DMD Fast Forward. Published on May 15, 2009 as doi:10.1124/dmd.109.026500

CYP2D6-CYP2C9 Protein-Protein Interactions and Isoform-Selective Effects on Substrate Binding and Catalysis

Murali Subramanian, Ph.D.

Michael Low, B.S.

Charles W. Locuson, Ph.D.

Timothy S. Tracy, Ph.D.

Department of Experimental and Clinical Pharmacology, College of Pharmacy,

University of Minnesota (M.S., M.L., T.S.T.)

Pfizer Animal Health, Veterinary Medicine Research and Development, Kalamazoo, MI (C.W.L.)

Running Title

CYP2C9-CYPD6 Protein Interactions

Correspondence:
Timothy S. Tracy, Ph.D.
Dept. of Experimental and Clinical Pharmacology
College of Pharmacy
University of Minnesota
7-115B Weaver-Densford Hall
308 Harvard St. SE
Minneapolis, MN 55126

Direct: 612-625-7665
Fax; 612-625-3927
e-mail: tracy017@umn.edu

Number of text pages: 29

Number of tables: 2

Number of figures: 8

Words in abstract: 241

Words in introduction: 643

Words in discussion: 1,487

Non-Standard Abbreviations

Cytochrome P450 (P450)

NADPH-cytochrome P450 reductase (CPR)

cytochrome b5 (b5)

dilauroylphosphatidylcholine (DLPC)

spectrally determined binding affinity (K_S)

ABSTRACT Cytochrome P450 (P450) protein-protein interactions have been observed with various in-vitro systems. Interestingly, these interactions appear to be isoform dependent, with some combinations producing no effect, increased or decreased catalytic activity. With some exceptions, most of the work to date has involved P450s from rabbit, rat and other animal species, with few studies including human P450s. In the studies presented herein, the interactions of two key drug metabolizing enzymes CYP2C9 and CYP2D6 were analyzed in a purified, reconstituted enzyme system for both changes in substrate binding affinity and rates of catalysis. In addition, an extensive study was conducted as to the "order of mixing" for the reconstituted enzyme system and the impact on the observations. CYP2D6 co-incubation inhibited CYP2C9 mediated S-flurbiprofen metabolism in a protein concentration dependent manner. V_{max} values were reduced by up to 50%, but no appreciable effect on K_m was observed. Spectral binding studies revealed a 20-fold increase in the K_s of CYP2C9 towards S-flurbiprofen in the presence of CYP2D6. CYP2C9 co-incubation had no effect on CYP2D6-mediated dextromethorphan O-demethylation. The order of combination of the proteins (CYP2C9, CYP2D6 and cytochrome P450 reductase (CPR)) influenced the magnitude of catalysis inhibition as well as the ability of increased cytochrome P450 reductase to attenuate the change in activity. A simple scheme, congruent with current results and those of others, is proposed to explain oligomer formation. In summary, CYP2C9-CYP2D6 interactions can alter catalytic activity and thus, influence in vitro-in vivo correlation predictions.

DMD Fast Forward. Published on May 15, 2009 as DOI: 10.1124/dmd.109.026500
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Cytochrome P450s (P450) are responsible for the metabolism of over 75% of the drugs in the market (Guengerich, 2006). Multiple subfamilies of cytochrome P450s can activate and metabolize a wide spectrum of substrates by a two electron transfer catalytic cycle, in which electrons are provided by either NADPH-cytochrome P450 reductase (CPR) or cytochrome b5 (b5) depending on the stage of the cycle (Guengerich, 2001). A number of studies have investigated the role of CPR and b5, and the nature of P450, CPR and b5 interactions. While CPR is indispensable for metabolism, cytochrome b5 either has no effect or significantly enhances metabolism (Locuson, et al., 2006;Shimada, et al., 1994). Yamazaki et al. (Yamazaki, et al., 1997) demonstrated that b5 stimulated the metabolism of tolbutamide and S-warfarin by CYP2C10, but had no effect on bufuralol hydroxylation by CYP1A1 and CYP2D6. Additionally, both apo and holo-b5 enhanced metabolism by CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4 and CYP3A5 in reconstituted systems (Shimada, et al., 1994) suggesting that the effect of these protein-protein interactions of the redox partner with P450 may involve enzyme conformational changes or other mechanisms besides just provision of electrons.

In addition to interactions with CPR and b5, P450s can also interact with each other resulting in changes in metabolism rates (Alston, et al., 1991;Backes and Cawley, 1999;Cawley, et al., 2001;Hazai and Kupfer, 2005;Kaminsky and Guengerich, 1985;Kelley, et al., 2005;Kelley, et al., 2006;Li, et al., 1999;Shimada, et al., 1994;Tan, et al., 1997;Yamazaki, et al., 1997;Backes, et al., 1998;Cawley, et al., 1995) . Reconstituted expressed enzymes (human CYP2C9-CYP2C19 (Hazai and Kupfer, 2005), rabbit CYP1A2-CYP2B4 (Yamazaki, et al., 1997), rabbit CYP1A2-CYP2E1 (Kelley, et al., 2006), human CYP3A4-CYP1A1 (Yamazaki, et al., 1997), human CYP3A4-CYP1A2 (Yamazaki, et al., 1997)), microsomes from induced animals (Cawley, et al.,

2001;Kaminsky and Guengerich, 1985), and triple-expression systems (CYP3A4, CYP2D6 and CPR (Li, et al., 1999), CYP2E1, CYP2A6 and CPR (Tan, et al., 1997)) have exhibited protein-protein interactions. However, P450-P450 interactions are not universal, as studies with reconstituted enzymes (Kelley, et al., 2006) or microsomes from rats (Dutton, et al., 1987) did not demonstrate interactions between CYP2E1 and CYP2B4. Initial suggestions that protein-protein interactions occurred because of competition for CPR (West and Lu, 1977) were disproven in subsequent studies utilizing saturating concentrations of CPR (Kaminsky and Guengerich, 1985). CPR activity in the form of cytochrome c was also measured to discount loss of CPR activity as a reason for the observed substrate inhibition.

Understanding and elucidating mechanisms underlying P450-P450 interactions are important for several reasons. First, they may impact upon predictions of in vivo clearance from in vitro data. Many times, these predictions are carried out using expressed enzyme systems that may not contain the full complement of P450s and thus, the potential for interactions. Secondly, a better understanding of P450-P450 interactions may provide insights into the organization of these proteins within the cell, their aggregation characteristics, and how these features affect catalytic activity. Thirdly, P450-P450 interactions may provide valuable insights into protein structure-function relationships and how the presence of different proteins may impact individual protein function.

Several possible mechanisms of altered P450 function resulting from these interactions have been explored. The role of ionic interactions between P450 pairs have been evaluated (Kelley, et al., 2005) using high concentrations of buffer salts to disrupt possible ionic interactions of CYP2B4 and CYP1A2 and restore most of the P450 activity. Investigators also have used molecular modeling of protein-protein interactions to evaluate how proteins might interact to

form heteromers that may alter activity (Hazai, et al., 2005;Backes, et al., 1998). However, it remains unclear whether conformational changes, altered catalytic cycle functioning, or other mechanisms are responsible for the observed changes in P450 function.

CYP2C9 comprises of about 10% of the liver, while CYP2D6 comprises of 2-3% of the liver but is involved in the metabolism of 30% of all drugs (Shimada, et al., 1994). 1-5% of the Caucasian population has multiple copies of CYP2D6 leading to greater CYP2D6 proteins, and lower CYP2C9:CYP2D6 ratios in these individuals (Meijerman, et al., 2007). The current study examined the consequence of CYP2C9 - CYP2D6 interactions and the resulting impact on metabolism rates. In addition, the role of CPR on these interactions was evaluated. Previous work (Gorsky and Coon, 1986) has demonstrated that order of mixing of CYP proteins with redox transfer proteins can alter the rate of metabolism and thus, the order of combination of these enzymes (CYP2C9, CYP2D6 and CPR) in a reconstituted system was also explored to evaluate what role this might play in the degree of interactions observed.

DMD Fast Forward. Published on May 15, 2009 as DOI: 10.1124/dmd.109.026500
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Materials

Acetonitrile, dibasic potassium phosphate, methanol and phosphoric acid were purchased from Fisher Scientific Co. (Pittsburgh, PA). Dextromethorphan, dilauroylphosphatidylcholine (DLPC), levallorphan (internal standard), NADPH and triethylamine were purchased from Sigma-Aldrich (St. Louis, MO). Dextrorphan was purchased from Research Biochemicals International (Natick, MA). (S)-Flurbiprofen, 4'-hydroxyflurbiprofen, and 2-fluoro-4-biphenyl acetic acid (internal standard) were gifts from the former Pharmacia, Inc. (Kalamazoo, MI). Human CYP2D6.1 and human P450 CPR were purchased from Invitrogen (Carlsbad, CA). CYP2C9.1 was expressed and purified as previously described (Locuson, et al., 2006).

Enzyme Reconstitution

Incubations contained P450s, extruded DLPC vesicles (10 µg/incubation), CPR and substrate. Different mixing schemes of CYP2C9, CYP2D6 and CPR were explored - sequential and simultaneous. In the sequential mixing schemes, two components were mixed and allowed to equilibrate on ice for 5 minutes, followed by the addition of the third component and further equilibration on ice for 5 minutes. In the simultaneous mixing scheme, all three components were mixed consecutively and allowed to equilibrate for 10 minutes on ice. The order of mixing of the P450s was also investigated, and all experimental mixing conditions are summarized in Table 1. Once all three components had equilibrated on ice, they were was reconstituted in DLPC (extruded through a 200-nm pore size membrane) and allowed to further equilibrate for 5 or 10 minutes in the case of sequential or simultaneous mixing, respectively. Enzyme mixtures

were then added to substrate and buffer, and pre-incubated at 37°C for 8 minutes prior to initiation of the reaction.

(S)-Flurbiprofen Metabolism by CYP2C9 in the Presence of CYP2D6

(S)-Flurbiprofen (2, 5, 10, 25, 50, 100, 200 and 300 μM) was incubated with CYP2C9 (5 pmol/incubation), CPR (10 or 20 pmol/incubation for sub-saturating or saturating conditions) and CYP2D6 (0 to 5 pmol/incubation). The ratios of CYP2C9:CYP2D6 ranged from 10:1 to 1:1 in an attempt to mimic in-vivo ratios of these enzymes across the range of expression (poor to extensive to ultra-rapid metabolizers). All experiments were carried out in 50 mM potassium phosphate buffer, pH 7.4 at 37°C. Following initiation of the reaction with NADPH (1 mM final concentration), at 20 minutes incubation time the reactions were terminated by adding 200μl of 180ng/mL 2-fluoro-4-biphenyl acetic acid (internal standard) in acetonitrile followed by 40μl of half strength phosphoric acid to adjust to pH ~3.0 for HPLC separation. All experiments were performed three times on separate days.

Dextromethorphan Metabolism by CYP2D6 in the Presence of CYP2C9

Dextromethorphan (1, 2, 5, 10, 20, 50, 100, 150 and 200μM), CYP2D6 (10 pmol per incubation), CYP2C9 (0 or 10 pmol per incubation) and CPR (either 20 pmol or 40 pmol per incubation) were incubated together in 50 mM potassium phosphate buffer, pH 7.4 at 37°C. Reactions were initiated with NADPH (1 mM final concentration). After 20 minutes, the reactions were terminated by adding 50 μl of half strength phosphoric acid followed by 20μl of 8μg/ml levallorphan (internal standard) in water. Again, all experiments were performed three times on separate days.

DMD Fast Forward. Published on May 15, 2009 as DOI: 10.1124/dmd.109.026500
This article has not been copyedited and formatted. The final version may differ from this version.

Analysis of 4'-Hydroxyflurbiprofen

Quantitation of 4'-hydroxyflurbiprofen formation was carried out exactly as described previously (Tracy, et al., 2002).

Analysis of Dextrorphan

Dextrorphan was analyzed by HPLC with fluorescence detection. The HPLC system consisted of a Waters Alliance 2695XE pumps/autosampler and a Waters 2495 fluorescence detector (Waters Corp., Milford, MA) set at an excitation wavelength of 280 nm and an emission wavelength of 310 nm. The mobile phase consisted of de-ionized water, methanol, acetonitrile and triethylamine, pH 3.0 (60:34.3:5.7:0.25 v/v) pumped at 1 ml/min through a 4.6 X 150mm Cyano Column (Regis Technologies). Dextrorphan and internal standard eluted at 6.0 and 7.3 min, respectively.

(S)-Flurbiprofen Spectral Binding

Difference spectra were obtained to evaluate enzyme-substrate affinity based on the alteration in heme iron spin state that occurs when active site water(s) are displaced through *(S)*-flurbiprofen binding. Briefly, varying ratios of CYP2C9 (0 and 0.5 μM concentrations) and CYP2D6 (0 and 0.5 μM concentrations), and 0.5 μg DLPC/pmol-P450 in 50 mM potassium phosphate buffer, pH 7.4 were mixed into the sample and reference cuvettes. Increasing concentrations of (S)-flurbiprofen, dissolved in 50 mM potassium phosphate (pH 7.4) with a small amount of acetonitrile, were added into the sample cuvette (acetonitrile concentration did not exceed 1%) and phosphate buffer to the reference cuvette and a 3-minute equilibrium interval

DMD Fast Forward. Published on May 15, 2009 as DOI: 10.1124/dmd.109.026500
This article has not been copyedited and formatted. The final version may differ from this version.

allowed between readings. Spectral binding experiments were conducted on an Aminco DW2000 spectrophotometer with Olis upgrade (Olis, Inc., Bogart, GA) set to record spectra from 340 to 500 nm wavelength with a slit-width of 0.5 mm and 20 scans per datum at 1.0 nm steps. The temperature of the cell was held constant at 30 °C with a Julabo F12 compact refrigerated circulator with an external Julabo ED temperature controller (Julabo USA, Inc., Allentown, PA). The difference in absorbance between high spin (390 nm) and low spin (418 nm) components was calculated, plotted against *(S)*-flurbiprofen concentration and the data fit by nonlinear regression SigmaPlot 8.0 (Systat Software, Point Richmond CA) to estimate the Ks (spectral binding constant) from the titration binding curve using the following equation:

$$\Delta A = \frac{B\max \bullet [S]}{K_S + [S]}$$

DMD Fast Forward. Published on May 15, 2009 as DOI: 10.1124/dmd.109.026500
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Effect of CYP2D6 on CYP2C9 metabolism of S-Flurbiprofen

The effect of varying amounts of co-incubated CYP2D6 and CPR on the metabolism of (S)-flurbiprofen presented in Figures 1 and 2. Figure 1 shows the metabolism of (S)-flurbiprofen when three concentrations of CYP2D6 and 10 pmol of CPR was used in each incubation, while Figure 2 shows the same when 20 pmol of CPR was used in each incubation. In all cases, a CYP2D6 concentration-dependent inhibition was observed resulting in a decrease in V_{max} but less change in K_m. When 2.5 or 5 pmol of CYP2D6 was preincubated with CYP2C9, significant inhibition ($p<0.05$) was observed. Figures 3 and 4 summarize the effect of CYP2D6 on V_{max} of (S)-flurbiprofen metabolism by CYP2C9 when the order of combination of the enzymes were varied. When CYP2C9 was combined with CYP2D6 followed by the addition of CPR (SeqAdd1), a reduction in V_{max} was observed (Figure 3A). Increasing the levels of CPR did not restore the CYP2C9 activity. A similar trend was observed when CYP2C9 and CPR were initially combined and then CYP2D6 was added (SeqAdd2, Figure 3B). However, when CYP2D6 and CPR were initially combined, followed by the addition of CYP2C9, addition of more CPR reduced the amount of inhibition (SeqAdd3, Figure 3C), demonstrating that order of mixing can impact the nature of inhibition. Figure 4 depicts the inhibition of CYP2C9-mediated metabolism of (S)-flurbiprofen when CYP2C9, CYP2D6 and CPR were mixed together simultaneously and preincubated prior to the addition of DLPC. Figure 4A depicts the data obtained when the order of mixing was CYP2C9, then CPR, followed by CYP2D6 (Allmix1), and Figure 4B shows data when the order of mixing was CYP2C9, then CYP2D6, followed by CPR (Allmix2). Inhibition was observed at all levels of CYP2D6 and this inhibition was significantly reduced ($p<0.05$) by the addition of more CPR. There was no difference observed

between Allmix1 and Allmix2, wherein the order of addition of CYP2D6 and CPR were reversed. In all cases, as the amount of CYP2D6 was increased, the degree of inhibition of (S)-flurbiprofen metabolism was also increased. However, at low CYP2D6/CYP2C9 ratios (0.2:1), less than 10-20% inhibition was observed. At the highest tested CYP2D6/CYP2C9 ratio of 1:1, the ratios of $V_{max+2D6}/V_{max-control}$ ranged from 50 to 80%. Hence, a halving of maximal velocity was observed at the highest ratio of CYP2D6/CYP2C9.The effect of saturating levels of CPR on the amount of inhibition in various mixing schemes is summarized in Table 2. Control incubations of CYP2D6 with (S)-flurbiprofen (no CYP2C9 present) demonstrated that CYP2D6 was unable to metabolize (S)-flurbiprofen to the 4'-hydroxy metabolite and no additional peaks were noted in the chromatograms (data not shown).

Effects of CYP2C9 on Dextromethorphan Metabolism by CYP2D6

The presence of equimolar ratios of CYP2C9 had no effect on the metabolism of dextromethorphan by CYP2D6 (Figure 5). Furthermore, control incubations confirmed that no detectable dextrorphan was formed when dextromethorphan was incubated alone with CYP2C9. The V_{max} values for dextromethorphan O-demethylation by CYP2D6 ranged from 0.2-0.4 pmol/min/pmol-CYP2D6, while K_m was between 4-8 μM.

Spectral Binding Studies

To determine whether the protein-protein interactions between CYP2C9 and CYP2D6 resulted in an alteration in binding affinity for the substrate, P450 spectral binding titration studies were performed. In the presence of CYP2C9 alone (control) the spectrally determined binding affinity (K_S) for (S)-flurbiprofen was 6 μM (Figure 6). The addition of equimolar

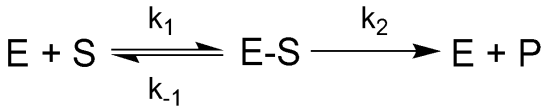
CYP2D6 shifted the K_S for (S)-flurbiprofen to 118 μM (Figure 7), resulting in a decrease in binding affinity. A control binding experiment with (S)-flurbiprofen and CYP2D6 alone confirmed that (S)-flurbiprofen was not causing a spin state change in CYP2D6, suggesting that (S)-flurbiprofen did not bind to CYP2D6 (Figure 8). To further confirm that (S)-flurbiprofen does not bind to the CYP2D6 active site, the metabolism of dextromethorphan by CYP2D6 in the presence of (S)-flurbiprofen was evaluated and (S)-flurbiprofen had no effect on dextromethorphan metabolism by CYP2D6 (data not shown). This absence of competitive inhibition, along with the spectral binding data presented above, further reinforces that (S)-flurbiprofen does not enter the CYP2D6 active site, ruling out the possibility that (S)-flurbiprofen binding to the CYP2D6 heme contributed to the alterations in the observed binding affinity of (S)-flurbiprofen for CYP2C9. The 20-fold increase in K_S toward (S)-flurbiprofen in the presence of CYP2D6 indicates an increased dissociation of the flurbiprofen-CYP2C9 complex when CYP2D6 is present, and is consistent with the inhibitory results from the kinetic studies in the reconstituted systems.

Discussion

Protein-protein interactions involving cytochrome P450 proteins can affect catalytic turnover in either a positive or negative manner and consequently affect in vitro – in vivo correlations of drug disposition. Several previous studies have demonstrated either inhibition or stimulation of P450 mediated metabolism due to protein-protein interactions e.g., (Hazai and Kupfer, 2005;Cawley, et al., 2001;Yamazaki, et al., 1997;Cawley, et al., 1995) but others have not observed an effect (Kelley, et al., 2006) suggesting that the effects may be isoform and/or substrate dependent in a manner that affects the rate of metabolism. In the present work, it was demonstrated that CYP2D6 co-incubation with CYP2C9 results in a gene-dose dependent inhibition of CYP2C9-mediated flurbiprofen hydroxylation with no effect on K_m. This effect was not completely reversed by addition of more cytochrome P450 reductase (CPR). Furthermore, the order of mixing of the constituents had a measurable effect on the degree of inhibition noted. Interestingly, CYP2C9 had no effect on CYP2D6 catalytic activity. Because CYP2D6 expression is polymorphic resulting in substantially different levels of expression of active protein among individuals, this may impact CYP2C9 activity in individuals and complicate in vitro – in vivo correlations.

While some of the parameters affecting protein-protein interactions have been explored, other factors such as the role of interactions on K_S (affinity) of an enzyme, and the role of order of addition of interacting enzymes have not been evaluated in a systematic manner. When equimolar amounts of CYP2D6 were added to CYP2C9, the spectral binding constant K_S increased 20-fold, but K_m remained unchanged. This apparent paradox can be rationalized by examining the factors that make up K_S and K_m (see kinetic scheme below).

DMD Fast Forward. Published on May 15, 2009 as DOI: 10.1124/dmd.109.026500
This article has not been copyedited and formatted. The final version may differ from this version.



K_S is the ratio of k_{-1}/k_1, where k_{-1} is the dissociation of the E-S complex and k_1 is the formation of the E-S complex, where E=enzyme and S=substrate and k_2 is the rate of product formation. Since K_m is a sum of K_S and the fraction k_2/k_1, for K_m to remain the same while K_S increases 20-fold would require that k_1 remains unchanged while k_{-1} increases and k_2 decreases. This suggests that CYP2D6 destabilizes the E-S complex, causing its dissociation to the individual components (E and S). It should be noted that $K_m \cong K_s$ when k_2 is very small compared to k_{-1}/k_1, however, these values ($K_m \cong K_s$) do not approach equality but rather Ks becomes larger than K_m. The reasons for this are unclear but may be related to the slightly different conditions in which the two experiments (K_m vs. Ks determinations) were carried out such as slight differences in lipid concentrations.

The order of combination of the enzymes also has not been explored in a systematic manner, previously. Kaminsky and Guengerich (Kaminsky and Guengerich, 1985) briefly evaluated the order of addition of the competing P450 after the addition of CPR, lipids and substrate. This order of addition was found to have no influence on the amount of inhibition observed. In the current work presented herein, K_m was unaffected by protein-protein interactions, however, the order of combination of enzymes influenced the effect CPR had on the inhibition. The degree of inhibition was similar at subsaturating levels of CPR, regardless of mixing scheme (Table 2). However, differences in the degree of inhibition were noted at saturating levels of CPR. In the Allmix1 and Allmix2 cases (enzymes + CPR mixed concurrently, sit for 10 min. then add lipids), less inhibition was noted when additional CPR was added. These findings suggest that some portion of the reduction in CYP2C9 activity is due to the two enzymes competing for the CPR,

since all constituents are allowed to equilibrate simultaneously. However, with sequential mixing (two of the components added first, allowed to equilibrate, then the third component added) in all cases (SeqAdd1 and SeqAdd2) but one (SeqAdd3), additional CPR had no effect on the degree of inhibition.

While the order of addition of enzymes does not possess any in-vivo significance, it can provide information on whether irreversible or tightly bound quasi-irreversible heteromers are formed. Gorsky and Coon (Gorsky and Coon, 1986)demonstrated that when only P450 and CPR are combined, the order of mixing the enzymes and lipids was insignificant. However, when P450, CPR and b5 were combined, the order of addition of b5 had a significant effect on the extent of metabolism. In the present study, three enzyme proteins were also being combined (CYP2C9, CYP2D6 and CPR) and the order of mixing of these enzymes also appeared to affect the rate of metabolism. In theory, reversible interactions between proteins should not be influenced by the order of addition, whereas the formation of tightly bound heteromers (quasi-irreversible interactions) would be affected by the order of mixing. In the current study this was also able to be probed by altering mixing order. Since mixing order affected the rate of metabolism in the current study, these results suggest that quasi-irreversible interactions are occurring when CYP2C9, CYP2D6 and CPR are combined.

In an attempt to explain the effects of order of addition observed in our system, a simplistic kinetic scheme is proposed below. The basis for the proposed model lies in the following assumptions: 1. P450s mixed together form heteromers with each other and CPR (Hazai, et al., 2005;Backes, et al., 1998;Alston, et al., 1991;Backes and Kelley, 2003), 2. Heteromer complexes retain catalytic activity (Backes, et al., 1998), 3. Only one P450 binding sites exists on a single CPR molecule (Backes, et al., 1998), and 4. Additional combinations of proteins (e.g., CPR-

DMD Fast Forward. Published on May 15, 2009 as DOI: 10.1124/dmd.109.026500
This article has not been copyedited and formatted. The final version may differ from this version.

CYP2C9-CYP2D6-CPR) can be formed but are in lower amounts than the primary species (CYP2D6-CYP2C9-CPR) (Backes and Kelley, 2003).

Equations representing the order of addition (only primary species presumed to be formed are shown)

1) SeqAdd1: 2C9_2D6_5mins_CPR

$$2C9 + 2D6 \rightarrow 2C9\text{-}2D6 \xrightarrow{+CPR} 2C9\text{-}2D6\text{-}CPR + CPR\text{-}2C9\text{-}2D6$$

$$2C9 + 2D6 \rightarrow 2C9\text{-}2D6 \xrightarrow{+2[CPR]} 2C9\text{-}2D6\text{-}CPR + CPR\text{-}2C9\text{-}2D6$$

In this scheme, doubling the amount of CPR does not produce a new species, but simply more of the same two species.

2) SeqAdd2: 2C9_CPR_5mins_2D6

$$2C9 + CPR \rightarrow 2C9\text{-}CPR \xrightarrow{+2D6} 2D6\text{-}2C9\text{-}CPR$$

$$2C9 + 2[CPR] \rightarrow 2C9\text{-}CPR + CPR \xrightarrow{+2D6} 2D6\text{-}2C9\text{-}CPR + 2D6\text{-}CPR$$

Doubling the amount of CPR results in a new species being formed, namely 2D6-CPR. However, no CYP2D6 substrate is present.

3) SeqAdd3: 2D6_CPR_5mins_2C9

$$2D6 + CPR \rightarrow 2D6\text{-}CPR \xrightarrow{+2C9} 2D6\text{-}2C9\text{-}CPR$$

$$2D6 + 2[CPR] \rightarrow 2D6\text{-}CPR + CPR \xrightarrow{+2C9} 2D6\text{-}2C9\text{-}CPR + 2C9\text{-}CPR$$

DMD Fast Forward. Published on May 15, 2009 as DOI: 10.1124/dmd.109.026500
This article has not been copyedited and formatted. The final version may differ from this version.

Analogous to #2 above, doubling the amount of CPR results in a new species, namely 2C9-CPR. This is an active species since substrate is present and thus, this 2C9-CPR species also contributes to the formation of the 4-hydroxyflurbiprofen metabolite. Hence, in this scheme, increasing the amount of CPR reduces the amount of inhibition since a "non-inhibited species" i.e., 2C9-CPR is present, whereas no change in the degree of inhibition would be observed with schemes 1 and 2 under the conditions of added CPR.

Inconsistent or differential incorporation of enzymes and CPR into the lipid matrix (DLPC) when two enzymes are co-incubated in reconstituted systems could lead to changes in activity. Furthermore, differences in mobility of aggregated heteromers, as compared to monomers, into the lipid matrix could also affect activity. Differences observed among the various mixing schemes could, in part, be due to lipid incorporation differences. However, triple-expression (Li, et al., 1999;Tan, et al., 1997) systems and microsomal systems (Cawley, et al., 2001;Kaminsky and Guengerich, 1985) have also exhibited inhibition and activation, suggesting that differences in incorporation into lipids provides an incomplete explanation for the observed changes. It has been suggested that in vivo the ratio of total P450:CPR is 20:1, while in reconstituted systems the ratio utilized is 1:2 (Estabrook, et al., 1971). What role the ratio (*in vivo* vs. *in vitro*) of P450 to its redox partner CPR plays in this interaction is unclear. However, two studies (Kaminsky and Guengerich, 1985;Cawley, et al., 2001) did observe a protein-protein interaction in microsomes from rats treated *in vivo* with enzyme specific enzyme inducers, suggesting that the observations are not an artifact of the *in vitro* system. The other P450 redox partner, cytochrome b5, also can alter the catalytic activity and kinetic profile of P450 metabolism through mechanisms independent of provision of electrons (Jushchyshyn, et al., 2005;Locuson, et al., 2007), purportedly via conformational changes in the P450 induced by cytochrome b5. For

simplicity, cytochrome b5 was omitted from the experiments described herein, but it should be recognized that the situation may be even more complicated than currently presented.

Several explanations have been put forth to explain, at least in part, P450 protein-protein interactions, including the role of ionic bonds between P450 proteins. Kelley et al., (Kelley, et al., 2005) used high concentrations of buffer salts to eliminate any ionic interactions between CYP1A2-CYP2B4, and were able to regain most of the activity that had been diminished by the purported interactions. However, circular dichroism studies have demonstrated conformational changes in proteins at high salt concentrations (Yun, et al., 1996), rendering the aforementioned studies somewhat ambiguous. It is possible that ionic interactions play a role in P450-P450 interactions but additional work in this area is needed.

Studies of P450 protein-protein interactions using either denatured or apo-proteins also demonstrated inhibition of catalytic activity (Kaminsky and Guengerich, 1985), lending additional credence to the hypothesis that conformational changes occur as a result of the protein interactions. However, it must be recognized that these inactive proteins may also bind CPR, albeit to a non-productive endpoint. Locuson et al., demonstrated that apo-b5 could stimulate CYP2C9 mediated metabolism despite its inability to provide electrons (Locuson, et al., 2007). In addition, these results demonstrated that the apo-protein was not competing with the CPR for binding to the enzyme, since activity was stimulated rather than inhibited.

Cytochrome P450 2C9 has a relative large and flexible active site architecture that has been proposed to accommodate more than one substrate molecule simultaneously (Hummel, et al., 2003), whereas it has been reported that CYP2D6 is characterized by a smaller active site volume and accepts primarily basic nitrogen and planar aromatic ring substrates (Rowland, et al., 2006). This larger active site volume and flexibility of CYP2C9 as compared to CYP2D6, may

in part explain the differential inhibition of CYP2C9 if conformational changes induced by P450-P450 interactions are critical to the observed effects. The change in CYP2C9 affinity (K_S) for substrate observed in the current studies is consistent with a conformational change in the active site architecture.

The current studies demonstrate in vitro interactions between two important human P450 enzymes (CYP2C9 and CYP2D6), resulting in a differential reduction in catalytic activity. The order of combining these enzymes yielded unexpectedly different results that could be explained by a simple kinetic scheme consistent with previous modeling results and thus, providing indirect evidence for the formation of CYP2C9-CYP2D6 heteromers, that further complex with CPR to form a reactive entity that results in altered metabolism rates and the potential to confound in vitro-in vivo correlations.

References

Alston K, Robinson RC, Park SS, Gelboin HV and Friedman FK (1991) Interactions among cytochromes P-450 in the endoplasmic reticulum. Detection of chemically cross-linked complexes with monoclonal antibodies. *J Biol Chem* **266**:735-739.

Backes WL, Batie CJ and Cawley GF (1998) Interactions among P450 enzymes when combined in reconstituted systems: formation of a 2B4-1A2 complex with a high affinity for NADPH-cytochrome P450 reductase. *Biochemistry* **37**:12852-12859.

Backes WL and Cawley GF (1999) Interactions among NADPH-cytochrome P450 reductase, P450 1A1, and P4502B4. *Faseb Journal* **13**:A811.

Backes WL and Kelley RW (2003) Organization of multiple cytochrome P450s with NADPH-cytochrome P450 reductase in membranes. *Pharmacol Ther* **98**:221-233.

Cawley GF, Batie CJ and Backes WL (1995) Substrate-dependent competition of different P450 isozymes for limiting NADPH-cytochrome P450 reductase. *Biochemistry* **34**:1244-1247.

Cawley GF, Zhang S, Kelley RW and Backes WL (2001) Evidence supporting the interaction of CYP2B4 and CYP1A2 in microsomal preparations. *Drug Metab Dispos* **29**:1529-1534.

Dutton DR, McMillen SK, Sonderfan AJ, Thomas PE and Parkinson A (1987) Studies on the rate-determining factor in testosterone hydroxylation by rat liver microsomal cytochrome P-450: evidence against cytochrome P-450 isozyme:isozyme interactions. *Arch Biochem Biophys* **255**:316-328.

Estabrook RW, Franklin MR, Cohen B, Shigamatzu A and Hildebrandt AG (1971) Biochemical and genetic factors influencing drug metabolism. Influence of hepatic microsomal mixed function oxidation reactions on cellular metabolic control. *Metabolism* **20**:187-199.

Gorsky LD and Coon MJ (1986) Effects of conditions of reconstitution with cytochrome b5 on the formation of products in cytochrome P450-catalyzed reactions. *Drug Metab Dispos* **14**:89-96.

Guengerich FP (2001) Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem Res Toxicol* **14**:611-650.

Guengerich FP (2006) Cytochrome P450s and other enzymes in drug metabolism and toxicity. *AAPS J* **8**:E101-E111.

Hazai E, Bikadi Z, Simonyi M and Kupfer D (2005) Association of cytochrome P450 enzymes is a determining factor in their catalytic activity. *J Comput Aided Mol Des* **19**:271-285.

Hazai E and Kupfer D (2005) Interactions between CYP2C9 and CYP2C19 in reconstituted binary systems influence their catalytic activity: possible rationale for the inability of CYP2C19 to catalyze methoxychlor demethylation in human liver microsomes. *Drug Metab Dispos* **33**:157-164.

Hummel MA, Gannett PM and Tracy TS. Evaluation of proton to heme distances of flurbiprofen and dapsone within the active-site of CYP2C9. 12th North American ISSX Meeting , 201. 2003.

Jushchyshyn MI, Hutzler JM, Schrag ML and Wienkers LC (2005) Catalytic turnover of pyrene by CYP3A4: evidence that cytochrome b5 directly induces positive cooperativity. *Arch Biochem Biophys* **438**:21-28.

Kaminsky LS and Guengerich FP (1985) Cytochrome P-450 isozyme/isozyme functional interactions and NADPH-cytochrome P-450 reductase concentrations as factors in microsomal metabolism of warfarin. *Eur J Biochem* **149**:479-489.

Kelley RW, Cheng D and Backes WL (2006) Heteromeric complex formation between CYP2E1 and CYP1A2: evidence for the involvement of electrostatic interactions. *Biochemistry* **45**:15807-15816.

Kelley RW, Reed JR and Backes WL (2005) Effects of ionic strength on the functional interactions between CYP2B4 and CYP1A2. *Biochemistry* **44**:2632-2641.

Li DN, Pritchard MP, Hanlon SP, Burchell B, Wolf CR and Friedberg T (1999) Competition between cytochrome P-450 isozymes for NADPH-cytochrome P-450 oxidoreductase affects drug metabolism. *J Pharmacol Exp Ther* **289**:661-667.

Locuson CW, Gannett PM and Tracy TS (2006) Heteroactivator effects on the coupling and spin state equilibrium of CYP2C9. *Arch Biochem Biophys* **449**:115-129.

Locuson CW, Wienkers LC, Jones JP and Tracy TS (2007) CYP2C9 protein interactions with cytochrome b(5): effects on the coupling of catalysis. *Drug Metab Dispos* **35**:1174-1181.

Meijerman I, Sanderson LM, Smits PH, Beijnen JH and Schellens JH (2007) Pharmacogenetic screening of the gene deletion and duplications of CYP2D6. *Drug Metab Rev* **39**:45-60.

Rowland P, Blaney FE, Smyth MG, Jones JJ, Leydon VR, Oxbrow AK, Lewis CJ, Tennant MG, Modi S, Eggleston DS, Chenery RJ and Bridges AM (2006) Crystal structure of human cytochrome P450 2D6. *J Biol Chem* **281**:7614-7622.

Shimada T, Yamazaki H, Mimura M, Inui Y and Guengerich FP (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* **270**:414-423.

Tan Y, Patten CJ, Smith T and Yang CS (1997) Competitive interactions between cytochromes P450 2A6 and 2E1 for NADPH-cytochrome P450 oxidoreductase in the microsomal membranes produced by a baculovirus expression system. *Arch Biochem Biophys* **342**:82-91.

Tracy TS, Hutzler JM, Haining RL, Rettie AE, Hummel MA and Dickmann LJ (2002) Polymorphic variants (CYP2C9*3 and CYP2C9*5) and the F114L active site mutation of CYP2C9: effect on atypical kinetic metabolism profiles. *Drug Metab Dispos* **30**:385-390.

West SB and Lu AY (1977) Liver microsomal electron transport systems. Properties of a reconstituted, NADH-mediated benzo[a]pyrene hydroxylation system. *Arch Biochem Biophys* **182**:369-378.

Yamazaki H, Gillam EM, Dong MS, Johnson WW, Guengerich FP and Shimada T (1997) Reconstitution of recombinant cytochrome P450 2C10(2C9) and comparison with cytochrome P450 3A4 and other forms: Effects of cytochrome P450-P450 and cytochrome P450-b(5) interactions. *Arch Biochem Biophys* **342**:329-337.

Yun CH, Song M, Ahn T and Kim H (1996) Conformational change of cytochrome P450 1A2 induced by sodium chloride. *J Biol Chem* **271**:31312-31316.

Footnote

This work was supported by the National Institutes of Health [Grant #GM 063215].

Figure Legends:

Figure 1: Effect of CYP2D6 on (S)-flurbiprofen metabolism by CYP2C9 at 10 pmol CPR/incubation. Figures 1A, 1B, 1C, 1D and 1E represent SeqAdd1, SeqAdd2, SeqAdd3, Allmix1 and Allmix2 respectively. Data fitting for the plots are shown. For clarity, representative data from a single experiment are presented. However, all experiments were conducted in triplicate over three separate days and the mean values used for inhibition degree determination. Single factor analysis of variance was used for statistical comparisons.

Figure 2: Effect of CYP2D6 on (S)-flurbiprofen metabolism by CYP2C9 at 20 pmol CPR/incubation. Figures 2A, 2B, 2C, 2D and 2E represent SeqAdd1, SeqAdd2, SeqAdd3, Allmix1 and Allmix2 respectively. Data fitting for the plots are shown. For clarity, representative data from a single experiment are presented. However, all experiments were conducted in triplicate over three separate days and the mean values used for inhibition degree determination. Single factor analysis of variance was used for statistical comparisons.

Figure 3: Effect of CYP2D6 on CYP2C9-mediated (S)-flurbiprofen metabolism for three levels of CYP2D6 and two levels of CPR. Figure 3A, 3B and 3C depict the effect of CPR on the amount of inhibition for SeqAdd1, SeqAdd2 and SeqAdd3 respectively. The ratios of V_{max} are significantly different ($p<0.05$) from unity when the CYP2D6 levels are 2.5 and 5 pmol/incubation. In Figures 3A and 3B, increasing CPR (20 pmol vs. 10 pmol) did not affect the degree of inhibition. In Figure 3C (SeqAdd3), a significant ($p<0.05$) decrease in degree of inhibition was observed when CPR levels were 20 pmol and CYP2D6 was at 2.5

pmol/incubation, as compared to 10 pmol of CPR. Single factor analysis of variance was used for statistical comparisons.

Figure 4: Inhibition of CYP2C9-mediated metabolism of (S)-flurbiprofen when CYP2C9, CYP2D6 and CPR were mixed together simultaneously. Figure 4A and 4B show the effect of CPR on the amount of inhibition for Allmix1 and Allmix2 respectively. V_{max} ratios were significantly different ($p<0.05$) from unity when the CYP2D6 levels were 2.5 and 5 pmol/incubation. In all cases, the degree of inhibition was reduced significantly ($p<0.05$) when 20 pmol of CPR was used per incubation, as compared to 10 pmol of CPR. Single factor analysis of variance was used for statistical comparisons.

Figure 5: Effect of CYP2C9 on dextromethorphan metabolism by CYP2D6 at 20 and 40 pmol/incubation of CPR. No CYP2C9 (control) and 1:1 CYP2C9:CYP2D6 ratios were tested.

Figure 6: Spectral binding studies of (S)-flurbiprofen with CYP2C9. Inset shows the absorbance difference as a function of (S)-flurbiprofen concentration used to calculate Ks.

Figure 7: Spectral binding studies of (S)-flurbiprofen with CYP2C9 in the presence of CYP2D6. Inset shows the absorbance difference as a function of (S)-flurbiprofen concentration used to calculate Ks.

Figure 8: Spectral difference binding studies of (S)-flurbiprofen with CYP2D6. No change in binding states was observed.

DMD Fast Forward. Published on May 15, 2009 as DOI: 10.1124/dmd.109.026500
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1: Order of combination of enzymes in the metabolism of (S)-flurbiprofen by CYP2C9, and the effect of CPR.

Mixing Scheme	Order of Addition	Effect of Saturating CPR
SeqAdd1	2C9_2D6_5min_CPR_5min_DLPC	No effect
SeqAdd2	2C9_CPR_5min_2D6_5min_DLPC	No effect
SeqAdd3	2D6_CPR_5min_2C9_5min_DLPC	Reduced Inhibition
Allmix1	2C9_CPR_2D6_10min_DLPC	Reduced Inhibition
Allmix2	2C9_2D6_CPR_10min_DLPC	Reduced Inhibition

DMD Fast Forward. Published on May 15, 2009 as DOI: 10.1124/dmd.109.026500
This article has not been copyedited and formatted. The final version may differ from this version.

Table 2: Effect of CPR on 1:1 CYP2C9:CYP2D6 mediated (S)-flurbiprofen metabolism for various mixing schemes. The units of CPR concentration are pmol/incubation, and the V_{max} ratios are for incubations with and without CYP2D6.

.

	CPR	$V_{max+2D6}$ / $V_{max-control}$ mean ± s.d.	$K_{m+2D6}/K_{m-control}$ mean ± s.d.	CPR	$V_{max+2D6}$ / $V_{max-control}$ mean ± s.d.	$K_{m+2D6}/K_{m-control}$ mean ± s.d.
SeqAdd1	10	0.60 ± 0.03	0.98 ± 0.23	20	0.61 ± 0.03	0.99 ± 0.12
SeqAdd2	10	0.69 ± 0.1	1.06 ± 0.27	20	0.78 ± 0.03	0.97 ± 0.26
SeqAdd3	10	0.52 ± 0.1	1.32 ± 0.65	20	0.69 ± 0.1	1.07 ± 0.32
Allmix1	10	0.54 ± 0.01	1.43 ± 0.56	20	0.71 ± 0.05	1.05 ± 0.11
Allmix2	10	0.56 ± 0.04	0.78 ± 0.10	20	0.8 ± 0.05	1.01 ± 0.27

Addition of excess (20 pmol) CPR significantly ($p<0.05$) increased the V_{max} ratios in Allmix 1 and Allmix 2, but not in SeqAdd1 and SeqAdd2. In SeqAdd3, a significant ($p<0.05$) increase in V_{max} ratios was seen at 2.5 pmol of CYP2D6 only between the two CPR levels. No significant changes were observed in the K_m ratios upon addition of excess CPR in all systems. Single factor analysis of variance was used for statistical comparisons.

Figure 1

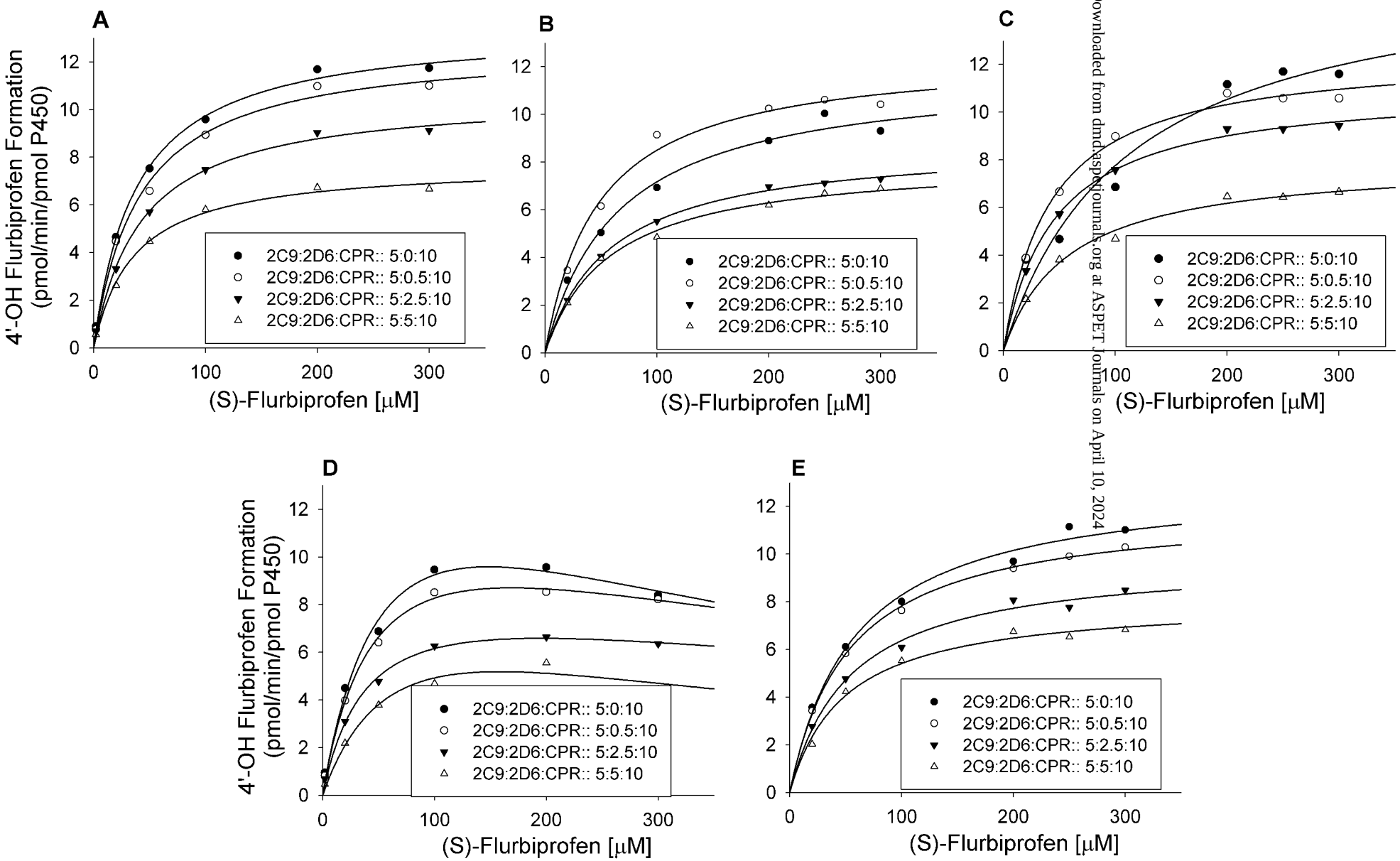


Figure 2

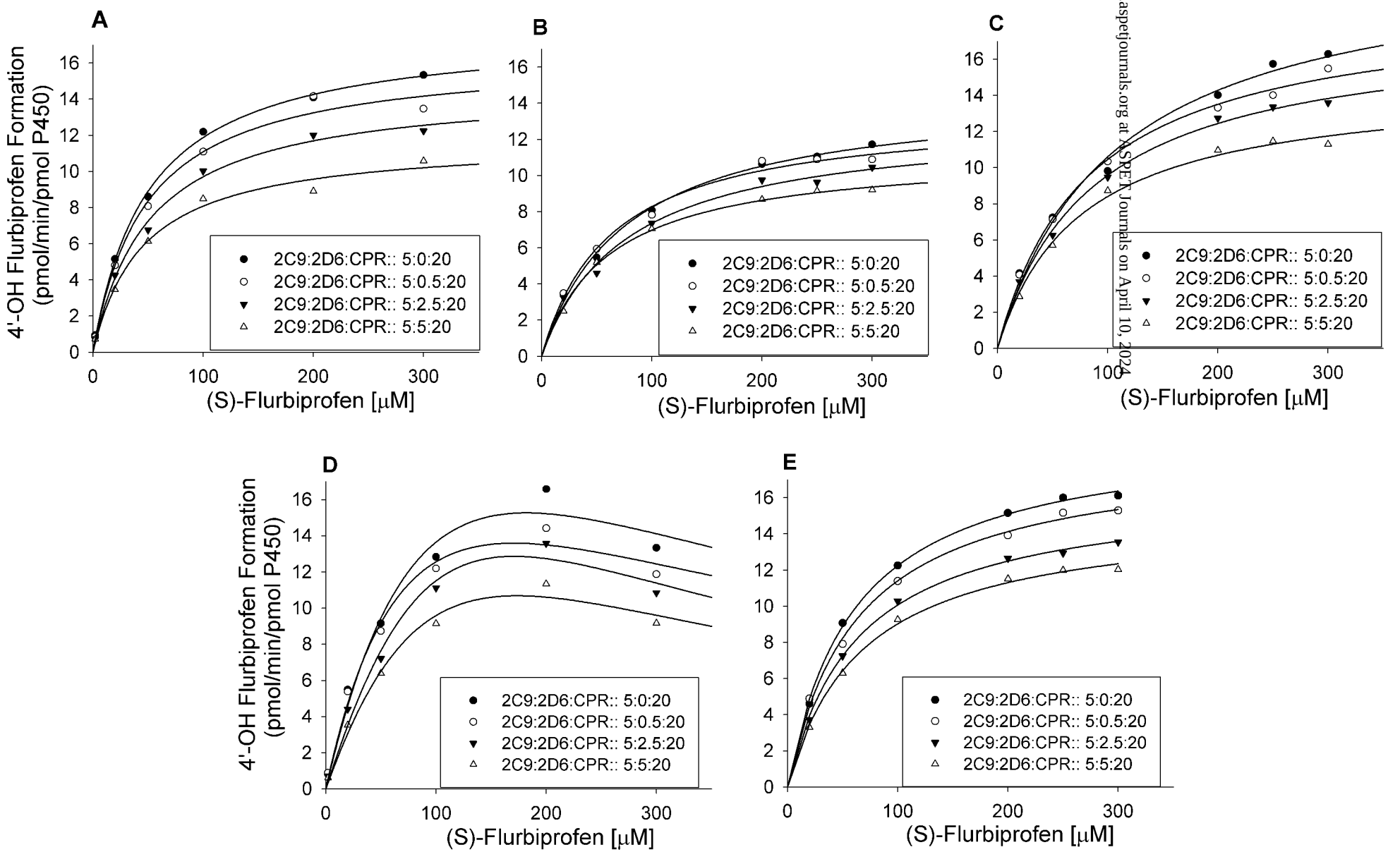
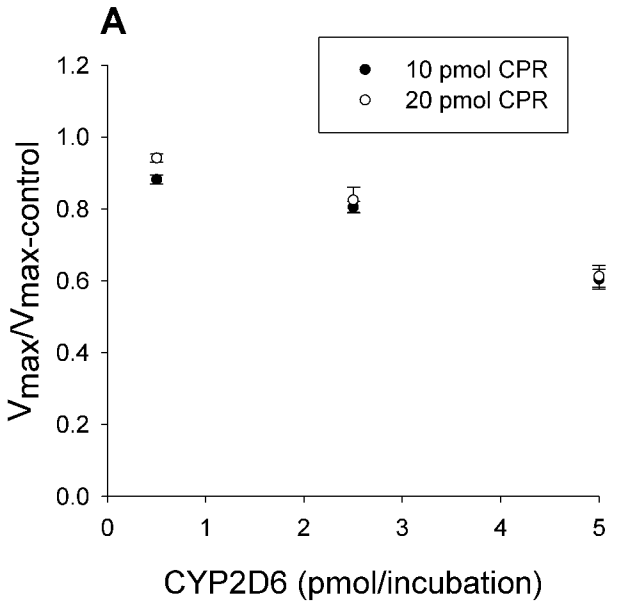
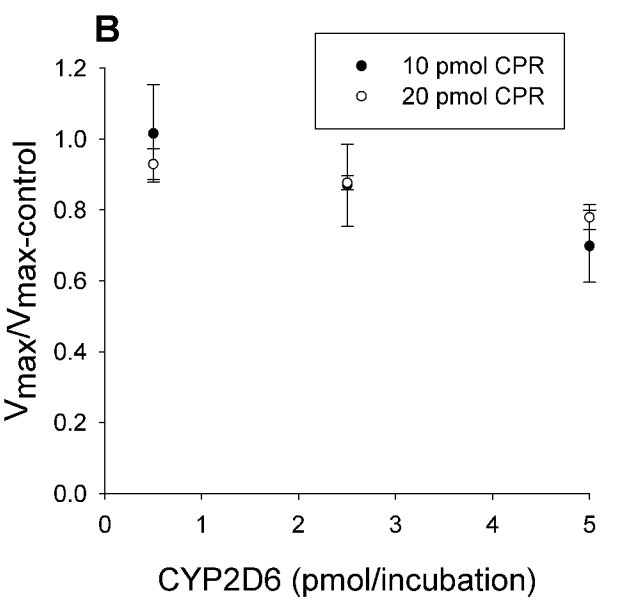


Figure 3





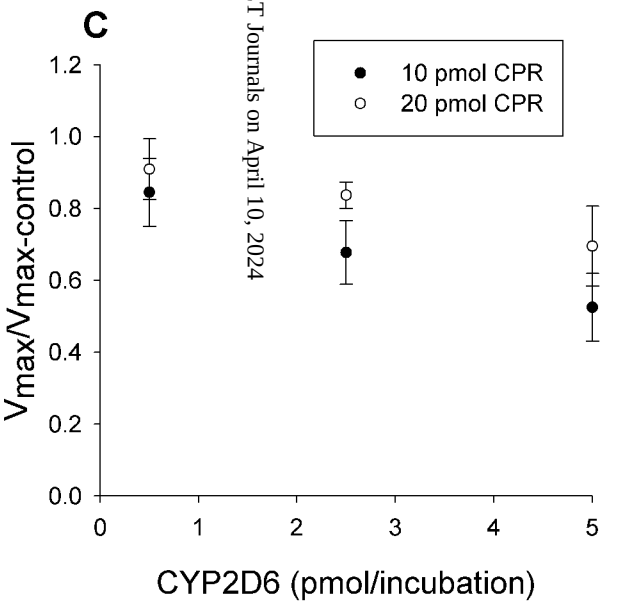
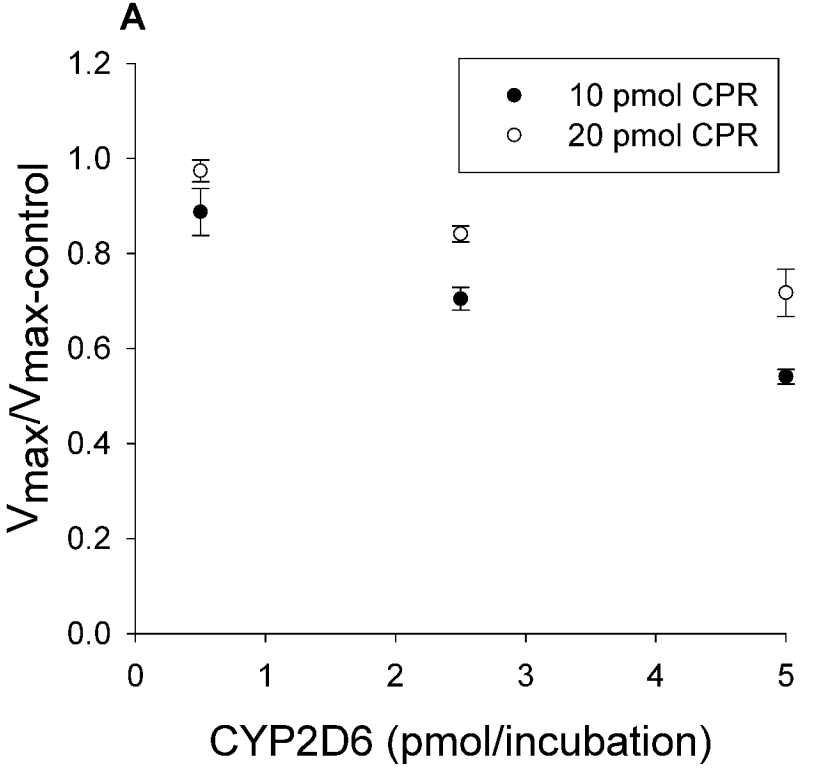


Figure 4



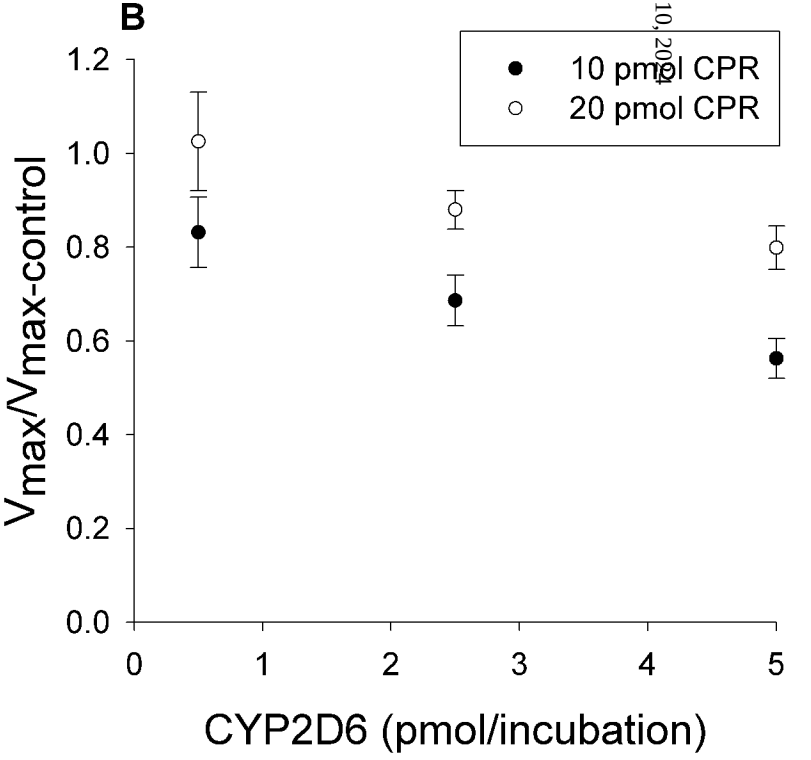


Figure 5

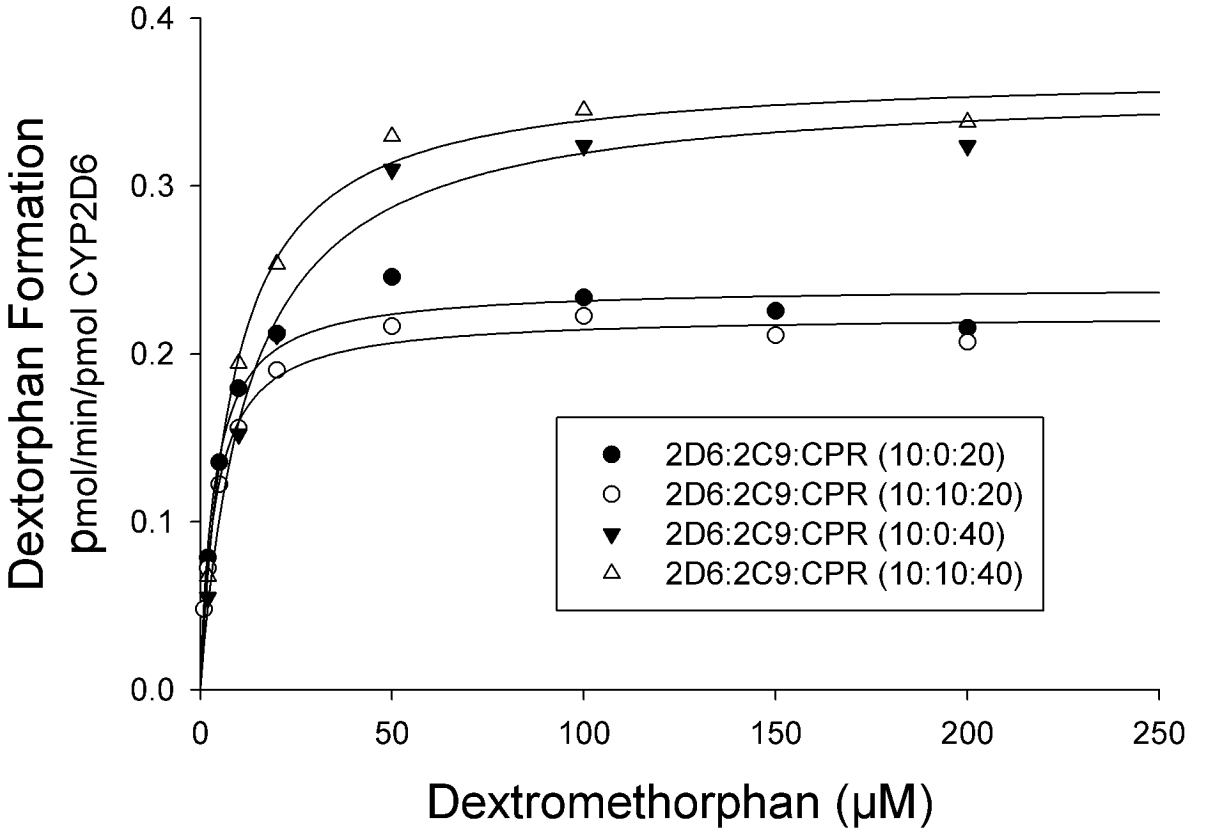

Dextorphan Formation
pmol/min/pmol CYP2D6
0.4
0.3
0.2
0.1
0.0
0
50
100
150
200
250
Dextromethorphan (μM)
2D6:2C9:CPR (10:0:20)
2D6:2C9:CPR (10:10:20)
2D6:2C9:CPR (10:0:40)
2D6:2C9:CPR (10:10:40)

Figure 6

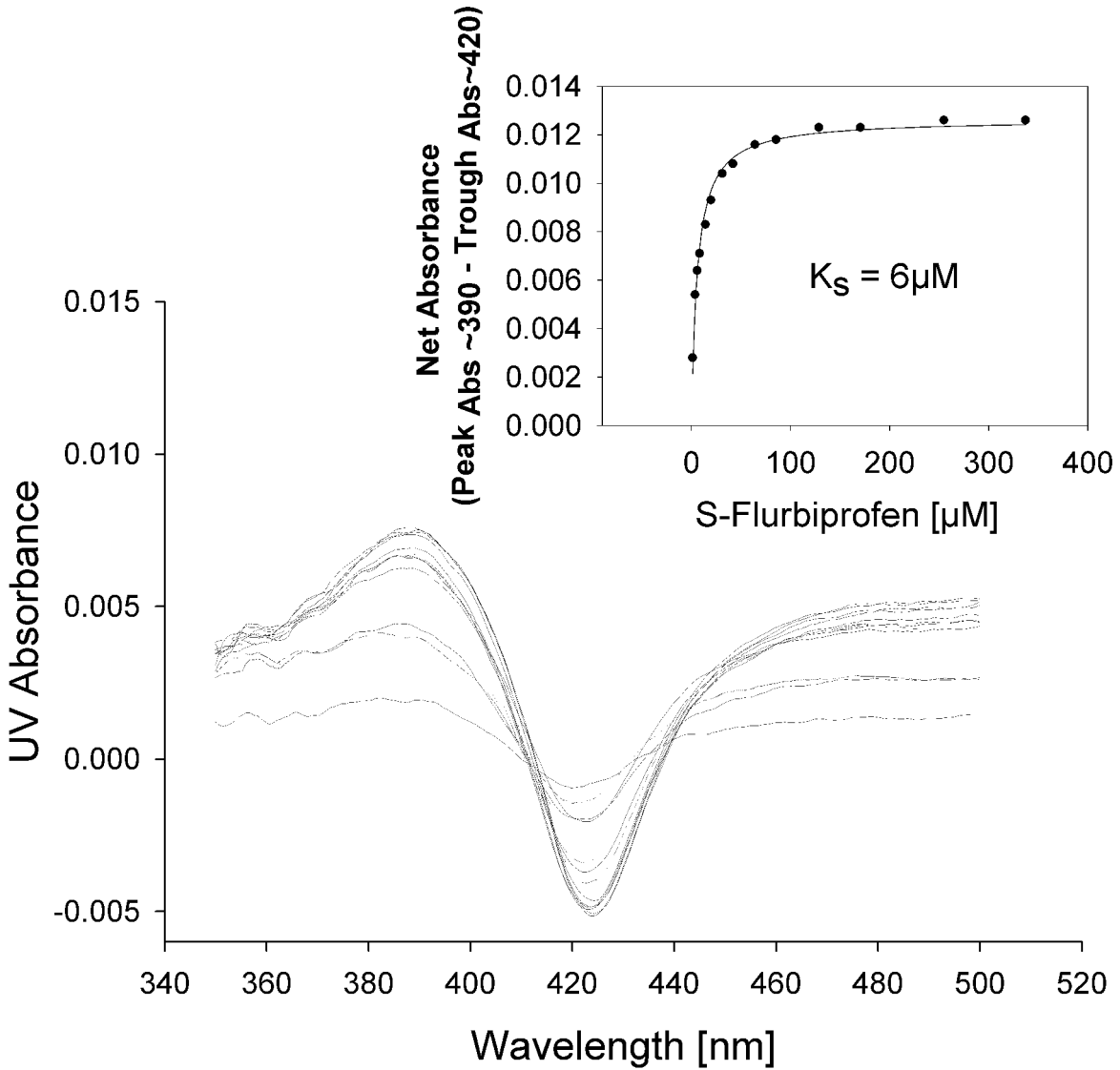

Net Absorbance
(Peak Abs ~390 - Trough Abs~420)
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0
100
200
300
400
S-Flurbiprofen [µM]
K_S = 6µM
UV Absorbance
0.015
0.010
0.005
0.000
-0.005
340
360
380
400
420
440
460
480
500
520
Wavelength [nm]

Figure 7

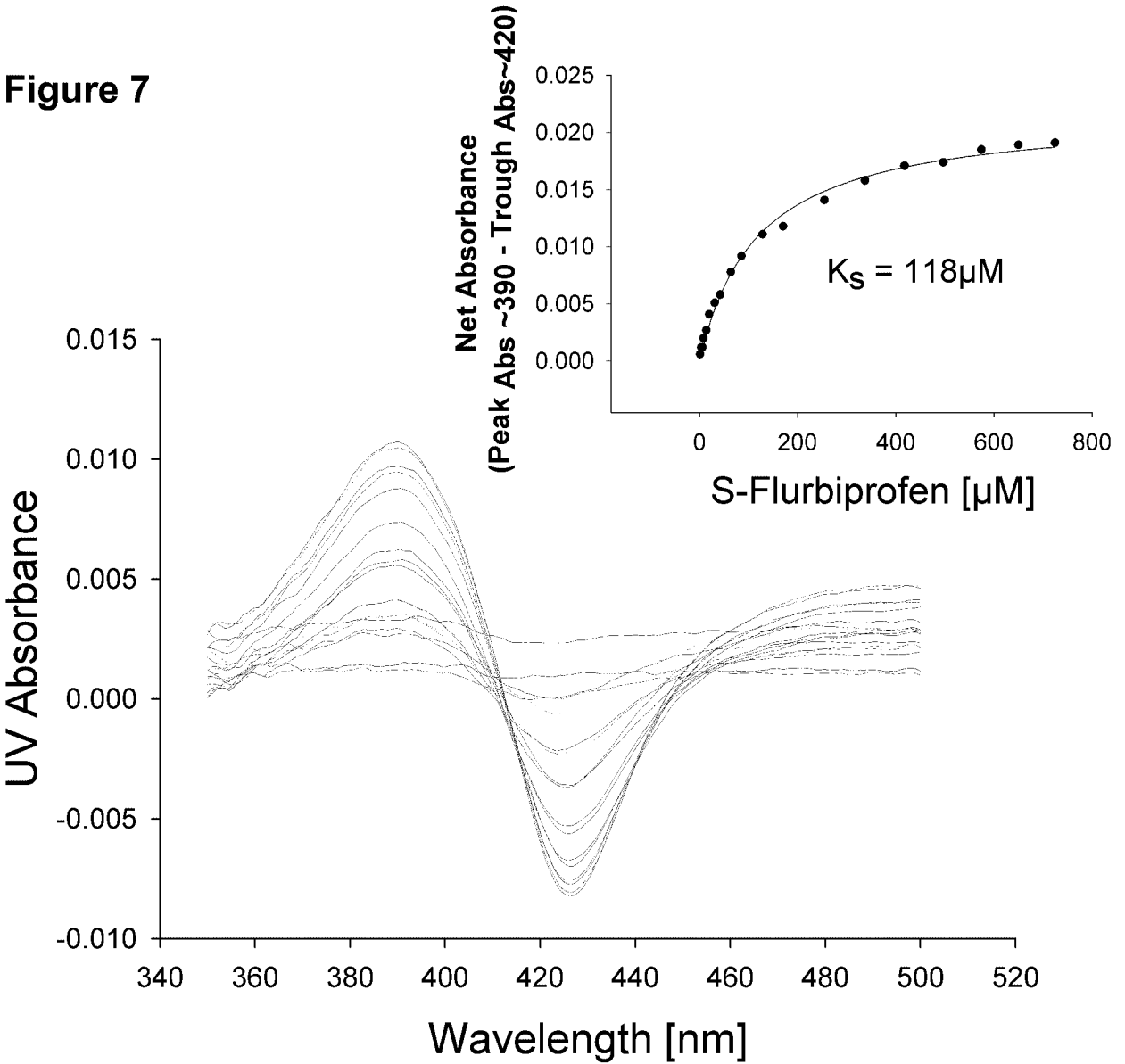
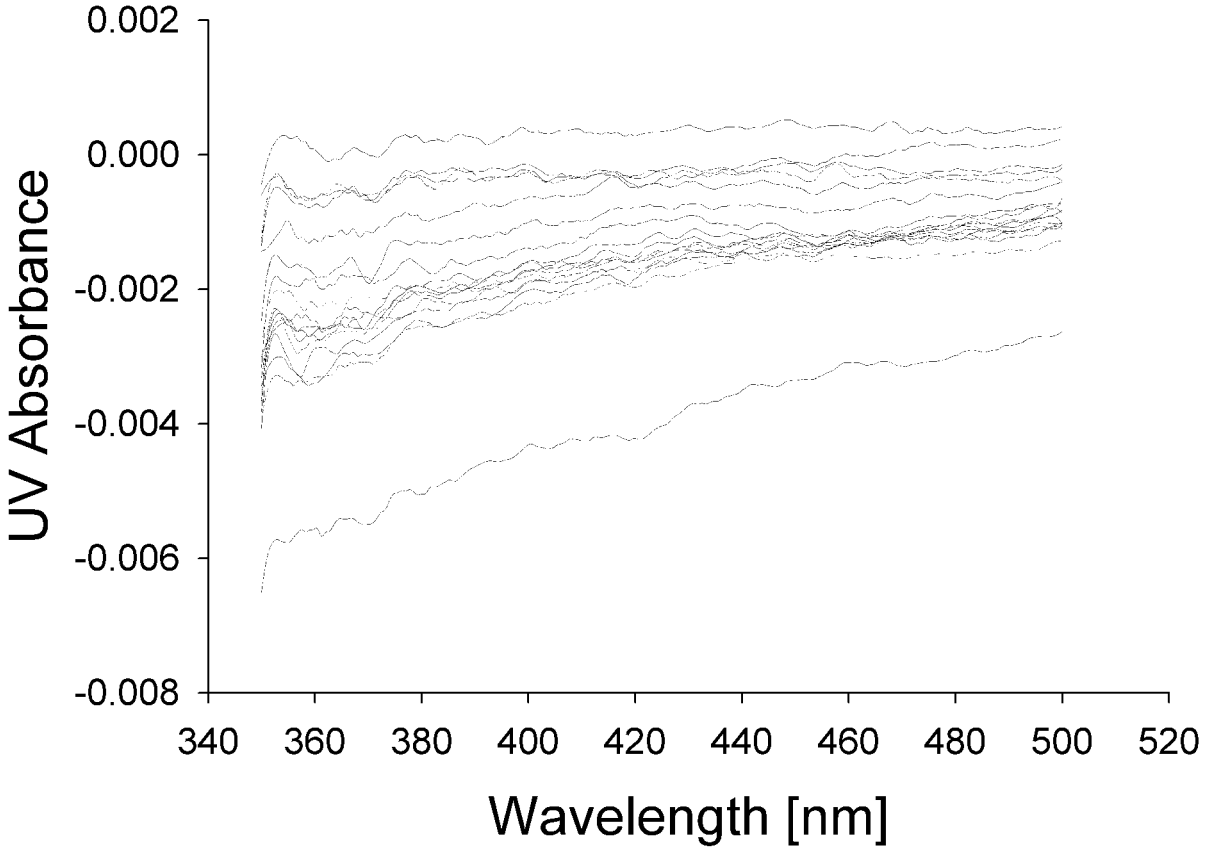

Net Absorbance
(Peak Abs ~390 - Trough Abs~420)
0.025
0.020
0.015
0.010
0.005
0.000
0
200
400
600
800
K_S = 118µM
S-Flurbiprofen [µM]
UV Absorbance
0.015
0.010
0.005
0.000
-0.005
-0.010
340
360
380
400
420
440
460
480
500
520
Wavelength [nm]

Figure 8


0.002
0.000
-0.002
-0.004
-0.006
-0.008
UV Absorbance
340
360
380
400
420
440
460
480
500
520
Wavelength [nm]