and baicalein, and one flavonol, quercetin, have been found to be potent inhibitors (Kim et al., 2002; von Moltke et al., 2004; Kumar et al., 2006; Foti et al., 2007).

In the present study, we have investigated the inhibition of CYP2C9-mediated diclofenac 4'-hydroxylation by a series of flavones and flavonols. As shown in Table 1, tested flavones include flavone, 6-hydroxyflavone, 7-hydroxyflavone, chrysin, baicalein, apigenin, luteolin, scutellarein, and wogonin as well as the two flavone glucuronides, scutellarin and baicalin. Tested flavonols include galangin, fisetin, kaempferol, morin, and quercetin. We were particularly interested to establish the mechanism of inhibition of CYP2C9 through enzyme kinetic studies, molecular dynamic and computer docking simulation, and subsequent construction of site-directed mutants. The main goal of our study was to determine the potential for flavonoids to interact with therapeutic drugs metabolized by CYP2C9.

Materials and Methods

Materials. Materials (purity) and suppliers were as follows: flavone ($\geq 99.0\%$), 7-hydroxyflavone, 6-hydroxyflavone (98%), chrysin ($\geq 96.0\%$), baicalein (98%), apigenin (95%), luteolin ($\geq 99.0\%$), galangin (95%), fisetin ($\geq 99.0\%$), kaempferol ($\geq 96.0\%$), and morin and quercetin ($> 99.0\%$), Sigma-Aldrich (St. Louis, MO); scutellarein (98%), National Pharmaceutical Engineering Center (Nanchang, China); wogonin ($\geq 99.0\%$), National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); scutellarin (96.5%) and baicalin (98.0%), Liaoning Institute for the Control of Pharmaceutical Products (Shenyang, China); diclofenac and 4'-hydroxydiclofenac, Merck Biosciences (Darmstadt, Germany); Dulbecco's modified Eagle's medium, pcDNA3.1(+) plasmid, RECO purified, reconstituted

This work was supported by the National Science Foundation of China [Grants 30472062, 20673044, 20333050].

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

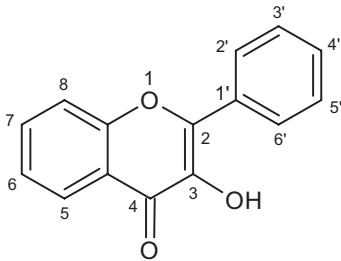
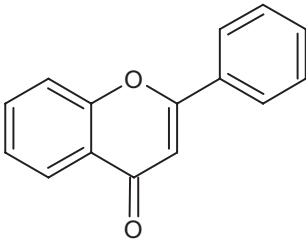
doi:10.1124/dmd.108.023416.

ABBREVIATIONS: P450, cytochrome P450; MD, molecular dynamics.

TABLE 1

Structure of the flavonols and flavones and their mean K_i values (micromolar) as inhibitors of diclofenac 4'-hydroxylase activity in the CYP2C9 RECO system

Incubations performed using 0.3 pmol of RECO CYP2C9, 2.5 to 100 μ M diclofenac, and a test compound with a concentration in the range 0 to approximately 6 times K_i . 4'-Hydroxydiclofenac/diclofenac was <1/20 in all assays. Global standard error for data fitting was less than 30% and $\gamma^2 > 0.90$ for each effector.

			
Flavonols	K_i	Flavones	K_i
Galangin: 5,7=OH	0.15	Flavone	17
Fisetin: 7,3',4'=OH	1.7	7-Hydroxyflavone: 7=OH	2.0
Kaempferol: 5,7,4'=OH	1.1	6-Hydroxyflavone: 6=OH	2.2 ^a
Morin: 5,7,2',4'=OH	1.8	Chrysin: 5,7=OH	1.0
Quercetin: 5,7,3',4'=OH	2.0	Baicalein: 5,6,7=OH	0.91
		Apigenin: 5,7,4'=OH	2.0
		Luteolin: 5,7,3',4'=OH	1.3
		Scutellarein: 5,6,7,4'=OH	1.7
		Wogonin: 5,7 = OH, 8=OCH ₃	1.0
		Scutellarin (scutellarein 7-O- β -D-glucuronide)	75
		Baicalin (baicalein 7-O- β -D-glucuronide)	40

^a Noncompetitive inhibitor; all others are competitive inhibitors.

CYP2C9 enzymes, and Lipofectamine 2000, Invitrogen (Carlsbad, CA); fetal bovine serum, Tianjin H&Y Bio Co. Ltd. (Tianjing, China); DpnI restriction enzyme, New England BioLabs (Ipswich, MA); Prime Star DNA polymerase, Takara Biotechnology (Dalian, China); rabbit anti-human CYP2C9 antibody, Abcam Inc. (Cambridge, MA); NADPH, Roche Diagnostics (Basel, Switzerland); and pooled human liver microsomes, BD Biosciences (San Jose, CA). COS-7 cell were donated by the Vaccination Center, Jilin University (Changchun, China). All other reagents were of analytical grade.

Enzyme Assays. To determine whether the tested compounds were irreversible mechanism-based inhibitors of CYP2C9, time-dependent inhibition by flavones and flavonols was evaluated using a method similar to that described by Sridar et al. (2004).

The inhibitory effect of flavonoids on CYP2C9 mediated 4'-hydroxylation of diclofenac both in the CYP2C9 RECO system (a purified, reconstituted enzyme system containing recombinant human CYP2C9, P450 reductase, cytochrome *b*₅, and liposomes) and in pooled human liver microsomes was evaluated. Incubations were performed using 0.3 pmol of RECO CYP2C9 or S9 fraction containing 0.3 pmol of CYP2C9 or 0.2 μ l of pooled human liver microsomes in a final volume of 200 μ l. The incubation mixture containing 100 mM Tris buffer, pH 7.5, 200 μ M NADPH, 2.5 to 100 μ M diclofenac, and 0 to 40 μ M test compound was maintained at 37°C for 20 min after which reaction was terminated by placing the incubation tube on ice and adding 500

μ l of ice-cold methanol. Tubes were then stored overnight at -20°C to allow complete protein precipitation to occur. After centrifugation for 30 min at 12,000 rpm 4°C, the supernatants were analyzed for 4'-hydroxydiclofenac by high-performance liquid chromatography.

High-Performance Liquid Chromatography Analysis. Separation was carried out on a SB-300A C₁₈ column (4.6 \times 200 mm, 10 μ m; Agilent Technologies, Santa Clara, CA) using 0.1 M potassium phosphate buffer, pH 7.4:acetonitrile (8:3) at 0.8 ml/min as mobile phase. Detection was by UV absorption at 280 nm. The retention times of 4'-hydroxydiclofenac and diclofenac were 7 and 22 min, respectively. Standard curves for the assays were prepared using incubation mixtures spiked with 4'-hydroxydiclofenac. The assay was linear in the range 0.13 to 100 μ M (Guo et al., 2005a).

Kinetic Analysis. The mechanism of flavonoid inhibition of diclofenac 4'-hydroxylase was determined by nonlinear regression analysis of the initial velocity-substrate concentration data (4'-hydroxydiclofenac/diclofenac, <1/20) and by Lineweaver-Burke plots using SigmaPlot software (Systat Software, Inc., San Jose, CA). Apparent inhibitory constants (K_i) for competitive inhibitors were calculated by nonlinear regression of the fit of data to the competitive inhibition eq. 1 using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). The K_i value for noncompetitive inhibitor was determined by the fit of the data to the noncompetitive inhibition eq. 2. The

TABLE 2

Mean K_i values (micromolar) for inhibition of diclofenac 4'-hydroxylase activity by baicalein, galangin, and 6-hydroxyflavone in the CYP2C9 RECO system, in human liver microsomes, and in S9 fraction from wild-type CYP2C9.1 and two variants transiently expressed in COS-7 cells

Rate of diclofenac 4'-hydroxylation by each enzyme system was 11.5, 18.8, 28.6, 25.9, and 26.7 nmol/min/nmol CYP2C9. Incubations performed using 0.3 pmol of RECO CYP2C9, S9 fraction containing 0.3 pmol of CYP2C9, or 0.2 μ l of pooled human liver microsomes, 2.5 to 100 μ M diclofenac, and a test compound with a concentration in the range 0 to approximately 6 times K_i . 4'-Hydroxydiclofenac/diclofenac <1/20 in all the assays. Global standard error for data fitting was less than 30% and $\gamma^2 > 0.90$ for each effector.

	RECO CYP2C9	Human Liver Microsomes	CYP2C9 Expressed in COS-7 Cells		
			CYP2C9.1	CYP2C9 Phe100Asp	CYP2C9 Phe100Trp
Flavonoid					
Baicalein	0.91	4.0	1.0		
Galangin	0.15	0.73	0.50		
6-Hydroxyflavone	2.2 ^a	11 ^a	17 ^a	25	24
Diclofenac 4'-hydroxylation kinetic parameters					
K_m (μ M)	14	4.0	2.3	2.5	2.6
V_{max} (nmol/min/nmol CYP2C9)	13.1	19.6	29.3	26.5	27.4

^a Noncompetitive inhibition; in all other situations, competitive inhibition.

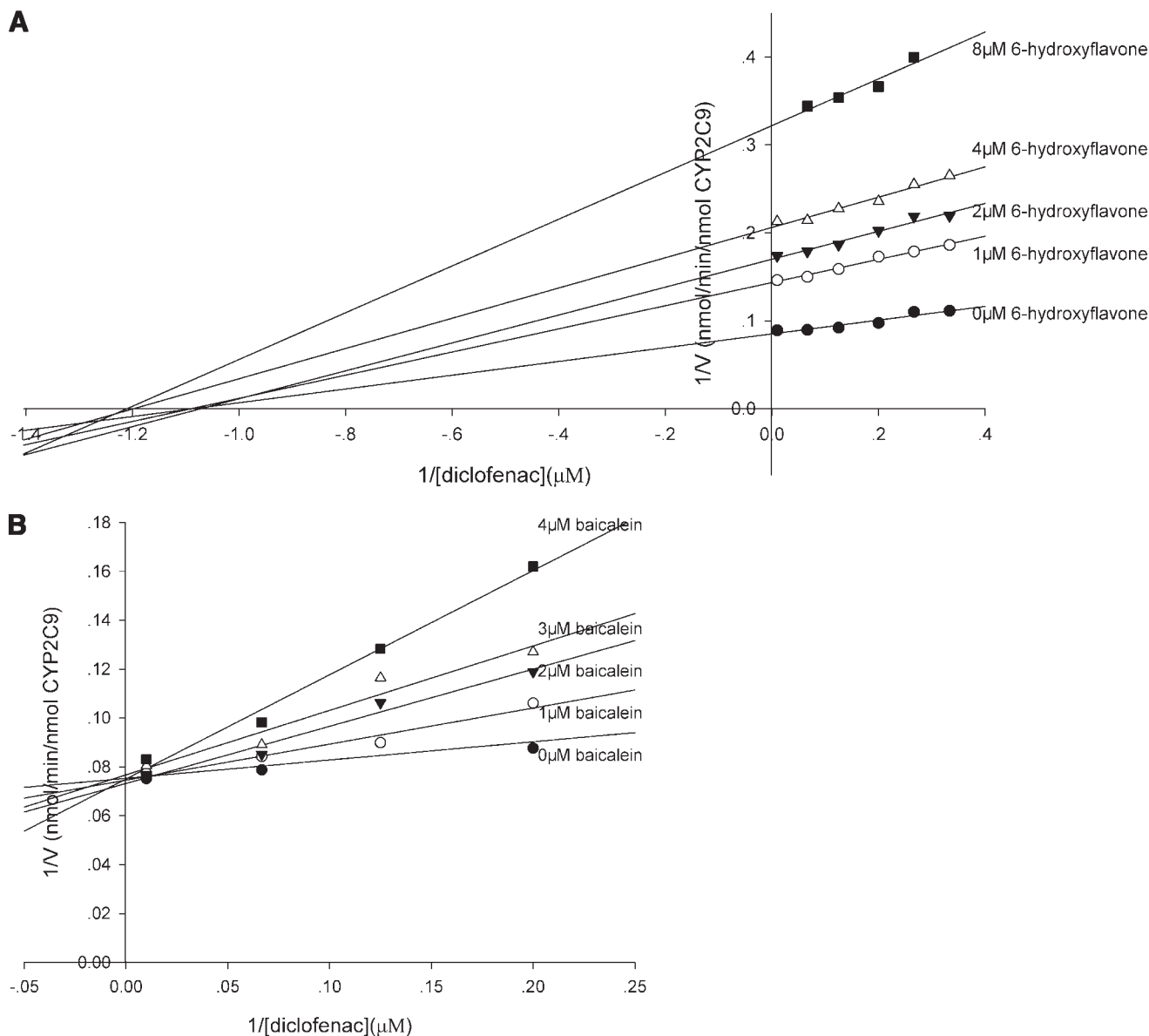


FIG. 1. Lineweaver-Burke plots for 4'-hydroxydiclofenac formation from diclofenac in the CYP2C9 RECO system in the presence of 6-hydroxyflavone (A) and baicalein (B). As shown in A, 6-hydroxyflavone is a noncompetitive inhibitor of RECO CYP2C9, with lines intersecting on x-axes, whereas in B, baicalein is a competitive inhibitor, with lines intersecting on y-axes. Line fit was by linear regression of reciprocal data.

goodness of fit was determined by visual inspection of the data with the Dixon plot and r^2 values.

$$V = V_{\max}/(1 + K_m/S/(1 + I/K_i)) \quad (1)$$

$$V = V_{\max}/(1 + K_s/S)(1 + I/K_i) \quad (2)$$

where K_s , the dissociation constant of the enzyme-substrate complex, is approximately equal to K_m .

Molecular Dynamic Simulation and Flexible Docking. Molecular modeling studies were performed on a SGI O3800 workstation using Gaussian 03 (Frisch et al., 2003) and the Insight II software package, version 98.0 MSI (Accelrys, San Diego, CA). The consistent-valence force field was used for energy minimization and MD simulation. A three-dimensional structure of substrate-free CYP2C9.1 was constructed based on the X-ray crystal structure of the CYP2C9-flurbiprofen complex (Protein Data Bank code 1R9O) (Wester et al., 2004) and was used to characterize the explicit enzyme complexed with baicalein, quercetin, apigenin, luteolin, morin, and 6-hydroxyflavone. The Insight II/binding-site module was used to search residues on the surface of the enzyme for inhibitor accessing based on the known substrate binding site of

1R9O. To consider the solvent effect, enzyme-ligand complexes were solvated in a sphere of TIP3P water molecules with a radius of 10 Å in the docking process. The docked receptor-ligand complex was selected using the criteria of interacting energy combined with the geometrical matching quality and Ludi score calculated using the Ludi/Insight II module (Oda et al., 2004). The methods and parameters of MD simulation and docking have been described previously (Zhou et al., 2006).

Site-Directed Mutagenesis and Construction of Plasmids. The pcDNA3.1(+) plasmid containing human *CYP2C9*1* cDNA was constructed in our laboratory (Guo et al., 2005b). Site-directed mutagenesis to introduce the TTC→GAC, TGG, and AAG transitions at position 298 to 300 (leading to F100D, F100W, and F100K substitution) was performed using pcDNA3.1(+) plasmids carrying CYP2C9*1 cDNA as the template for polymerase chain reaction amplification by Prime Star DNA polymerase (Takara Biotechnology). The specific base transition was introduced into the amplification products by a pair of completely complementary primers containing substituted base. The oligonucleotide primers for production of the CYP2C9 F100D, F100W, and F100K mutants (mutations underlined) were 5'-TCTGGAAGAGGCATTGACCCACTGGCT-GAAAGAG-3', 5'-TCTGGAAGAGGCATTTGGCCACTGGCTGAAAGAG-3'

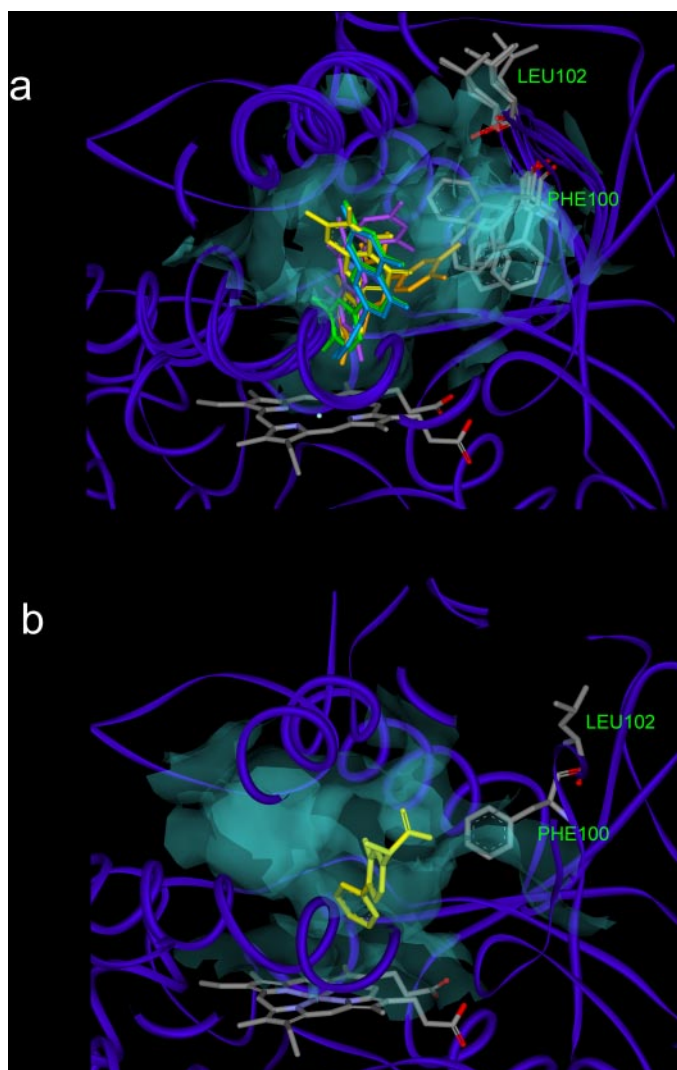


FIG. 2. Computer docking simulation of CYP2C9 enzyme complexes with luteolin, apigenin, baicalein, quercetin, and morin (a) and the similar region of the CYP2C9 crystal structure of 1R9O (b). The heme (at the bottom), Phe100 and Leu102 are rendered as sticks with carbon atoms colored gray. In a, overlapped luteolin, apigenin, baicalein, quercetin, and morin are in green, cyan, yellow, orange, and purple, respectively, whereas in b, flurbiprofen is in yellow. The solvent-accessible surface around the flavonoids is calculated using a 1.3-Å probe with the DS Visualizer, version 2.0 (Accelrys).

and 5'-TCTGGAAGAGGCATTAAGCCACTGGCTGAAAGAG-3', respectively. After incubation with DpnI to remove the original templates, the newly amplified polymerase chain reaction products containing substituted bases were transformed to *Escherichia coli* JM109-competent cells. Clones containing the desired nucleotide changes were identified by sequencing carried out by Sangon Co. Ltd. (Shanghai, China).

Expression of CYP2C9 Protein in COS Cells. The pcDNA3.1(+) plasmids containing the gene of human wild-type CYP2C9 and the three mutants were transiently transfected into COS-7 cells using Lipofectamine 2000 (Invitrogen). Expression was undertaken as described previously (Guo et al., 2005b), and the S9 fraction containing wild-type CYP2C9 and three variants was collected for assay or stored at -80°C . The quantity of expressed CYP2C9 protein was assayed by Western blotting as described previously (Guo et al., 2005b).

Results

Inhibition of CYP2C9 Activity by Flavonoids. The apparent inhibitory constants (K_i) for the inhibition of RECO CYP2C9-mediated diclofenac 4'-hydroxylation activity by flavones and flavonols

are presented in Table 1. With the exception of flavone, all flavonoids tested were found to inhibit RECO CYP2C9, with $K_i \leq 2.2 \mu\text{M}$. Galangin was the most potent inhibitor, with $K_i = 0.15 \mu\text{M}$. In contrast, the glucuronidated flavones were weak CYP2C9 inhibitors ($K_i > 40 \mu\text{M}$) consistent with previous reports (von Moltke et al., 2004; Liu et al., 2006). In terms of the inhibition of CYP2C9 in other enzyme systems, Table 2 shows galangin, baicalein, and 6-hydroxyflavone were potent inhibitors of CYP2C9 in all the systems examined (RECO CYP2C9, the S9 fraction of COS-7 cells containing transiently expressed CYP2C9 and pooled human liver microsomes).

Mechanism of Inhibition of CYP2C9. All the flavonoids tested were found to be reversible inhibitors of human CYP2C9-mediated diclofenac 4'-hydroxylation because no time-dependent inhibition was observed. Kinetic analysis of diclofenac 4'-hydroxylation formation revealed that 6-hydroxyflavone was a noncompetitive inhibitor of CYP2C9 in all the CYP2C9 enzyme systems tested, whereas the other flavonoids were competitive inhibitors (Fig. 1; Tables 1 and 2).

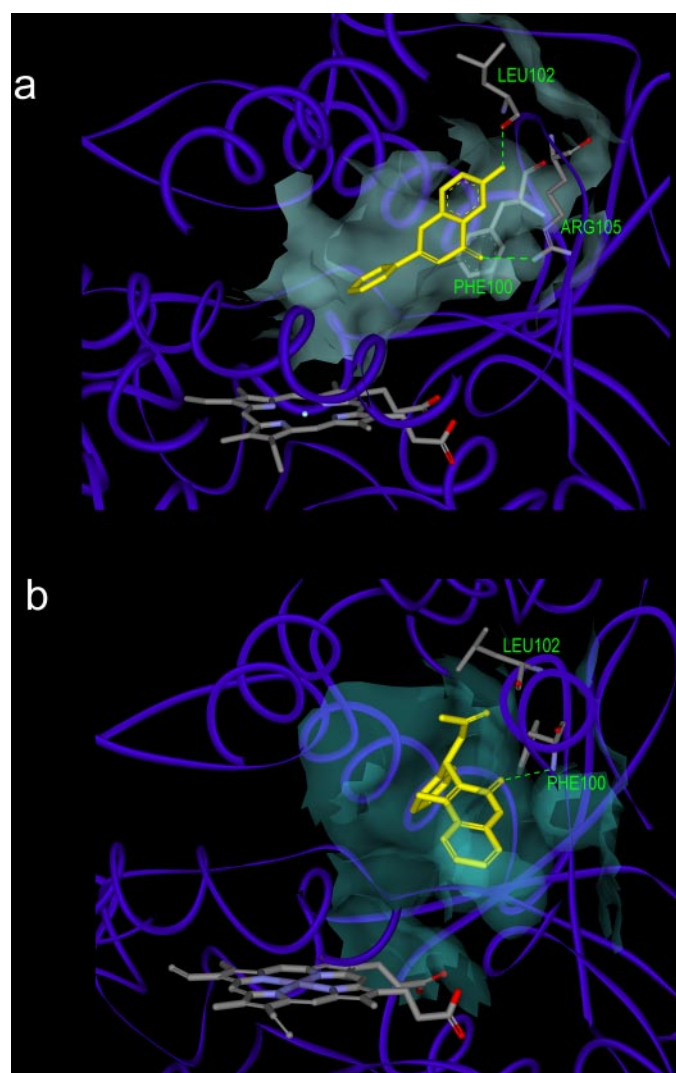


FIG. 3. Computer docking simulation of CYP2C9 enzyme complexes with 6-hydroxyflavone (a) and the similar region in the CYP2C9 crystal structure of 1OG5 (b). Phe100, Leu102, and Arg105 are rendered as sticks with carbon atoms colored gray. In a, 6-hydroxyflavone and in b, warfarin are shown as stick figures colored yellow. In a, hydrogen bonds between 6-hydroxyflavone and the backbone oxygen atoms of Leu102 and the side chain of Arg105 are depicted as green dashed lines. The π - π stacking interaction between 6-hydroxyflavone and Phe100 is also shown. In b, hydrogen bonds between (S)-warfarin and the backbone nitrogen amide atoms of Phe100 and Ala103 are depicted.

TABLE 3

Ludi scores, theoretical K_i values (micromolar; Ludi score = $-100 \log K_i$), and mean experimental K_i values (micromolar) for inhibition of CYP2C9-mediated diclofenac 4'-hydroxylase by flavonols and flavones in the RECO CYP2C9 system

Compound	Ludi Score	Theoretical K_i	Experimental K_i (RECO CYP2C9)
6-Hydroxyflavone	563 ^a	2.34 ^a	2.2 ^a
Quercetin	578	1.66	2.0
Luteolin	587	1.35	1.3
Baicalein	596	1.10	0.9
Apigenin	640	0.398	2.0
Morin	641	0.389	1.8

^a Noncompetitive inhibition; in all other situations, competitive inhibition.

MD Simulation and Flexible Docking of CYP2C9. As shown in Fig. 2, the competitive inhibitors luteolin, apigenin, baicalein, quercetin, and morin bind close to the heme and occupy the same binding site as that of flurbiprofen in the 1R9O crystal structure (Wester et al., 2004). In contrast, docking simulation of the noncompetitive inhibitor, 6-hydroxyflavone, presented in Fig. 3a shows it binds to a site further from the heme with a different orientation. This site is in a corner of the substrate binding cavity similar to the reported binding site of warfarin in the 1OG5 crystal structure shown in Fig. 3b (Williams et al., 2003). The complex of CYP2C9 with 6-hydroxyflavone defined by docking simulation indicates that 6-hydroxyflavone lies in a predominantly hydrophobic pocket bound by a π - π stacking interaction with the phenyl group of Phe100, hydrogen bonding between the 6-hydroxy group and the backbone oxygen atoms of Leu102 and hydrogen bonding between the 4-carbonyl group and the side chain of Arg105.

The enzyme-ligand complexes by docking simulation were analyzed by the Insight II/Ludi program to characterize the affinities of the inhibitors. Ludi scores and theoretical K_i values calculated from them are listed in Table 3. Theoretical K_i values agreed closely with experimental K_i values, except for morin and apigenin in which theoretical values were lower than experimental values.

Inhibition of CYP2C9 Mutants Substituted at Phe100. To further characterize the 6-hydroxyflavone binding site of CYP2C9, constructed mutants substituted at Phe100 were transiently expressed in COS-7 cells generating F100D, F100W, and F100K mutants. The F100K mutant, which has not been investigated previously, showed no detectable enzyme activity, possibly because of incorrect folding. In contrast, the F100D and F100W variants catalyzed diclofenac 4'-hydroxylation at a rate similar to that of CYP2C9.1. A subsequent inhibition study and kinetic analysis showed that the inhibition of diclofenac 4'-hydroxylation by 6-hydroxyflavone in the CYP2C9 F100D and F100W variants was competitive (Fig. 4; Table 2). This confirms that the noncompetitive binding site of 6-hydroxyflavone lies beside Phe100 of CYP2C9.1 and that alteration of this site leads 6-hydroxyflavone to bind to the CYP2C9 substrate binding site and shows competitive inhibition.

Discussion

In recent years, scientific and public interest in flavonoids has grown enormously because of their putative beneficial effects against atherosclerosis, osteoporosis, diabetes mellitus, and certain cancers (Cermak, 2008). Flavonoid intake in the form of dietary supplements and plant extracts has been steadily increasing, with little awareness of the potential for drug interactions with conventional drugs. Moreover, some flavonoids are administered orally or intravenously as drugs (2008 State Food and Drug Administration RPC, <http://app1.sfda.gov.cn/datasearch/face3/dir.html>). Although most flavonoids are rapidly metabolized in the intestinal mucosa and the liver, and the

bioavailability of flavonoids and their metabolites is generally low, with peak values of plasma concentration in the low micromolar range (Manach et al., 2005; Cermak and Wolfram, 2006), some clinical studies have demonstrated that flavonoids can affect the metabolism of other drugs (Peng et al., 2003; Rajnarayana et al., 2003; Choi et al., 2004). In the current investigation of the inhibition of CYP2C9-mediated diclofenac 4'-hydroxylation, we have shown that many flavonoids are potent inhibitors of CYP2C9, with K_i values $\leq 2.2 \mu\text{M}$, and, for galangin, as low as $0.15 \mu\text{M}$. These findings raise concerns about possible drug interactions between flavonoids and the some 100 therapeutic drugs metabolized by CYP2C9 (Kirchheiner and Brockmüller, 2005).

Many noncompetitive inhibitors of CYP450 enzymes have been reported, particularly of CYP1A2 and CYP2C9. Noncompetitive inhibitors of CYP2C9 include nifedipine (Bourri  et al., 1999), tranylcyproline (Salsali et al., 2004), phenethyl isothiocyanate (Nakajima et al., 2001), and medroxyprogesterone acetate (Zhang et al., 2006). Nevertheless, the molecular basis of these P450 noncompetitive inhibitions was unknown. It is interesting to note that some exogenous substances, including dapsone and its analogs have been shown to activate CYP2C9 metabolism of flurbiprofen, piroxicam, and naproxen by binding to an allosteric site of the enzyme (Korzekwa et al., 1998; Hutzler et al., 2001, 2002; Hummel et al., 2004a,b). However, such allosteric binding has not been implicated previously in explaining the noncompetitive inhibition of CYP2C9.

In the current investigation, all the flavones and flavonols except

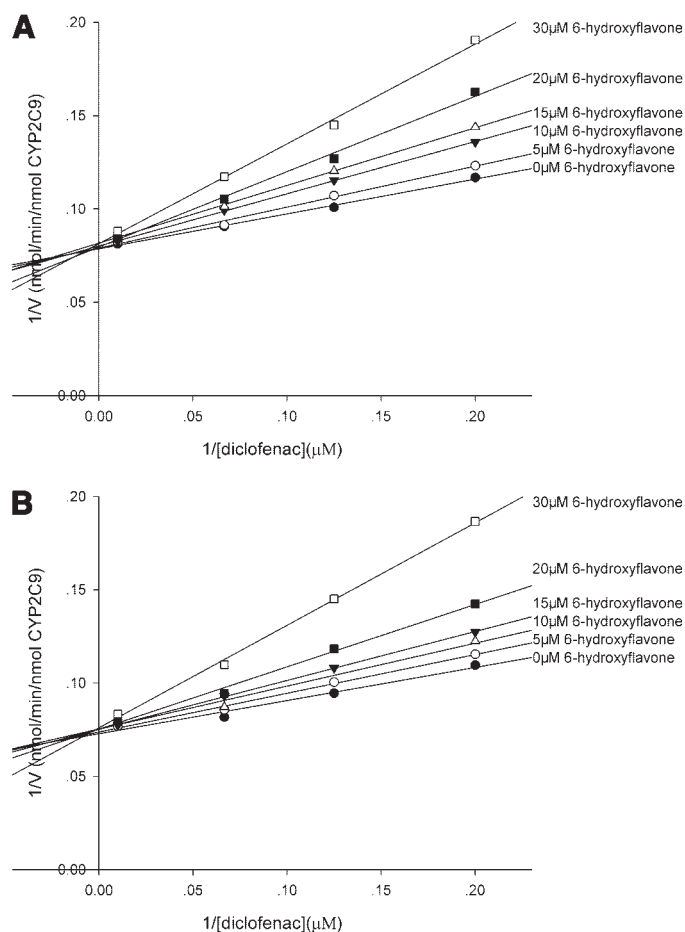


FIG. 4. Lineweaver-Burke plots for 4'-hydroxydiclofenac formation from diclofenac in the CYP2C9 F100D (A) and F100W (B) variants in the presence of 6-hydroxyflavone. Line fit was by linear regression of reciprocal data.

6-hydroxyflavone were found to be competitive inhibitors of CYP2C9, indicating they bind to its substrate binding site. On the basis of docking simulation studies using the 1R9O crystal structure, this binding site was shown to be close to the heme and the same site as occupied by flurbiprofen in the 1R9O crystal structure (Wester et al., 2004). Moreover, in our previous diclofenac docking study using methods similar to those used in this study, diclofenac was shown to bind to the same substrate binding site of substrate-free CYP2C9 constructed on the basis of the 1R9O crystal structure (Zhou et al., 2006). In contrast, the noncompetitive inhibitor 6-hydroxyflavone was shown to bind to a site further from the heme and oriented away from that used by the other flavones and flavonols. This site seems to be the same as the reported allosteric binding site of warfarin revealed in the crystal structure of 1OG5 (Williams et al., 2003) and the allosteric site of dapsone that leads to activation of CYP2C9-mediated flurbiprofen 4'-hydroxylation (Hummel et al., 2004a,b). Overall, these results indicate that the noncompetitive inhibition of CYP2C9 by 6-hydroxyflavone is because of its occupation of an allosteric binding site next to the substrate binding site.

The CYP2C9-6-hydroxyflavone complex defined by docking simulation indicates that 6-hydroxyflavone is bound by a π - π stacking interaction with the phenyl group of Phe100, and by two hydrogen bonding interactions with Leu102 and Phe100. Using the CYP2C9 variants F100D and F100W generated by site-directed mutagenesis, diclofenac 4'-hydroxylase activity was found to be similar to that of CYP2C9.1 and to be competitively inhibited by 6-hydroxyflavone. This confirms the presence of a direct interaction between 6-hydroxyflavone and Phe100 in the CYP2C9-noncompetitive inhibition.

In summary, we have shown that a series of structurally related flavones and flavonols are potent inhibitors of CYP2C9-mediated diclofenac 4'-hydroxylation. The flavonoids inhibiting CYP2C9 activity may increase the risk of toxicity from coadministered drugs that are CYP2C9 substrates with narrow therapeutic indices such as (S)-warfarin, tolbutamide, and phenytoin. However, the clinical relevance of this putative drug interaction remains to be revealed. In terms of the mechanism of inhibition, we have shown that flavonoids can act as competitive or noncompetitive inhibitors of CYP2C9, depending on whether they bind to the substrate binding site or an allosteric binding site of the enzyme.

References

- Bourri   M, Meunier V, Berger Y, and Fabre G (1999) Role of cytochrome P-450C9 in irbesartan oxidation by human liver microsomes. *Drug Metab Dispos* **27**:288–296.
- Bravo L (1998) Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Rev* **56**:317–333.
- Buening MK, Chang RL, Huang MT, Fortner JG, Wood AW, and Conney AH (1981) Activation and inhibition of benzo(a) pyrene and aflatoxin B1 metabolism in human liver microsomes by naturally occurring flavonoids. *Cancer Res* **41**:67–72.
- Cermak R (2008) Effect of dietary flavonoids on pathways involved in drug metabolism. *Expert Opin Drug Metab Toxicol* **4**:17–35.
- Cermak R and Wolfram S (2006) The potential of flavonoids to influence drug metabolism and pharmacokinetics by local gastrointestinal mechanisms. *Curr Drug Metab* **7**:729–744.
- Choi JS, Choi BC, and Choi KE (2004) Effect of quercetin on the pharmacokinetics of oral cyclosporine. *Am J Health Syst Pharm* **61**:2406–2409.
- Foti RS, Wahlstrom JL, and Wieners LC (2007) The in vitro drug interaction potential of dietary supplements containing multiple herbal components. *Drug Metab Dispos* **35**:185–188.
- Frisch MJ, Trucks GW, and Schlegel HB (2003) *Gaussian 03 (revision A.1)*. Gaussian, Inc., Pittsburgh, PA.

- Guo Y, Wang Y, Si D, Fawcett PJ, Zhong D, and Zhou H (2005a) Catalytic activities of human cytochrome P450 2C9*1, 2C9*3 and 2C9*13. *Xenobiotica* **35**:853–861.
- Guo Y, Zhang Y, Wang Y, Chen X, Si D, Zhong D, Fawcett JP, and Zhou H (2005b) Role of CYP2C9 and its variants (CYP2C9*3 and CYP2C9*13) in the metabolism of lornoxicam in humans. *Drug Metab Dispos* **33**:749–753.
- Hertog MGL, Hollman PCH, and van de Putte B (1993) Content of potentially anticarcinogenic flavonoids of tea infusions, wines, and fruit juices. *J Agric Food Chem* **41**:1242–1246.
- Hummel MA, Dickmann LJ, Rettie AE, Haining RL, and Tracy TS (2004a) Differential activation of CYP2C9 variants by dapsone. *Biochem Pharmacol* **67**:1831–1841.
- Hummel MA, Gannett PM, Aguilar JS, and Tracy TS (2004b) Effector-mediated alteration of substrate orientation in cytochrome P450 2C9. *Biochemistry* **43**:7207–7214.
- Hutzler JM, Hauer MJ, and Tracy TS (2001) Dapsone activation of CYP2C9-mediated metabolism: evidence for activation of multiple substrates and a two-site model. *Drug Metab Dispos* **29**:1029–1034.
- Hutzler JM, Kolwankar D, Hummel MA, and Tracy TS (2002) Activation of CYP2C9-mediated metabolism by a series of dapsone analogs: kinetics and structural requirements. *Drug Metab Dispos* **30**:1194–1200.
- Kim JY, Lee S, Kim DH, Kim BR, Park R, and Lee BM (2002) Effects of flavonoids isolated from *Scutellariae radix* on cytochrome P-450 activities in human liver microsomes. *J Toxicol Environ Health A* **65**:373–381.
- Kirchheiner J and Brockm  ller J (2005) Clinical consequences of cytochrome P450 2C9 polymorphisms. *Clin Pharmacol Ther* **77**:1–16.
- Korzekwa KR, Krishnamachary N, Shou M, Ogai A, Parise RA, Rettie AE, Gonzalez FJ, and Tracy TS (1998) Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry* **37**:4137–4147.
- Kuhnau J (1976) The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev Nutr Diet* **24**:117–191.
- Kumar V, Wahlstrom JL, Rock DA, Warren CJ, Gorman LA, and Tracy TS (2006) CYP2C9 inhibition: impact of probe selection and pharmacogenetics on in vitro inhibition profiles. *Drug Metab Dispos* **34**:1966–1975.
- Lasker JM, Huang M-T, and Conney AH (1982) In vivo activation of zoxazolamine metabolism by flavone. *Science* **216**:1419–1421.
- Liu KH, Kim MJ, Jeon BH, Shon JH, Cha JJ, Cho KH, Lee SS, and Shin JG (2006) Inhibition of human cytochrome P450 isoforms and NADPH-CYP reductase in vitro by 15 herbal medicines, including *Epimedium herba*. *J Clin Pharm Ther* **31**:83–91.
- Manach C, Williamson G, Morand C, Scalbert A, and Remesy C (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* **81**(1 Suppl):230S–242S.
- Nakajima M, Yoshida R, Shimada N, Yamazaki H, and Yokoi T (2001) Inhibition and inactivation of human cytochrome P450 isoforms by phenethyl isothiocyanate. *Drug Metab Dispos* **29**:1110–1113.
- Oda A, Yamamoto N, and Hirono S (2004) Studies of binding modes of (S)-mephenytoin to wild types and mutants of cytochrome P450 2C19 and 2C9 using homology modeling and computational docking. *Pharm Res* **21**:2270–2278.
- Peng WX, Li HD, and Zhou HH (2003) Effect of daidzein on CYP1A2 activity and pharmacokinetics of theophylline in healthy volunteers. *Eur J Clin Pharmacol* **59**:237–241.
- Peterson J and Dwyer J (1998) Flavonoids: dietary occurrence and biochemical activity. *Nutr Res* **18**:1995–2018.
- Rajnarayana K, Reddy MS, and Krishna DR (2003) Diosmin pretreatment affects bioavailability of metronidazole. *Eur J Clin Pharmacol* **58**:803–807.
- Salsali M, Holt A, and Baker GB (2004) Inhibitory effects of the monoamine oxidase inhibitor tranlylcypromine on the cytochrome P450 enzymes CYP2C19, CYP2C9, and CYP2D6. *Cell Mol Neurobiol* **24**:63–76.
- Sridar C, Goosen TC, Kent UM, Williams JA, and Hollenberg PF (2004) Silybin inactivates cytochromes P450 3A4 and 2C9 and inhibits major hepatic glucuronosyltransferases. *Drug Metab Dispos* **32**:587–594.
- von Moltke LL, Weemhoff JL, Bedir E, Khan IA, Hartzel JS, Goldman P, and Greenblatt DJ (2004) Inhibition of human cytochromes P450 by components of Ginkgo biloba. *J Pharm Pharmacol* **56**:1039–1044.
- Wester MR, Yano JK, Schoch GA, Yang C, Griffin KJ, Stout CD, and Johnson EF (2004) The structure of human cytochrome P450 2C9 complexed with flurbiprofen at 2.0-  resolution. *J Biol Chem* **279**:35630–35637.
- Williams PA, Cosme J, Ward A, Angove HC, Matak Vinkovi   D, and Jhoti H (2003) Crystal structure of human cytochrome P450 2C9 with bound warfarin. *Nature* **424**:464–468.
- Zhang JW, Liu Y, Li W, Hao DC, and Yang L (2006) Inhibitory effect of medroxyprogesterone acetate on human liver cytochrome P450 enzymes. *Eur J Clin Pharmacol* **62**:497–502.
- Zhou YH, Zheng QC, Li ZS, Zhang Y, Sun M, Sun CC, Si D, Cai L, Guo Y, and Zhou H (2006) On the human CYP2C9*13 variant activity reduction: a molecular dynamics simulation and docking study. *Biochimie* **88**:1457–1465.

Address correspondence to: Dr. Hui Zhou, College of Life Science, Jilin University, Changchun, 130023, China. E-mail: zhouhui@jlu.edu.cn