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Mechanism of CYP2C9 inhibition by flavones and flavonols

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CYP2C9, cytochrome p450 2C9; MD, Molecular dynamics; K_i , apparent inhibitory constant; K_s , dissociation constant of enzyme-substrate complex.

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Abstract

This paper describes an *in vitro* investigation of the inhibition of cytochrome p450 2C9 (CYP2C9) by a series of flavonoids made up of flavones (flavone, 6-hydroxyflavone, 7-hydroxyflavone, chrysin, baicalein, apigenin, luteolin, scutellarein, wogonin) and flavonols (galangin, fisetin, kaempferol, morin, quercetin). With the exception of flavone, all flavonoids were shown to inhibit CYP2C9-mediated diclofenac 4'-hydroxylation in the CYP2C9 RECO[®] system with $K_i \leq 2.2\mu\text{M}$. In terms of the mechanism of inhibition, 6-hydroxyflavone was found to be a noncompetitive inhibitor of CYP2C9 whereas the other flavonoids were competitive 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.

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Introduction

Flavonoids are polyphenolic secondary metabolites which are widely distributed in higher plants and ingested by humans in their regular food (Bravo, 1998; Kuhnau, 1976). Flavones and flavonols are two major classes of flavonoids (Table 1). Flavonols are present in a wide variety of fruits and vegetables whereas flavones are mainly found in cereals and herbs (Bravo, 1998; Hertog et al., 1993; Peterson and Dwyer, 1998). In the West, the estimated daily intake of both flavonols and flavones is in the range 20-50 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 (State Food and Drug Administration, 2008), 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 (CYP450) 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* (Choi et al., 2004; Peng et al., 2003; Rajnarayana et al., 2003). However, for CYP2C9, which ranks among the most

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

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.

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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 ($\geq 96.0\%$), morin and quercetin ($>99.0\%$), Sigma-Aldrich, Inc. (St. Louis, MO, USA); scutellarein (98%), the National Pharmaceutical Engineering Center (Nanchang, China); wogonin ($\geq 99.0\%$), the National institute for the Control of Pharmaceutical and Biological Products (Beijing, China); scutellarin (96.5%) and baicalin (98.0%), the 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 CYP2C9 enzymes and Lipofectamine[™] 2000, Invitrogen (Carlsbad, CA, USA); fetal bovine serum, Tianjin H&Y Bio Co. Ltd. (Tianjing, China); *DpnI* restriction enzyme, New England BioLabs (Beverly, MA, USA); Prime Star DNA polymerase, Takara Biotechnology (Dalian, China); rabbit anti-human CYP2C9 antibody, Abcam Ltd. (Cambridge, MA, USA); NADPH, Roche Molecular Biochemicals (Basel, Switzerland); pooled human liver microsomes, BD Bioscience (San Jose, CA, USA). COS-7 cell were donated by the Vaccination Center, Jilin University (Changchun, China). All other reagents were of analytical grade.

Enzyme assays

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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. (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 b5 and liposomes) and in pooled human liver microsomes was evaluated. Incubations were performed using 0.3 pmol RECO[®] CYP2C9 or S9 fraction containing 0.3 pmol CYP2C9 or 0.2 μ L 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, 2.5-100 μ M diclofenac and 0-40 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 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 analysed for 4'-hydroxydiclofenac by HPLC.

HPLC analysis

Separation was carried out on a SB-300A C₁₈ column (4.6×200 mm, 10 μ m, Agilent Technologies, CA, USA) 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

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min, respectively. Standard curves for the assays were prepared using incubation mixtures spiked with 4'-hydroxydiclofenac. The assay was linear in the range 0.13-100 μM (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 equation (1) using GraphPad Prism 5 software (GraphPad, San Diego, CA). The K_i for noncompetitive inhibitor was determined by the fit of the data to the noncompetitive inhibition equation (2). The goodness of fit was determined by visual inspection of the data with the Dixon plot and r^2 values.

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

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

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

Molecular dynamic (MD) simulation and flexible docking

Molecular modeling studies were performed on a SGI O3800 workstation using

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Gaussian 03 (Frisch et al., 2003) and the Insight II software package (Version 98.0 MSI, Accelrys, Inc. CA, USA). 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 (PDB 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 298-300 (leading to Phe100Asp, Phe100Trp and Phe100Lys substitution) was performed using pcDNA3.1(+) plasmids carrying *CYP2C9*1* cDNA as the template for polymerase chain reaction (PCR) amplification

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by Prime Star DNA polymerase. 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 Phe100Asp, Phe100Trp and Phe100Lys mutants (mutations underlined) were 5'-TCTGGAAGAGGCATTGACCCACTGGCTGAAAGAG-3', 5'-TCTGGAAGAGGCATTTGGCCACTGGCTGAAAGAG-3' and 5'-TCTGGAAGAGGCATTAAAGCCACTGGCTGAAAGAG-3', respectively. After incubation with *DpnI* to remove the original templates, the newly amplified PCR products containing substituted bases were transformed to *E. 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. Expression was undertaken as previously described (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 previously described (Guo et al., 2005b).

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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 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 (Liu et al., 2006; von Moltke et al., 2004). 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 since 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 (Figure 1, Tables 1 and 2).

MD simulation and flexible docking of CYP2C9

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As shown in Figure 3, 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 Figure 4a 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 Figure 4b (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 pi-pi 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 agreed closely with experimental K_i except for morin and apigenin where 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 Phe100Asp, Phe100Trp and Phe100Lys mutants. The Phe100Lys mutant, which has

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never been investigated before, showed no detectable enzyme activity, possibly due to incorrect folding. In contrast, the Phe100Asp and Phe100Trp 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 Phe100Asp and Phe100Trp variants was competitive (Figure 2, 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 show competitive inhibition.

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Discussion

In recent years, scientific and public interest in flavonoids has grown enormously due to 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). Although most flavonoids are intensely 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 lower micromolar range (Cermak and Wolfram, 2006; Manach et al., 2005), some clinical studies have demonstrated that flavonoids can affect the metabolism of other drugs (Choi et al., 2004; Peng et al., 2003; Rajnarayana et al., 2003). 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 $K_i \leq 2.2 \mu\text{M}$ and, in the case of 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 Brockmoller, 2005).

Many noncompetitive inhibitors of CYP450 enzymes have been reported particularly of CYP1A2 and CYP2C9. Noncompetitive inhibitors of CYP2C9 include nifedipine

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(Bourrie et al., 1999), tranylcyromine (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. Interestingly, 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 (Hummel et al., 2004a; Hummel et al., 2004b; Hutzler et al., 2001; Hutzler et al., 2002; Korzekwa et al., 1998). However, such allosteric binding has not been previously implicated in explaining the noncompetitive inhibition of CYP2C9.

In the current investigation, all the flavones and flavonols except 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 paper, 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 appears to be the same as the reported allosteric binding site of warfarin revealed in the crystal structure of 1OG5 (Williams et al.,

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2003) and the allosteric site of dapsone that leads to activation of CYP2C9-mediated flurbiprofen 4'-hydroxylation (Hummel et al., 2004a; Hummel et al., 2004b). Overall, these results indicate that the noncompetitive inhibition of CYP2C9 by 6-hydroxyflavone is due to 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 pi-pi stacking interaction with the phenyl group of Phe100, and by two hydrogen bonding interactions with Leu102 and Phe100. Using the CYP2C9 variants Phe100Asp and Phe100Trp 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

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inhibitors of CYP2C9 depending on whether they bind to the substrate binding site or
an allosteric binding site of the enzyme

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Legends of figures

Figure 1. Lineweaver-Burke plots for 4'-hydroxydiclofenac formation from diclofenac in the CYP2C9 RECO[®] system in the presence of (a) 6-hydroxyflavone and (b) baicalein. As shown in (a), 6-hydroxyflavone is a noncompetitive inhibitor of RECO[®] CYP2C9 with lines intersected at x-axes, while in (b), baicalein is a competitive inhibitor with lines intersected at y-axes. Line fit was by linear regression of reciprocal data.

Figure 2. Lineweaver-Burke plots for 4'-hydroxydiclofenac formation from diclofenac in the CYP2C9 Phe100Asp (a) and Phe100Trp (b) in the presence of 6-hydroxyflavone. Line fit was by linear regression of reciprocal data.

Figure 3. Computer docking simulation of CYP2C9 enzyme complexes with (a) luteolin, apigenin, baicalein, quercetin and morin and (b) the similar region of the CYP2C9 crystal structure of 1R9O. The heme (at the bottom), Phe100 and Leu102 are rendered as sticks with carbons atoms colored grey. 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, Inc. CA, USA).

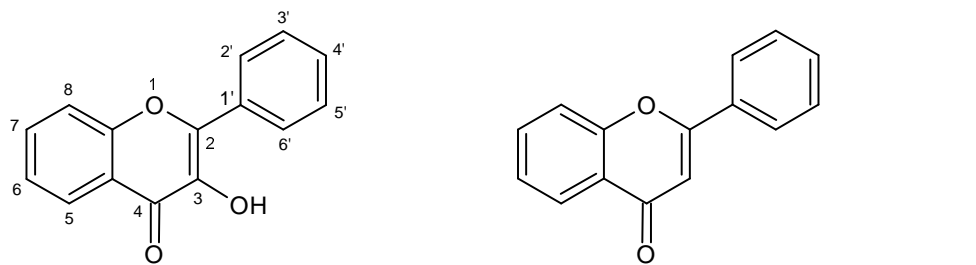
Figure 4. Computer docking simulation of CYP2C9 enzyme complexes with (a)

DMD #23416

6-hydroxyflavone and (b) the similar region in the CYP2C9 crystal structure of 1OG5. Phe100, Leu102 and Arg105 are rendered as sticks with carbons atoms colored grey. 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 pi-pi 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.

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Table 1. Structure of the flavonols and flavones and their mean K_i values (μM) as inhibitors of diclofenac 4'-hydroxylase activity in the CYP2C9 RECO[®] system*



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 **
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	0.9
		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	75
		(scutellarein 7-O- β -D-glucuronide)	
		baicalin	40
		(baicalein 7-O- β -D-glucuronide)	

*Incubations performed using 0.3 pmol RECO[®] CYP2C9, 2.5-100 μM diclofenac and a test compound

with a concentration in the range 0 to about 6 times K_i ; 4'-Hydroxydiclofenac / diclofenac < 1/20 in all

assays. Global standard error for data fitting was less than 30% and $\gamma^2 > 0.90$ for each effector.

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***Noncompetitive inhibitor; all others are competitive inhibitors*

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Table 2. Mean K_i values (μM) 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*

Flavonoids	RECO [®] CYP2C9	human liver microsomes	CYP2C9 expressed in COS-7 cells		
			CYP2C9.1	CYP2C9 Phe100Asp	CYP2C9 Phe100Trp
baicalein	0.91	4.0	1.0		
galangin	0.15	0.73	0.50		
6-hydroxyflavone	2.2**	11**	17**	25	24
Diclofenac 4'-Hydroxylation Kinetic Parameters					
K_m (μM)	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

* 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 RECO[®] CYP2C9, S9 fraction containing 0.3 pmol CYP2C9, or 0.2 μL pooled human liver microsomes, 2.5-100 μM diclofenac and a test compound with a concentration in the range 0 to about 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 effecter.

**Noncompetitive inhibition; in all other situations, competitive inhibition

DMD #23416

Table 3. Ludi scores, theoretical K_i values (μM , Ludi score = $-100 \log K_i$) and mean experimental K_i values (μM) 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*	2.34*	2.2*
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

*Noncompetitive inhibition; in all other situations, competitive inhibition

Figure 1a

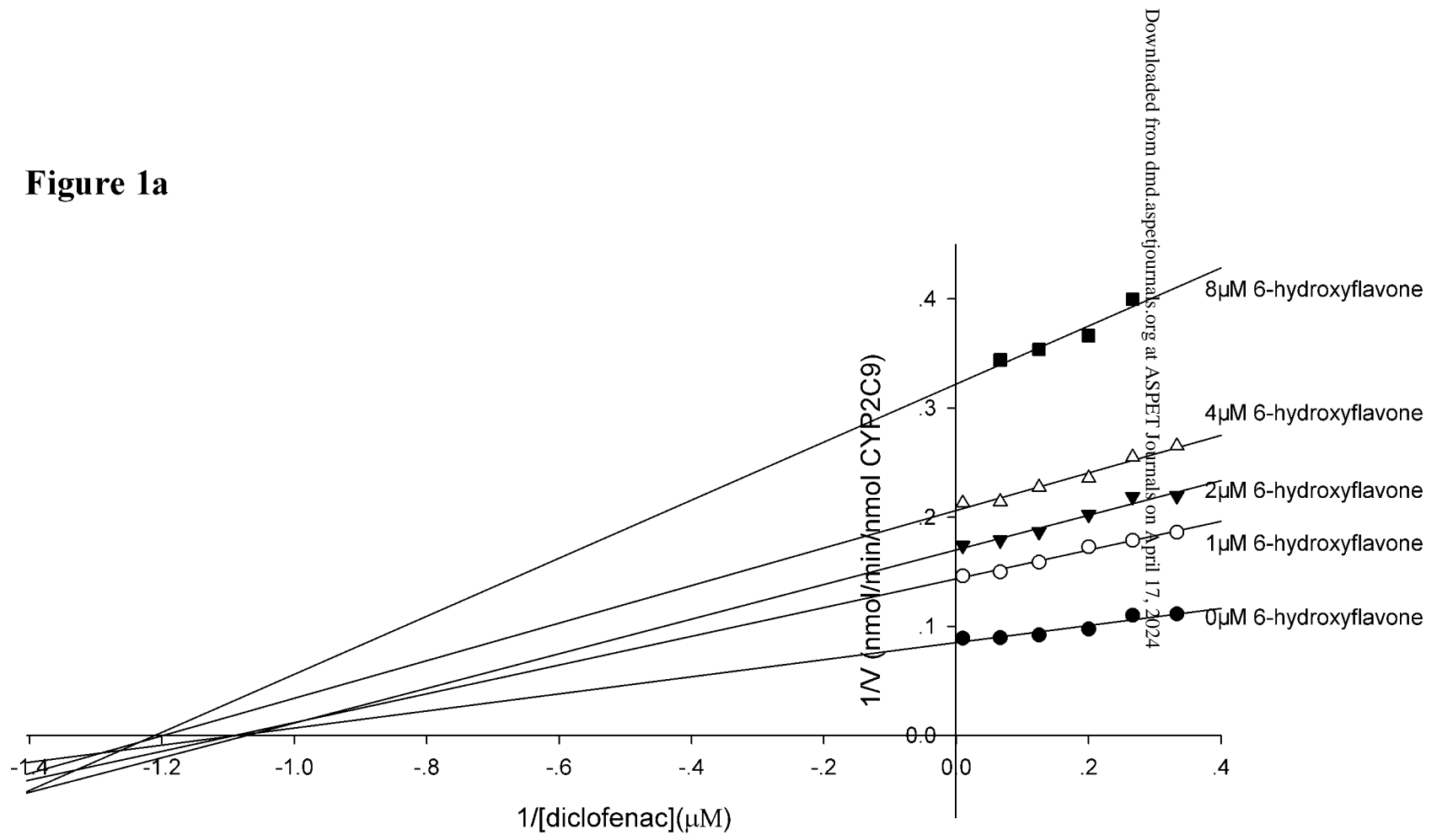


Figure 1b

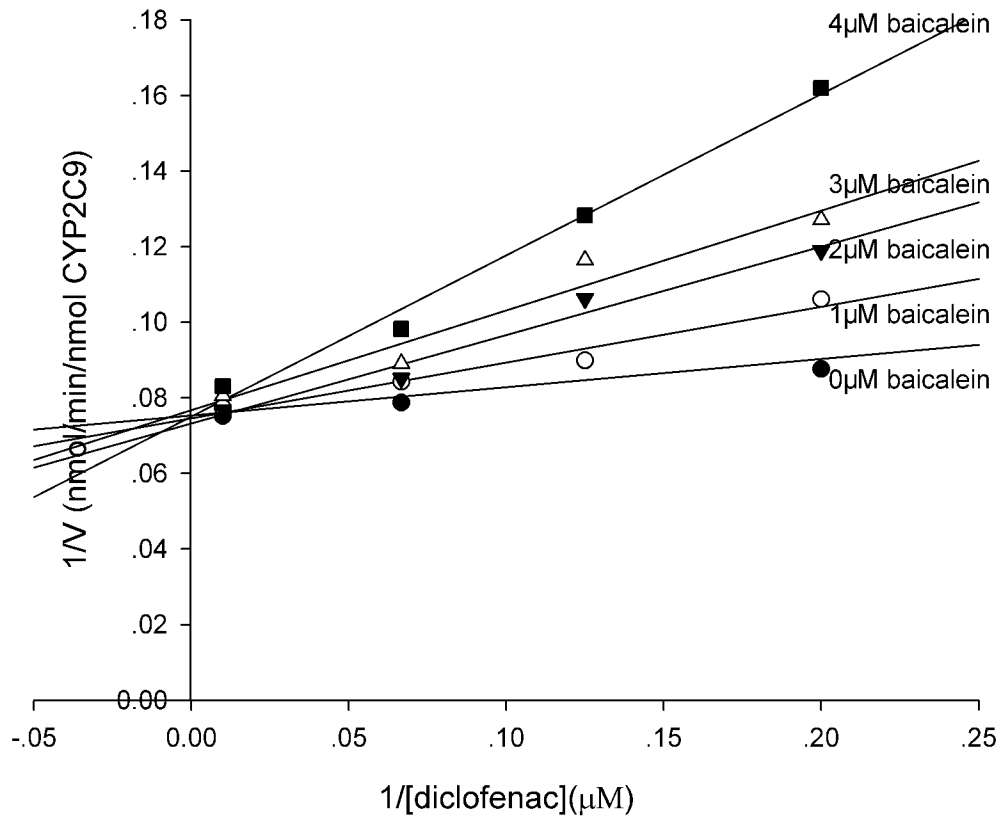


Figure 2a

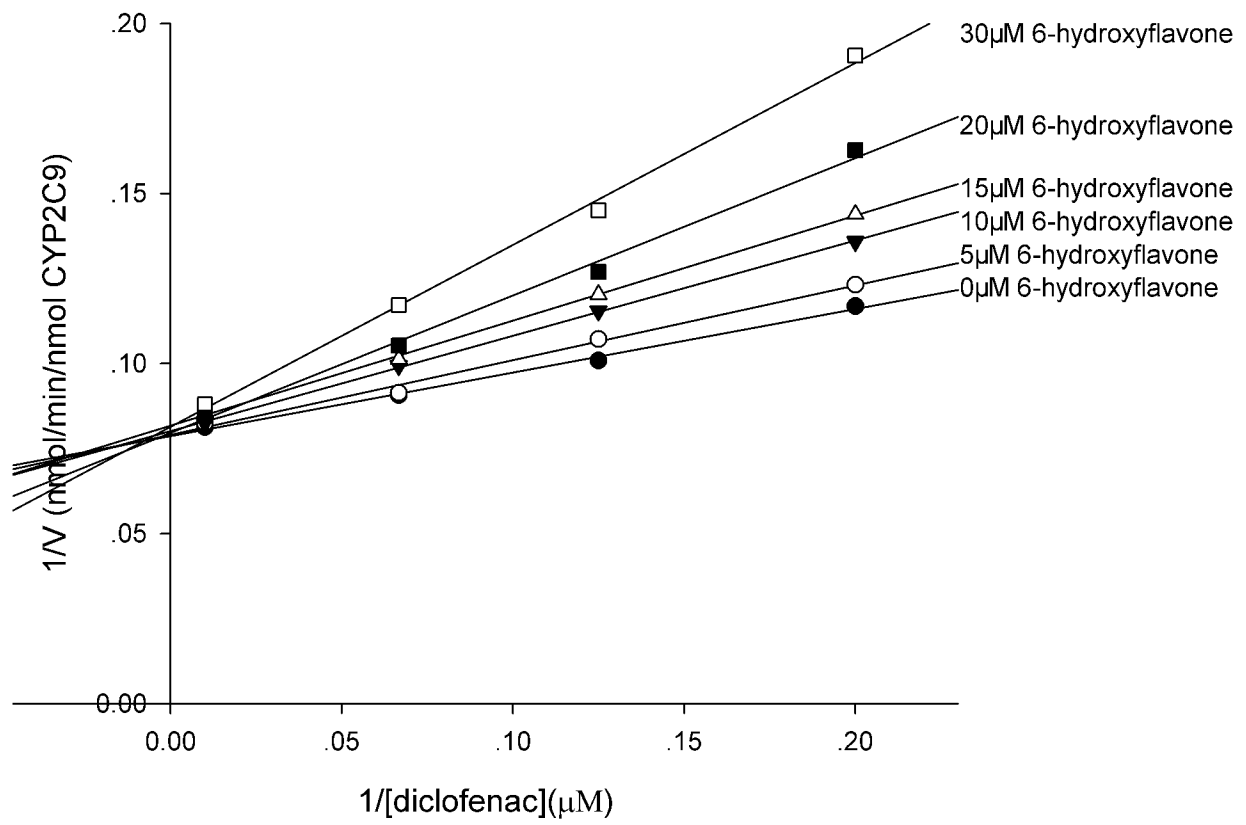


Figure 2b

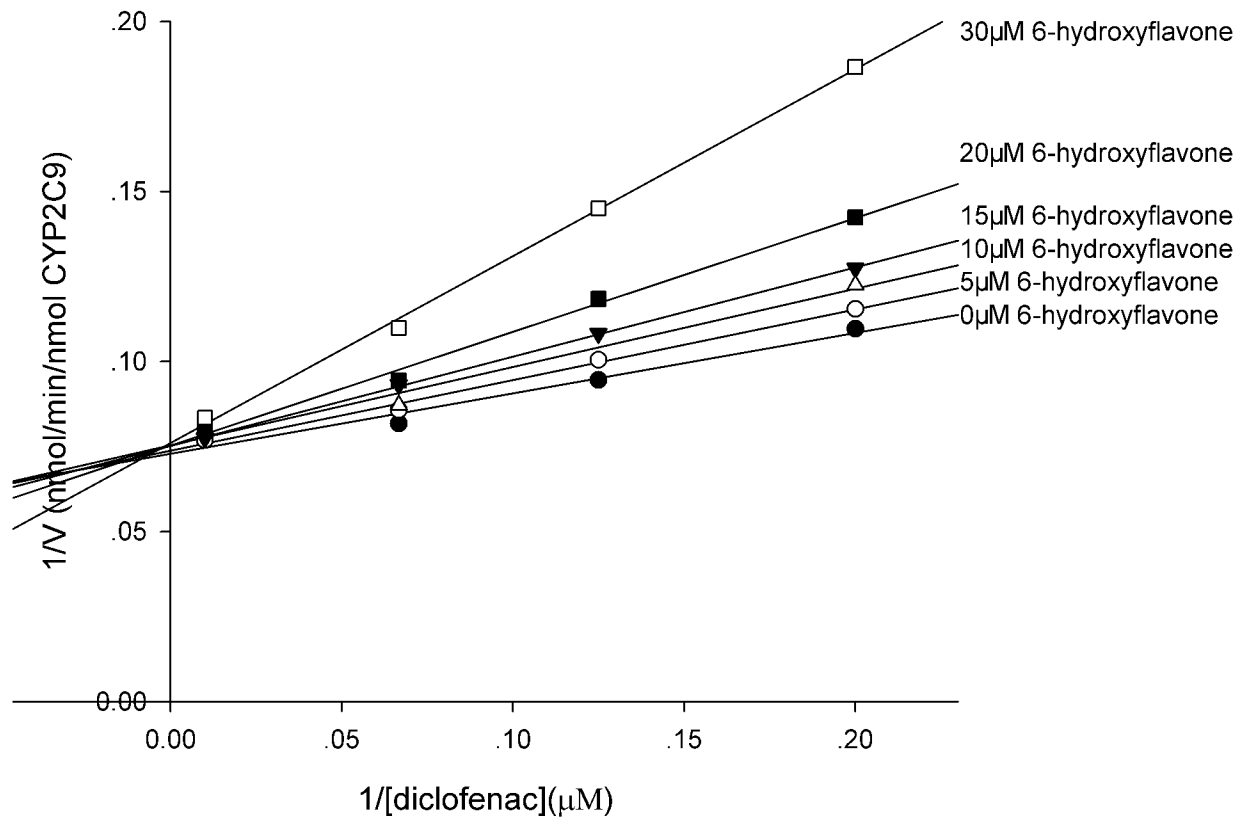
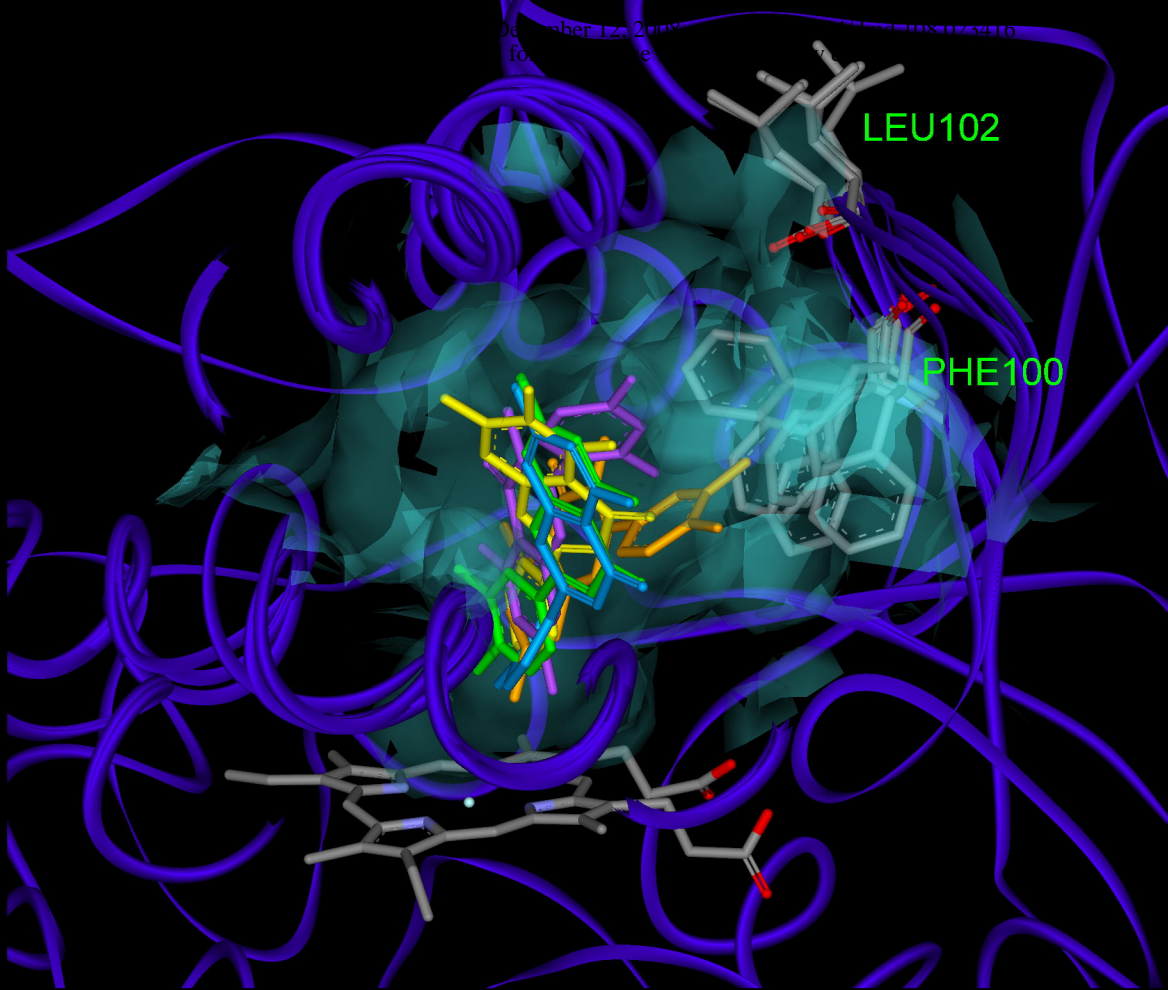


Figure 3

a



b

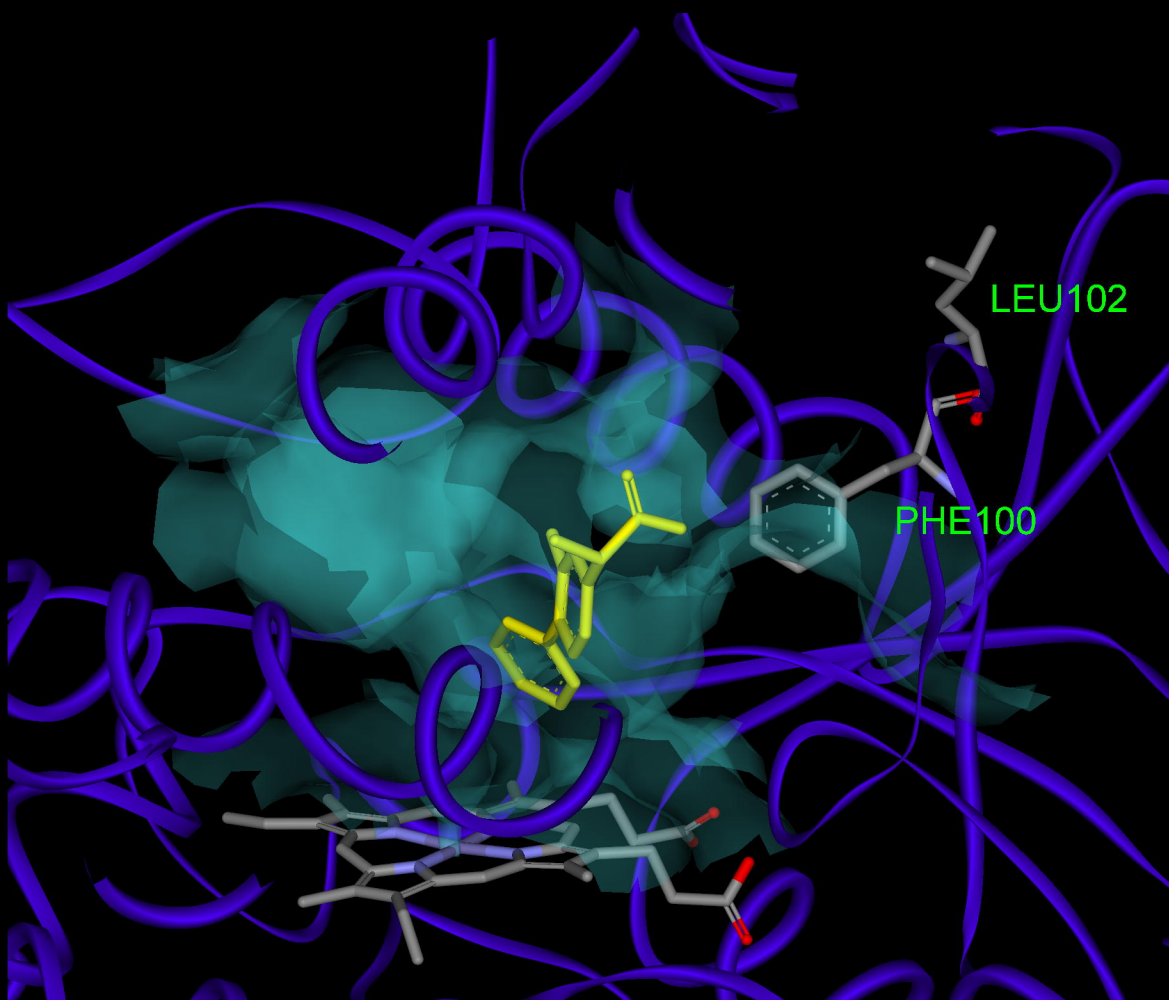
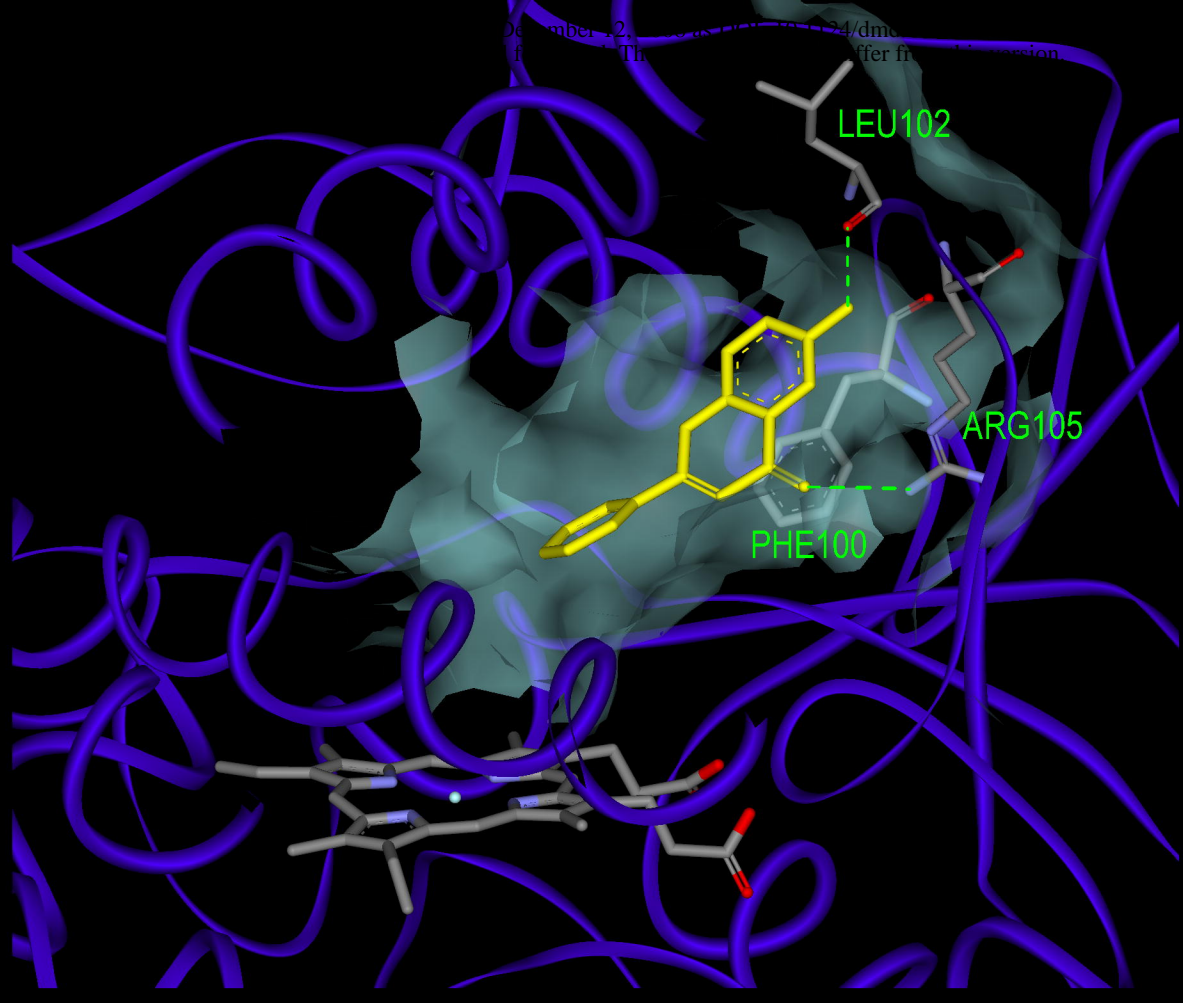


Figure 4

a



b

