Current cytochrome P450 phenotyping methods applied to metabolic drug-drug
interaction prediction in dogs

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Abstract

Recombinant cytochrome P450 phenotyping, different approaches for estimating fraction metabolized ($f_m$), and multiple measures of in vivo inhibitor exposure were tested for their ability to predict drug interaction magnitude in dogs. Previously, midazolam-ketoconazole interaction studies in dogs have been attributed to inhibition of CYP3A pathways. However, in vitro phenotyping studies demonstrated higher intrinsic clearances ($CL_{int,app}$) of midazolam with canine CYP2B11 and CYP2C21. Application of activity correction factors and isoform hepatic abundance to liver microsome $CL_{int,app}$ values further implicated CYP2B11 ($f_m \geq 0.89$) as the dog enzyme responsible for midazolam- and temazepam-ketoconazole interactions in vivo. Mean AUC$_i$/AUC ratios from intravenous and oral midazolam interaction studies were predicted well with unbound $K_i$ and estimates of unbound hepatic inlet inhibitor concentrations and intestinal metabolism using the AUC-competitive inhibitor relationship. No interactions were observed in vivo with bufuralol, although significant interactions with bufuralol were predicted with fluoxetine via CYP2D and CYP2C pathways (>2.45-fold) but not with clomipramine (<2-fold). The minor caffeine-fluvoxamine interaction (1.78-fold) was slightly higher than predicted values based on determination of a moderate $f_m$ value for CYP1A1, although CYP1A2 may also be involved in caffeine metabolism. The findings suggest promise for in vitro approaches to drug interaction assessment in dogs, but also highlight the need to identify improved substrate and inhibitor probes for canine P450s.
Introduction

Metabolism-mediated drug interactions are an important consideration during pre-clinical drug lead optimization. Inhibitors of drug metabolizing enzymes such as cytochromes P450 (P450) may be capable of decreasing the clearance of co-administered drugs when their clearance is metabolic. Therefore, it is important to evaluate new chemical entities as substrates and inhibitors of P450. To speed this evaluation, in vitro-in vivo extrapolation methods aimed at predicting metabolic drug interactions have continued to evolve. For instance, the choice of inhibition values (e.g. $K_i$ vs. unbound $K_i$) (Brown et al., 2006), inhibitor absorption rates (Brown et al., 2005; Kanamitsu et al., 2000), P450 induction (Fahmi et al., 2008), and the choice of in vivo concentrations of P450 inhibitors (Brown et al., 2005; Obach et al., 2005) have all been studied with respect to in vitro-based drug-drug interaction (DDI) predictions. Irreversible enzyme inhibition mechanisms, though recognized for some time, have increasingly been added to in vitro-based drug interaction extrapolation methods with assumptions about the turnover rates of P450 isoforms (Mayhew et al., 2000; Venkatakrishnan and Obach, 2007). Several of these in vitro findings or approaches have even been integrated into several commercial software packages as biology continues to advance towards more physiological-based modeling.

Other critical factors needed to predict drug interaction magnitude (e.g. AUCi/AUC ratio) are the relative contribution and inhibition levels of each metabolic pathway involved in clearance of a victim drug (Brown et al., 2005; Ito et al., 2005). Determining the relative fraction metabolized ($f_{in,P450}$) and the inhibition constant $K_i$ has traditionally required
inhibitors and substrates with high specificity for use in liver microsome incubations. Alternatively, recent studies have suggested that the in vitro fraction metabolized value for human P450s 3A4 and 2D6 determined with recombinant enzymes was similar to chemical inhibitors used with liver microsomes or hepatocytes at predicting the AUC/AUC ratio (McGinnity et al., 2008; Youdim et al., 2008).

Despite the advances in DDI prediction, the lack of probe substrates and probe inhibitors has limited the ability to associate DDIs in non-human species with specific P450 enzymes. In veterinary medicine where daily administered pharmaceutical agents are increasingly being pursued for dogs, various drug metabolizing enzymes have not been extensively studied. Furthermore, several drugs used as P450 probes for human enzymes appear to lack the same degree of specificity and affinity with the dog P450s. For example, quinidine was shown to be a weaker inhibitor of canine CYP2D15 than of human CYP2D6 (Roussel et al., 1998), canine liver microsomes demonstrate lower activity towards the human CYP2C9 substrate tolbutamide and less inhibition of CYP1A activity by furafylline (Chauret et al., 1997), and canine CYP2C isoforms demonstrate higher $K_m$ values for diclofenac and lower velocities for (S)-mephenytoin than the human CYP2C enzymes (Shou et al., 2003). These species differences do not preclude the use of liver microsomes for inhibition analysis in non-human species, but until specific P450 clearance pathways for drugs are identified, their use may be somewhat limited in discovery screening. With microsomes, all possible drugs that might be co-administered with new chemical entities would need to be evaluated as inhibitors and new chemical entities would need to be evaluated as inhibitors of co-administered drugs. On the other
hand, recombinant drug metabolizing enzymes allows one to catalogue drugs as substrates and/or inhibitors of specific P450s and to perform more targeted screening and DDI assessment.

Now that several of the dog hepatic P450s have been cloned and expressed, several new opportunities exist to screen compounds in early drug discovery efforts in order to characterize their P450 clearance and inhibition profiles. To date, seven P450 cDNA sequences cloned from canine liver have been expressed for study (Shou et al., 2003), but rP450s have not yet been evaluated for their ability to predict metabolic DDIs. Taking advantage of the *Escherichia coli* expressed canine P450s recently reported (Locuson et al., 2009), this report describes the use of current *in vitro* methods to predict *in vivo* drug interaction magnitude in dogs.
Materials and Methods

Chemicals and reagents. Substrates, inhibitors, and other reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified. Phosphate-buffered saline was purchased from Invitrogen (Carlsbad, CA). Metabolite standards for diclofenac, bufuralol, and midazolam were from BD Gentest (Franklin Lakes, NJ). All solvents were acquired from Honeywell Burdick and Jackson (Morristown, NJ). Midazolam solution (5 mg/mL, Abraxis Pharmaceutical), ketoconazole tablets (200 mg, Teva Pharmaceutical Industries, Ltd.), temazepam capsules (15 mg, Actavis Elizabeth), and fluvoxamine maleate tablets (50 mg, Barr Laboratories) were obtained by Pfizer Veterinary staff. Clomipramine HCl was purchased from Sigma. Racemic bufuralol-HCl and fluoxetine-HCl used for in vivo studies were synthesized within Pfizer. Bufuralol, clomipramine, fluoxetine, and caffeine were added to size #4 gelatin capsules for dosing. Pooled dog liver microsomes were from Xenotech LLC (Lanexa, KS) and beagle rP450 Bactosomes® containing beagle P450 reductase were prepared as described (Locuson et al., 2009). Control Escherichia coli Bactosomes were obtained from Cypex Ltd (Dundee, UK). Beagle plasma was obtained from potassium EDTA-treated blood (Bioreclamation, Inc., Westbury, NY).

Enzyme kinetics, ISEF, and fm,P450 values. Substrate depletion kinetics were carried out with rP450 (50 – 150 pmol/mL) or beagle liver microsomes (0.5 mg/mL) as described using six time points over a one hour incubation (Baratta et al., 2009). Caffeine metabolism studies were conducted with higher enzyme concentrations (500 pmol/mL rP450) due to its lower turnover. Low caffeine activity prevented full kinetic profiling,
but the relative rates of paraxanthine formation could be characterized with CYP1A1, 2B11, and 2C21 at 50 µM caffeine. Caffeine samples were pre-incubated at 37°C for 3 minutes with enzyme and all reactions were initiated with the addition of substrate to the reaction mixture. The reaction was terminated by the addition of 400 µl cold acetonitrile containing an internal standard and centrifuged at 20,000 x g for 5 minutes. A 10 µl aliquot of the supernatant was used for analysis.

Substrate titration kinetics for temazepam, diclofenac, bufuralol, and midazolam were determined previously with rP450 2B11, 2C21, 2D15, and 3A12, respectively (Locuson et al., 2009) and liver microsomes (Aidasani et al., 2008), which were converted to units of nmol/min/mg using the microsome lot P450 concentration of 0.462 nmol/mg. Temazepam (rP450 2C21), bufuralol (rP450 2C21), and midazolam (rP450 2B11 and CYP2C21) kinetics were determined for oxazepam, 1'OH-bufuralol, and 1'OH-midazolam to have \( V \) values of 3.2, 0.83, 3.2 and 2.8 nmol/min/nmol, and \( K_m \) values of 15.5, 2.2, 5.4, and 4.8 µM. Curves were fit using the Michaelis-Menten function, except for midazolam with CYP2C21, which demonstrated substrate inhibition and was fit with the function:

\[
\nu = \frac{(V \times [S])/(K_m+[S])+(S^2/K_{s,i})}{K/K}
\]

\( V/K \) and \( V \) ISEF values were determined from rP450 and microsome kinetics as described (Proctor et al., 2004; Youdim et al., 2008) using the hepatic expression estimates of 17, 48, 160, 160, and 69 pmol/mg for CYP1A, 2B, 2C, 2D, and 3A (Eguchi et al., 1996; Sakamoto et al., 1995). ISEF-corrected activities, or relative abundance-corrected activities were then used to calculate \( f_{m,P450} \) values. The relative abundance values used for CYP1A, 2B, 2C, 2D, and 3A were 0.035, 0.099, 0.331, 0.200, and 0.143 with CYP2D being lower than in pmol/mg units.
since the values were obtained from separate studies. Ascribing these values to P450 subfamilies should reinforce the fact that they may not differentiate between specific isoforms.

\textit{IC}_{50} and \textit{K}_i determination.  \textit{IC}_{50} values were determined as described from duplicate determinations except that rP450s were used in place of liver microsomes (25 pmol P450/mL) (Aidasani et al., 2008). Typically, \textit{K}_i was only determined for P450 isoforms exhibiting the lowest \textit{IC}_{50}s for a given victim-inhibitor combination. Experiments were performed in triplicate. \textit{K}_i was estimated from \textit{IC}_{50}, and then four inhibitor concentrations were selected for each substrate-enzyme pair (0, 0.5, 4, and 8 times the estimated value). Eight substrate concentrations were then chosen that spanned a range at least ten-fold above and five-fold below \textit{K}_m. Any co-solvents used in preparation of substrate and inhibitor solutions were normalized across all incubations. Incubations (0.1 mL) contained 25 pmol P450/mL, were initiated with a final concentration of 1.0 mM NADPH, and were quenched after 5 min with acetonitrile containing an internal standard. Non-linear, global regression fitting to the steady-state competitive inhibition model was then used to determine \textit{K}_i from plots of metabolite formation rate versus substrate concentration (\(v=(V\times[S])/(1+[I]/K_i\timesK_m+[S])\)). Reciprocal re-plot patterns suggested all inhibition mechanisms were competitive though it is recognized that there are reports of non-competitive inhibition with human rCYP3A4 and ketoconazole.

\textit{Protein binding.} Plasma protein binding and Bactosome protein binding of inhibitors were conducted with test compounds at 2 \(\mu\)M, near the average plasma levels observed in
pharmacokinetic studies. Bactosome protein binding was conducted with control Bactosomes at the same protein levels used in $K_i$ experiments (0.1 mg/mL). The Rapid Equilibrium Dialysis apparatus was used for binding studies according to the manufacturer’s instructions (Pierce Biosciences, Rockford, IL) and using phosphate-buffered saline in the buffer chambers. Experiments were conducted in quadruplicate and were automated on a Microlab STAR (Hamilton, Reno, NV). Plates were incubated on an orbital shaker (90 rpm) uncovered in a CO₂ incubator (5 %) at 37 °C and 90% humidity for 4 h. Buffer and protein chamber matrices were then matched before precipitating samples with acetonitrile containing an internal standard.

*Drug and dose selection.* All substrates were chosen based on a combination of metabolite selectivity by a particular P450 *in vitro* and high metabolic clearance *in vivo* in humans. Inhibitors were chosen primarily based on evidence from liver microsomes that they had high affinity for particular P450s, at least within an order of magnitude of human P450 IC$_{50}$s. Previous evidence for the ability of dogs to tolerate substrates and inhibitors was carefully considered. Some substrates were not thought to be promising probes. For instance, a possible CYP2C substrate such as an arylacetic acid profen was not evaluated due to toleration issues and non-P450 clearance routes, and because dog CYP2C enzymes have lower affinity and/or activity for other CYP2C probe substrates (Chauret et al., 1997; Shou et al., 2003). Dextromethorphan was not used as a CYP2D substrate due to potential toleration issues and to competing clearance by CYP3A.
Moderate substrate drug doses (near therapeutic range for dogs and/or humans) were chosen for safety reasons in the event of a large interaction, to limit pharmacological effects, and to limit metabolic clearance to the pathways with the highest efficiency. Although dose proportionality was not established for substrates, the doses are in or near the linear pharmacokinetic range for humans. Supratherapeutic doses of the selective serotonin re-uptake inhibitors and clomipramine were chosen to maximize the chance of drug interaction with CYP2D15, whereas the ketoconazole dose is within the therapeutic range for dogs. A >20% increase in the victim drug area-under-the-curve (AUC) was targeted. Doses were then calculated based on pharmacokinetics, IC\textsubscript{50}s determined with liver microsomes, and the assumption that \( f_{\text{m,in \, in vivo}} = 1 \) (Eq. 1). No correction for non-specific protein binding was made at this time since the free drug fraction in plasma and calculation of unbound IC\textsubscript{50} likely would offset each other to some degree.

\textit{Pharmacokinetic studies.} All pharmacokinetic studies were carried out in compliance with national legislation and subject to local ethical review by an IACUC committee consisting of internal and external members. Groups of four female research beagles 7 – 13 kg were selected in order to investigate the effects of multiple dosing of putative inhibitors on the pharmacokinetics of victim drugs. Adult dogs (>2 yr) were used since young dogs have underdeveloped xenobiotic metabolism capacity (Aldridge and Neims, 1980; Tanaka et al., 1998). Dogs were fed their daily allotment of dry food exactly 1 h before dosing. In Phase I, each dog was orally or intravenously (i.v.) administered the victim drugs at the beginning of each study (day 1) and serial blood samples were collected over 24 h for determination of the pharmacokinetics. Capsules and tablets were
administered near the back of the mouth. This was followed by 2 mL of water delivered to the mouth by syringe to aid in swallowing. Intravenous midazolam was dosed via the cephalic vein. In Phase II, beginning one day later, the inhibitor was orally administered once daily for three days. On the third day, one hour after the last dose of inhibitor, the victim was again administered and serial blood samples were collected. Typical collection times were pre-dose, 2 min (for i.v. dosing), 1.0, 1.5, 2.0, 3.0, 5.0, 8.0 and 24 h. Both victim and inhibitor concentrations were measured in plasma. Pharmacokinetic analysis was performed using the non-compartmental approach (linear trapezoidal rule for AUC calculation) over the dosing interval (0-24 h) with Watson (v7.2, Thermo Electron Corp., Philadelphia, PA).

Liquid chromatography tandem mass spectrometry. Enzyme kinetic, protein binding, and plasma pharmacokinetic study samples were all analyzed using standard sample solvent precipitation, gradient reverse phase ultra performance liquid chromatography, and positive mode tandem mass spectrometry methods similar to those described previously (Locuson et al., 2009). The analytical system consisted of a Waters Acquity UPLC system (Waters Corp., Milford, MA) coupled to an API4000 mass spectrometer (Applied Biosystems, Foster City, CA). Caffeine metabolism experiments required careful separation due to some desmethyl metabolites having the same masses and transitions. In this case, separation was accomplished isocratically using an Acquity UPLC® BEH HILIC column (2.1 x 50 mm, 1.7 μm, Waters Corp.) and a mobile phase consisting of 85% water containing 0.1% formic acid and 15% acetonitrile. After caffeine eluted at 1.07 min, the acetonitrile was increased to 70% to wash the column.
The flow rate was 0.8 mL/min. Mass spectrophotometric analysis was performed in the positive ion mode using multiple reaction monitoring of the transitions 181 → 124 m/z for paraxanthine (0.56 min) and theophylline (0.62 min), 181 → 138 m/z for theobromine (0.42 min) and 211 → 196 m/z for trimethyluric acid (0.70 min).

**Drug interaction magnitude prediction.** A common measure of DDI magnitude, the ratio of AUC in the presence of inhibitor to AUC in the absence of inhibitor (AUCi/AUC), was predicted using the competitive inhibitor relationship (Rowland and Matin, 1973). Inhibition of multiple P450 clearance pathways for a single substrate by a single inhibitor was considered with the use of $f_m$ and $K_i$ values for each P450 as demonstrated in Equation 1

$$\frac{AUC_i}{AUC} = \frac{1}{\sum_{x=1}^{n} \left( \frac{f_{m,x}}{1 + \left( \frac{[I]}{K_{i,x}} \right)} \right) + \left(1 - \sum_{x=1}^{n} f_{m,x} \right)}$$

where $x$ refers to a specific P450 clearance pathway. This approach has been well-documented and continues to be evaluated in the drug discovery process (Brown et al., 2005; Obach et al., 2006; Obach et al., 2005). Nevertheless, it is still important to recall some important assumptions, such as a well-stirred model with rapid inhibitor distribution through the liver, a constant inhibitor concentration in the liver during each pass, passive diffusion of inhibitor into the liver, the victim drug is orally administered and its concentrations < $K_m$ for each P450 metabolism pathway, and a competitive or non-competitive P450 inhibition mechanism. Additional terms are needed for intestinal
inhibition and assessments for i.v. administration assumes victims are low extraction drugs with unbound intrinsic clearances that are much lower than hepatic blood flow.

Input parameters were selected based on the following logic. \( K_i \) values used in predictions were determined as described above except that the values were converted to unbound \( K_i (K_{i,u}) \) according to the free fraction of inhibitor present in rP450 incubations. Four estimated values of \([I]_{in \, vivo}\) were tested from the last study day where victim and perpetrator drugs were co-administered because of the difficulty in characterizing this parameter: (1) free hepatic inlet concentration estimated using the average systemic free plasma concentration, \( C_{avg,u} \) (0-24 h), (2) free hepatic inlet concentration estimated using the maximum systemic free plasma concentration, \( C_{max,u} \), (3) systemic \( C_{avg,u} \) (0-24 h), and (4) systemic \( C_{max,u} \). The method of Kanamitsu was used for calculating hepatic inlet inhibitor concentrations (Kanamitsu et al., 2000) (Equation 2):

\[
C_{hep,inlet} = C_p + \left( \frac{D \times k_a \times F_a}{Q_h} \right) \times f_{u,b} \quad \text{Eq. 2}
\]

where \( C_p \) is either \( C_{avg,u} \) or \( C_{max,u} \) (corrected for blood partitioning where possible), \( D \) is dose, \( k_a \) is the absorption rate constant (0.03 min\(^{-1}\) used as the default value), \( F_a \) is the fraction absorbed, \( Q_h \) is hepatic blood flow, and \( f_{u,b} \) is the free blood fraction. Free inhibitor concentrations were corrected for blood partitioning using the human values for ketoconazole (B:P=0.69) (Shibata et al., 2008), fluoxetine (B:P=0.55), and fluvoxamine (B:P=0.55) (McGinnity et al., 2008). A value of B:P was not found for clomipramine so a value of 1 was used since the analogs amitriptyline and imipramine have ratios less than 1, but their inhibitory desalkyl metabolites have compensating ratios greater than 1 (Amitai et al., 1993). An intermediate value of 35 mL/min/kg for dog liver blood flow
was used. The value is similar to that suggested by Davies and Morris after their review of several published values (30.9 mL/min/kg) (Davies and Morris, 1993).

The $f_m$ value used in Eq. 1 for each P450-victim pair was derived using estimates of $f_{m,\text{in vivo}}$ and the relative $f_{m,P450}$ values determined using either rP450 ISEF or hepatic abundance-corrected $CL_{\text{int,app}}$ values as described above. A comprehensive listing of all the calculated $f_m$ values evaluated in Eq. 1 is provided in Supplementary Data. Since both $f_m$ values are fractions, the $f_{m,\text{in vivo}}$ value used in DDI extrapolation, which should reflect turnover to all metabolites by P450, is calculated by multiplication ($f_{m,\text{in vivo}} \times f_{m,P450}$).

Two estimates of $f_{m,\text{in vivo}}$ were evaluated. $f_{m,\text{in vivo}}=1$ was used as a conservative estimate and then compared to additional $f_{m,\text{in vivo}}$ values for oxidative metabolism that were acquired from published excretion studies. High midazolam $f_{m,\text{in vivo}}$ values have been observed in excretion studies (0.99) (Smith et al., 1981) and values $>0.90$ have typically been used for human DDI extrapolations. In dogs, no unchanged drug was observed in urine (Vree et al., 1981), and no reports of fecal excretion could be found. As a result, only $f_{m,\text{in vivo}}=1$ was evaluated for midazolam. A study using a high dose of temazepam in dogs found high levels of oxazepam, oxazepam glucuronide, and temazepam glucuronide (Schwarz, 1979). Less than half of the dose was recovered in urine, but approximately 50% of the dose, and 50% of total urinary constituents, was recovered as oxazepam and oxazepam glucuronide so an $f_{m,\text{in vivo}}=0.5$ was assumed. Bufuralol excretion data from dogs could not be found, but human studies have demonstrated a wide range of metabolic
clearances likely due to the polymorphic CYP2D6 enzyme (Dayer et al., 1986). The human extensive metabolizer value of $f_{m,\text{in vivo}} = 0.8$ was used due to the high *in vitro* and *in vivo* dog clearance determined here. Caffeine metabolism is extensive in humans (>95%) (Callahan et al., 1982) and dogs (96%) (Aldridge and Neims, 1979) where a majority of the dose is excreted in urine as oxidative metabolites. Sixty-six percent of the administered dose in dogs was previously recovered as characterized metabolites, but 96% was recovered as metabolites relative to total urinary constituents (Aldridge and Neims, 1979). Caffeine undergoes extensive sequential metabolism, but several of the metabolites have been shown to be formed by P450 so a value of $f_{m,\text{in vivo}} = 0.96$ was used.

The impact of intestinal metabolism on midazolam AUC was estimated using $F_a \times F_g$, calculated by oral bioavailability, the determined i.v. plasma clearance of 24.7 mL/min kg corrected using the human blood:plasma partitioning ratio of 0.86 (Gorski et al., 1998), and liver blood flow (35 mL/min/kg) (Benet et al., 1996). $F_a$, the fraction absorbed, is high for midazolam in humans after correction for its high extraction (Gorski et al., 1998) so a value of 1 was used allowing the estimation of $F_g$ as 0.334. This *in vivo* $F_g$ approximation assumes that intestinal metabolism of an i.v. administered drug is negligible, as is any active drug transport. *In vitro* methods have also been evaluated for estimating $F_{g,i}/F_g$, but both *in vivo* and *in vitro* methods have been useful for high intestinal extraction drugs with minimal active drug transport potential (Galetin et al., 2008). Complete intestinal inhibition ($F_{g,i} = 1$) was used as a conservative assumption based on the large doses of inhibitors present in the gut and because dissolved drug levels could not be characterized. The midazolam $F_{g,i}/F_g$ value (0.299) was also used for
temazepam based on the similar rP450 kinetics and high \textit{in vivo} clearances of the two compounds. The $F_{g,i}/F_g$ ratio for midazolam was then multiplied by the hepatic DDI parameters for midazolam and temazepam (Eq. 1). Bufuralol is a highly metabolized drug in humans (Balant et al., 1980), but does not appear to be subject to extensive intestinal metabolism as a value of $F_a \times F_g$ was estimated to be $>1.0$. Oral caffeine is completely absorbed intact in humans (Blanchard and Sawers, 1983). Therefore, $F_{g,i}/F_g$ ratios of 1 were used for bufuralol and caffeine.

Statistical measures of DDI prediction accuracy were not calculated due to the currently small number of DDI-positive studies reported here and elsewhere.
Results

Treatment toleration. Dosing of bufuralol produced no clinical observations. Temazepam and midazolam appeared to produce occasional mild sedation with euphoria. Caffeine appeared to produce mild excitation in some subjects. Side effects of substrates did not appear to worsen when dosed with inhibitors. Ketoconazole and clomipramine were well-tolerated. Fluoxetine and fluvoxamine caused temporary constipation and inappetence in all subjects. Although supra-therapeutic doses of fluoxetine and fluvoxamine were desired based on initial DDI estimates (see Methods), the side effects were not expected after three doses according to previous studies. Both drugs have been administered once-a-day orally to dogs at 10 mg/kg for periods longer than three days according to the fluoxetine dog product label and a metabolism study of fluvoxamine (Ruijten et al., 1984). Subjects were examined by veterinarians and recovered spontaneously.

Pharmacokinetics. Relevant pharmacokinetic parameters for P450 substrates and inhibitors used for evaluating rP450-based DDI projection methods are provided in Table 1. Drug interactions were observed with the substrates caffeine, midazolam, and temazepam, but not with bufuralol (Fig. 1). The pharmacokinetics of bufuralol were highly variable, somewhat limiting the power to distinguish weak drug interactions. For all positive interactions, $T_{\text{max,obs}}$ and half-life were largely unchanged compared to the absence of inhibitors, although the animal numbers and blood sampling times did not allow small changes to be discerned. $C_{\text{max,obs}}$, $AUC$, and clearance were altered, especially with ketoconazole. Four values of unbound in vivo inhibitor concentrations
were gathered or estimated from the pharmacokinetic studies for drug interaction extrapolation. The values generally followed the order $C_{\text{avg,}u} < C_{\text{max,obs,}u} < [I]_{\text{in,}u}$ estimated with $C_{\text{avg,}u} < [I]_{\text{in}}$ estimated with $C_{\text{max,}u}$ (Table 2).

**Plasma protein and Bactosome binding.** High plasma protein binding was observed for three of the inhibitors (Table 3). Fraction unbound values followed the order fluvoxamine > fluoxetine > ketoconazole > clomipramine. Fluvoxamine binding to plasma was much lower than the other inhibitors consistent with the moderate binding of fluvoxamine in human plasma. Significant non-specific binding was observed for P450 inhibitors in dialysis experiments with control Bactosomes, albeit the fraction unbound was higher than was measured in plasma at the protein concentration tested.

**rP450 inhibition.** Inhibition constants ($IC_{50}$ or $K_i$) were determined for several rP450-inhibitor combinations after determining the isoforms with the highest intrinsic clearances for each victim drug (see below) (Table 4). All but one inhibition constant was $< 10 \, \mu\text{M}$ and the majority of unbound inhibition constants were $< 1 \, \mu\text{M}$. Although suspected earlier based on liver microsome findings (Kuroha et al., 2002b), ketoconazole was verified to be a potent inhibitor of canine rP450 3A12 (Fig. 2). Unexpected findings include the higher inhibition of CYP2C21 over CYP2D15 by fluoxetine, and the inhibition of CYP2C21 by clomipramine. rP450 caffeine metabolic activity was very low and required high enzyme and caffeine concentrations to detect metabolites. Therefore, phenacetin was used a substrate in CYP1A1 inhibition experiments.
**In vitro drug clearance and \( f_{m,P450} \) determination.** Canine rP450s 2B11 and 2C21 demonstrated the highest activity towards midazolam and temazepam clearance in substrate depletion experiments (Table 5). *In vitro* bufuralol clearance values were similar with rP450 2D15 and 2C21. The midazolam and temazepam findings were consistent with previous 1'-hydroxy-midazolam and oxazepam metabolite formation rates (Locuson et al., 2009), but the involvement of 2C21 in bufuralol clearance may suggest it metabolizes a position other than the 1'-position metabolized selectively by 2D15 (Shou et al., 2003). Caffeine metabolism by liver microsomes and all rP450s was low so substrate titration kinetics and ISEF activity correction could not be performed. However, under high caffeine concentrations (50 µM), P450s 1A1, 2B11, and 2C21 were found to form paraxanthine. Low levels of trimethyluric acid and theophylline were also formed by CYP1A1 and CYP2B11, respectively. P450s 2D15 and 3A12 did not produce any detectable caffeine metabolites. Since paraxanthine was the major observed metabolite from rP450s, the relative formation rate of paraxanthine over the first 20 min was used to estimate the \( f_{m,P450} \) values for caffeine. (Liver microsomes were found to form all four major primary metabolites of caffeine).

With one exception, all of the canine Bactosome rP450 ISEF values for the studied substrates were no more than 10-fold above or below unity (Table 5). The rank order of ISEF values for midazolam metabolism by all active metabolic pathways were the same for \( V \) and \( V/K \) calculations when midazolam kinetics were used for each P450 isoform. \( V \) ISEF results are not shown because they tended to be more sensitive to the probe substrate used and therefore resulted in more heavily weighted \( f_m \) values and less accurate
DDI magnitude predictions than \( V/K \) ISEFs. The rank order of ISEF values for temazepam metabolism was the same in all cases. ISEF values for the two pathways involved in bufuralol metabolism (CYP2C21 and 2D15) provided very different results depending on whether bufuralol or diclofenac kinetic values were used in calculations for the CYP2C21 ISEF value. As a result, \( f_{m,P450} \) values derived from ISEF-corrected intrinsic clearances could be very different depending on the choice of substrate. \( f_{m,P450} \) values calculated only from hepatic enzyme abundance were moderate because of similar expression levels of CYP2B, 2C, and 3A, and because all substrates tested were cleared by more than one enzyme in vitro.

**Prediction of AUC\(_i\)/AUC.** The use of different \( f_{m,P450} \) (Table 5) and \( [I]_{in vivo} \) (Table 2) values derived using alternate methods were evaluated for predicting AUC\(_i\)/AUC. A commonly used estimate of \( [I]_{in vivo} \), hepatic inlet \( [I]_{in,u} \) calculated with \( C_{max} \), overestimated the DDI magnitudes for bufuralol, i.v. midazolam, and temazepam using ISEF-generated \( f_{m,P450} \). Overall, \( [I]_{in,u} \) calculated with \( C_{avg} \) provided the best DDI estimates across different \( f_{m,in vivo} \) values and victim drugs as shown in Table 6. Use of systemic \( C_{max,u} \) or \( C_{avg,u} \) alone underestimated all interactions. Using the abundance-only approach to derive \( f_{m,P450} \) with \( [I]_{in,u} (C_{avg}) \) resulted in overestimation of bufuralol and underestimation of caffeine, midazolam, and temazepam interactions.
Discussion

Dogs pose an interesting challenge in P450-mediated DDI assessments since most of the enzymes have not been adequately characterized, but also because dogs are often treated off-label with a number of human drugs with sometimes little idea of DDI risk. It is therefore suggested that more drugs administered to dogs be further profiled using \textit{in vitro} methods to identify potential DDI victims and perpetrators. The use of rP450s in particular provides data that, over time, could stimulate the investigation of pharmacokinetic DDIs in dogs. For example, reporting P450 intrinsic clearance and inhibition values in the literature helps investigators identify potential interacting drugs. It also helps the identification or design of better P450 probe substrates and inhibitors by building up structure-activity relationships specific to dog P450s. In addition, rP450 profiling has enabled the prediction of DDI magnitude, but this has been limited to human research. Currently, few examples of positive DDIs have been reported for dogs and even fewer attempts have been made to predict them in a quantitative manner. In order to evaluate the utility of canine rP450s in DDI projection, we have attempted to, retrospectively, relate the magnitude of pharmacokinetic interactions in dogs to the inhibition of specific P450s.

Interactions in dogs involving ketoconazole and proposed CYP3A substrates have previously been attributed to CYP3A pathways (Kuroha et al., 2002a; Kuroha et al., 2004). The human ketoconazole-midazolam interaction in particular is so established that it is usually the first substrate/inhibitor pair to be tested \textit{in vitro} with liver microsomes from lesser characterized species. In addition, CYP3A probes such as
midazolam are cleared quickly in dog liver microsomes and ketoconazole was demonstrated to have sub-micromolar inhibition constants against midazolam in liver microsomes (Aidasani et al., 2008; Kuroha et al., 2002b). Similar in vivo interactions were reproduced here with ketoconazole and midazolam (Table 5), and ketoconazole was verified to be a very potent inhibitor of rCYP3A12. However, the relatively low activity of CYP3A12 towards midazolam relative to CYP2B11 is notable. High plasma concentrations of ketoconazole coupled with a high $f_{m-P450}$ and moderate $K_i$ for CYP2B11 appeared to explain the majority of the observed midazolam AUC$_i$/AUC$_{total}$ ratios.

One observation that may explain the low turnover of midazolam by rCYP3A12 is the lower P450 oxidoreductase content of CYP3A12 versus CYP2B11 Bactosomes (Locuson et al., 2009) or lack of cytochrome $b_5$. However, the difference in relative P450:reductase ratios (~3.7-fold) is much smaller than the $CL_{int,app}$ differences between the two enzymes. Interestingly, lower activity of canine CYP3A12 towards the typical CYP3A substrates nifedipine and testosterone has also been shown independently with baculovirus-expressed CYP3A12 when compared to human CYP3A4 (Carr et al., 2006). Some amount of fortuitous prediction results also cannot be ruled out with the number of calculations and assumptions made in the DDI extrapolations. However, dog liver is known to express a significant amount of CYP2B11 enzyme (and CYP2C21), which is thought to influence the disposition of veterinary drugs.

As might be expected, the oral midazolam-ketoconazole interaction would be underestimated without the inclusion of an estimate for intestinal P450 inhibition.
Intestinal extraction is known to play a large role in human midazolam interactions. Dog CYP3A-like activities and messenger RNA levels have been reported (Kyokawa et al., 2001; Mealey et al., 2008), but the authors are not aware of other activities/isoforms having been quantitatively assessed in canine intestinal tissue. Therefore, it has been assumed here that there is an intestinal DDI component resulting from P450 inhibition with the realization that the exact contribution of CYP3A, CYP2B, or CYP2C enzymes remains unknown. Unfortunately, there are as many difficulties in estimating intestinal P450 inhibition (i.e. $F_{g,i}/F_g$ ratio) as there are to extrapolating hepatic P450 inhibition, including choice of blood flow values, in vivo enterocyte inhibitor concentrations, confounding drug transporter activities, drug permeability, and the potential for inhibitor metabolism (Galetin et al., 2008). Thus, the minimalistic approach using in vivo pharmacokinetic data were used to estimate $F_g$ and the most conservative estimate of $F_{g,i}=1$ was used to avoid over-interpretation. This approach appeared to explain the difference between DDI magnitude from i.v. and oral midazolam.

Large temazepam interactions were also noted with ketoconazole despite the expectation that there would be a larger contribution of conjugation clearance than is observed with midazolam (Schwarz, 1979). Still, the selective $CL_{int,app}$ (Table 5) and turnover of temazepam to oxazepam by CYP2B11 (Shou et al., 2003) suggested temazepam may be a suitable in vivo probe of this enzyme. As with midazolam, the estimates of $f_{m,P450}$ were high for CYP2B11. $K_{i,u}$ and $[I]_{in vivo,u}$ values provided surprisingly good DDI estimates using maximal P450 in vivo clearance and an intestinal first-pass effect of equal magnitude to midazolam. On the other hand, use of dog in vivo excretion data ($f_{m,in}$
vivo = 0.5) underestimated the temazepam-ketoconazole interaction due to glucuronide clearance. Excluding intestinal inhibition further underestimated the interaction regardless of the $[I]_{\text{in vivo}}$ value used. Without further data it is difficult to hypothesize the reason for low DDI prediction, but it is conjectured that intestinal P450 inhibition, as well as the following factors, may be involved: (1) intestinal or hepatic transporter interactions between temazepam and ketoconazole, (2) inhibition of glucuronidation by ketoconazole, or (3) underestimation the P450 $f_{\text{m,in vivo}}$ value at a 1 mg/kg dose because of P450 saturation by the 20 mg/kg dose used in the excretion study (Schwarz, 1979).

Very high plasma exposures of fluoxetine were observed, but no measurable bufuralol DDI was observed with fluoxetine as an inhibitor. This is despite the fact that the long-lived fluoxetine metabolite, norfluoxetine, is also a P450 inhibitor. Bufuralol is extensively metabolized in humans (Dayer et al., 1986) with involvement of P450 that appears to be sensitive to the numerous CYP2D6 genotypes. In dogs, little bufuralol is excreted unchanged (Francis et al., 1976), but the precise $f_{\text{m,in vivo}}$ value for oxidative P450 metabolism could not be estimated for dogs without quantitation of unchanged drug and metabolites. It is speculated that the choice of $f_{\text{m,in vivo}}$ value may be partly responsible for the overestimation of a fluoxetine interaction. For instance, while there are clearly multiple oxidative elimination pathways for bufuralol according to rP450 phenotyping, there is also evidence for direct conjugation in vivo (Francis et al., 1976). Since the approved canine drug clomipramine produced a low $IC_{50}$ in dog liver microsomes (~0.1 µM) (Aidasani et al., 2008), this drug was tested next as an inhibitor of bufuralol. However, inhibition of rCYP2D15 by clomipramine turned out to be less
potent than in liver microsomes (Table 4). Very high plasma protein binding also probably limits the P450 inhibition potential of clomipramine in vivo.

Fluvoxamine produced a small DDI with caffeine and in vitro-in vivo extrapolation provided a reasonable estimate of the true interaction magnitude using the CYP1A1 inhibition value. Despite fluvoxamine having the lowest plasma concentrations of all inhibitors the estimated unbound fluvoxamine concentration in the liver was high (Table 2). The interaction magnitude is suggested to be minor because of a moderate estimated $f_{m,P450}$ value for CYP1A1 resulting from contributing clearance by CYP2B11 and CYP2C21. A similar interaction was obtained with theophylline and enrofloxacin in dogs providing further evidence that the interaction does occur at a CYP1A pathway(s) in vivo (Intorre et al., 1995). Dog liver does express both CYP1A1 and CYP1A2 isoforms (Uchida et al., 1990), but the analysis of CYP1A1 alone does not appear to be responsible for the modest underestimation of the DDI. Instead, caffeine generally appears to be an insensitive marker of CYP1A activity in dogs based on in vitro data. Caffeine turnover in vitro was very low even with liver microsomes (containing CYP1A2). This finding was also reported by Mise et al. (Mise et al., 2008) who studied the effect of the 1117 C→T non-functional CYP1A2 genotype (Tenmizu et al., 2004) on liver microsome activity. Caffeine metabolism was low in both mutant and wild-type dog liver microsomes suggesting CYP1A2 provides a smaller contribution to caffeine metabolism in dogs relative to humans.
While the magnitude of dog DDIs could be rationalized in some of the cases presented, it is important to recall that there are many assumptions involved in the extrapolation methods. In addition, the drugs tested here have a long history and much is known about them. In early veterinary drug discovery, estimates of $f_{m, in vivo}$ are not available and the inhibition potential of metabolites may not be known. The conservative assumptions that $f_{m, in vivo}=1$ and $F_{g,i}=1$ would be expected to over-predict DDIs until victim drugs are further characterized. Further complexities also surround the assumptions and parameter estimates used for predicting intestinal metabolism and its inhibition, which can clearly impact DDI estimates. When using rP450, specific $f_{m, P450}$ values are integral in DDI predictions and this may be problematic because the only published hepatic P450 abundance levels may not be completely isoform specific. Therefore, differentiation of some isoforms within the same subfamily (e.g. CYP3A12 and CYP3A26) is not yet possible. The choice of substrates used to determine liver microsome ISEF values also requires further evaluation as they are used in the derivation of $f_{m, P450}$.

In summary, pharmaceutical development for companion animals continues to grow and expand into new therapeutic areas, but there are few methods for assessing pharmacokinetic drug interactions. Herein, methods commonly used to project P450-mediated DDI magnitude in humans were shown to provide reasonable DDI estimates in dogs for some victim drugs where in vivo parameters were available or could be estimated (e.g. $f_{m, in vivo}$, $F_{g,i}/F_g$). At minimum, it is proposed that the relative $f_{m, P450}$ values, the assumption of $f_{m, in vivo}=1$, $[I]_{in,u}$, and $K_{i,u}$ may be used to provide early discovery estimates of the worst-case scenario DDI for dogs. rP450 also reveals some of
the enzymes likely involved in dog DDIs – something that is severely lacking even for established drugs. Furthermore, the development of more selective in vivo substrates and inhibitors of dog P450s would benefit the testing of new drug leads and other agents used off-label, and would help in the refinement of DDI prediction methods. Given more selective P450 probes, the dog might even serve human health as a model in which to characterize the actual hepatic portal vein and intestinal drug concentrations that consistently yield the most accurate DDI extrapolations.
Acknowledgments

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References


Footnotes

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Figure Legends

Figure 1. Plasma concentration-time profiles of victim drugs in the absence (●) and presence of (○) inhibitors: (A) midazolam PO and ketoconazole, (B) midazolam IV and ketoconazole, (C) temazepam and ketoconazole, and (D) caffeine and fluvoxamine.

Figure 2. Representative inhibition plots of canine rP450. (A) CYP2C21 IC_{50} inhibition curves: (●) ketoconazole inhibition of temazepam metabolism to oxazepam, (▼) ketoconazole inhibition of midazolam metabolism to 1'OH-midazolam, and (○) clomipramine inhibition of bufuralol metabolism to 1'OH-bufuralol; (B) Non-linear regression fitting of CYP3A12 inhibition by ketoconazole to determine $K_i$ using the competitive inhibition model.
### Tables

Table 1. Summary of non-compartmental pharmacokinetic parameters of potential P450 substrates in dogs \((n=4)\) in the absence (Day 1) and presence of an inhibitor (Day 4) dosed once-a-day for three days\(^a\).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day</th>
<th>Caffeine (PO)</th>
<th>Midazolam (PO)</th>
<th>Midazolam (IV)</th>
<th>Temazepam</th>
<th>Bufuralol</th>
<th>Bufuralol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>1</td>
<td>1.3 ± 1.0</td>
<td>0.50 ± 0.10</td>
<td>0.50 ± 0.10</td>
<td>1.7 ± 0.3</td>
<td>0.78 ± 0.13</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.3 ± 1.0</td>
<td>0.50 ± 0.10</td>
<td>0.50 ± 0.10</td>
<td>1.7 ± 0.3</td>
<td>0.79 ± 0.13</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>(C_{\text{max,obs}}) (ng/mL)</td>
<td>1</td>
<td>1230 ± 265</td>
<td>88.9 ± 45.9</td>
<td>1860 ± 295</td>
<td>35.7 ± 31.0</td>
<td>407 ± 224</td>
<td>205 ± 120</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1790 ± 104</td>
<td>343 ± 127</td>
<td>1700 ± 1570</td>
<td>342 ± 180</td>
<td>216 ± 167</td>
<td>155 ± 164</td>
</tr>
<tr>
<td>(T_{\text{max,obs}}) (h)</td>
<td>1</td>
<td>2.3 ± 1.3</td>
<td>0.63 ± 0.25</td>
<td>0.033</td>
<td>0.63 ± 0.25</td>
<td>0.5</td>
<td>1.1 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.2 ± 0.8</td>
<td>0.5</td>
<td>0.033</td>
<td>0.75 ± 0.29</td>
<td>1.3 ± 0.6</td>
<td>1.3 ± 1.2</td>
</tr>
<tr>
<td>(t_{1/2}) (h)</td>
<td>1</td>
<td>4.2 ± 0.8</td>
<td>2.4 ± 1.7</td>
<td>1.3 ± 0.3</td>
<td>9.0 ± 7.3</td>
<td>1.3 ± 0.4</td>
<td>4.3 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.8 ± 1.2</td>
<td>1.7 ± 0.4</td>
<td>2.0 ± 0.8</td>
<td>3.2 ± 1.2</td>
<td>1.9 ± 0.2</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td>(AUC_{0-24}) (ng ⋅ h/mL)</td>
<td>1</td>
<td>10800 ± 857</td>
<td>92.5 ± 50.3</td>
<td>337 ± 55</td>
<td>54.7 ± 50.6</td>
<td>525 ± 264</td>
<td>408 ± 366</td>
</tr>
<tr>
<td>(F_{\text{oral}}) inh.</td>
<td></td>
<td>4</td>
<td>17900 ± 1220</td>
<td>760 ± 313</td>
<td>871 ± 359</td>
<td>685 ± 193</td>
<td>550 ± 391</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>9.2 ± 1.1</td>
<td>22.2 ± 4.5</td>
<td>22.2 ± 4.4</td>
<td>22.2 ± 4.4</td>
<td>9.7 ± 1.6</td>
<td>11.1 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>12.8 ± 2.8</td>
<td>272 ± 167</td>
<td>458 ± 314</td>
<td>379 ± 45</td>
<td>230 ± 77</td>
<td>24.3 ± 10.0</td>
<td></td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.864 ± 0.177</td>
<td>44.1 ± 27.3</td>
<td>42.8 ± 26.2</td>
<td>43.5 ± 16.0</td>
<td>14.4 ± 4.5</td>
<td>2.06 ± 0.73</td>
<td></td>
</tr>
<tr>
<td>Clomipramine</td>
<td>0.533</td>
<td>11.3</td>
<td>19.1</td>
<td>15.8</td>
<td>9.58</td>
<td>1.01</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)All values represent the mean ± standard deviation where applicable.
Table 2. Mean inhibitor concentrations evaluated for metabolic drug-drug interaction prediction.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Victim</th>
<th>Route</th>
<th>( C_{avg,u} )</th>
<th>( C_{max,obs,u} )</th>
<th>( a[I]<em>{in,u} ) (( C</em>{avg} ))</th>
<th>( a[I]<em>{in,u} ) (( C</em>{max} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluvoxamine</td>
<td>Caffeine</td>
<td>PO</td>
<td>0.231</td>
<td>0.375</td>
<td>6.71</td>
<td>6.85</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Midazolam</td>
<td>PO</td>
<td>0.362</td>
<td>1.41</td>
<td>1.24</td>
<td>2.29</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Midazolam</td>
<td>IV</td>
<td>0.883</td>
<td>1.98</td>
<td>1.77</td>
<td>2.86</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Temazepam</td>
<td>PO</td>
<td>0.505</td>
<td>1.39</td>
<td>1.39</td>
<td>2.28</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>Bufuralol</td>
<td>PO</td>
<td>0.589</td>
<td>0.885</td>
<td>1.52</td>
<td>1.81</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>Bufuralol</td>
<td>PO</td>
<td>0.016</td>
<td>0.033</td>
<td>0.167</td>
<td>0.184</td>
</tr>
</tbody>
</table>

*Estimates of hepatic vein inlet concentration (\( I_{in} \)) were corrected with published human blood:plasma partitioning values when possible (see Table 3).*
Table 3. Fraction of inhibitor drug unbound\(^a\).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Beagle Plasma (f_u)</th>
<th>(^b)Blood (f_u)</th>
<th>(^c)Control Bactosomes (f_u)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluvoxamine</td>
<td>0.132 ± 0.033</td>
<td>0.240</td>
<td>0.249 ± 0.017</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.017 ± 0.010</td>
<td>0.025</td>
<td>0.345 ± 0.044</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>0.019 ± 0.002</td>
<td>0.035</td>
<td>0.156 ± 0.015</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>0.005 ± 0.003</td>
<td>0.005</td>
<td>0.157 ± 0.002</td>
</tr>
</tbody>
</table>

\(^a\)Values represent the mean ± standard deviation.
\(^b\)Calculated from published human blood:plasma partition ratios for fluvoxamine and fluoxetine (McGinnity et al., 2008), and ketoconazole (Shibata et al., 2008), and beagle plasma binding.
\(^c\)Protein and drug concentrations were chosen to mimic the conditions of \(K_i\) experiments.
Table 4. Uncorrected and fraction unbound-corrected rP450 inhibition constants.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>CYP1A1</th>
<th>CYP2B11</th>
<th>CYP2C21</th>
<th>CYP2D15</th>
<th>CYP3A12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluvoxamine</td>
<td>Phenacetin</td>
<td>2.90</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Midazolam</td>
<td>ND</td>
<td>2.12 ± 0.96</td>
<td>ND</td>
<td>7.25</td>
<td>ND</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Temazepam</td>
<td>ND</td>
<td>0.96 ± 0.16</td>
<td>ND</td>
<td>18.9</td>
<td>ND</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>Bufuralol</td>
<td>ND</td>
<td>ND</td>
<td>0.44 ± 0.09</td>
<td>4.2 ± 1.3</td>
<td>ND</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>Bufuralol</td>
<td>ND</td>
<td>ND</td>
<td>2.32</td>
<td>1.16 ± 0.50</td>
<td>ND</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>Kᵢ,u (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluvoxamine</td>
<td>Phenacetin</td>
<td>0.72</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Midazolam</td>
<td>0.73</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>Temazepam</td>
<td>0.33</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>Bufuralol</td>
<td>0.069</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>Bufuralol</td>
<td>0.39</td>
</tr>
</tbody>
</table>

*Values represent either the inhibition constant determined from non-linear regression fitting to the competitive inhibition model (± error of the parameter estimate) or $K_i$ estimated as $IC_{50}/2$ with substrate=$K_m$. $IC_{50}$ was determined using the four-parameter logistic function.

*bReactions monitored for each rCYP: phenacetin deethylation, midazolam 1'-hydroxylation, temazepam N-demethylation, and bufuralol 1'-hydroxylation.

*cEstimated as $K_i = IC_{50}/2$ for enzymes demonstrating lower substrate clearance and/or less inhibition.

dNot determined; $K_i$ or $IC_{50}$ values were only determined for enzyme(s) demonstrating the highest in vitro clearance of the victim drug.

eEstimated from the control Bactosome fraction unbound in Table 3.
Table 5. Relative fraction metabolized (\( f_{m,P450} \)) values derived from hepatic abundance or ISEF methods.

<table>
<thead>
<tr>
<th>Victim Substrate</th>
<th>rP450</th>
<th>( ^a )Victim Depletion ( CL_{\text{int,app}} )</th>
<th>( f_{m,P450} ) (hepatic abundance method)</th>
<th>( ^b )ISEF Substrate</th>
<th>( ^c V/K ) ISEF Value</th>
<th>( ^d f_{m,P450} ) (ISEF method)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>1A1</td>
<td>1.0</td>
<td>0.36</td>
<td>( ^d ND )</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2B11</td>
<td>0.27</td>
<td>0.28</td>
<td>( ND )</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2C21</td>
<td>0.11</td>
<td>0.35</td>
<td>( ND )</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Midazolam</td>
<td>2B11</td>
<td>2.72</td>
<td>0.43</td>
<td>Temazepam</td>
<td>2.92</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Midazolam</td>
<td>1.68</td>
<td>0.91</td>
</tr>
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<td>0.22</td>
<td>Diclofenac</td>
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<td>0.44</td>
<td>Bufuralol</td>
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</table>

\( ^a \)\( \mu \)L/min/pmol P450.
\( ^b \)ISEFs and corresponding \( f_m \) values were calculated using the kinetics of the actual victim substrate or an alternate substrate for each rP450 as described in the Methods section.
\( ^c \)Due to low caffeine turnover, the relative rate of paraxanthine formation normalized to CYP1A1 is shown.
\( ^d \)Not determined due to low caffeine turnover.
Table 6. *In vitro-in vivo* DDI extrapolation results.

<table>
<thead>
<tr>
<th>Victim</th>
<th>Inhibitor</th>
<th>(^a)Estimated (F_{g,i}/F_g)</th>
<th>(^b)(f_{m,\text{in vivo}}) values evaluated</th>
<th>(^c)Predicted AUC/AUC (ISEF method)</th>
<th>(^c)Predicted AUC/AUC (hepatic abundance method)</th>
<th>Actual Mean AUC/AUC (n=4)</th>
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</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>Fluvoxamine</td>
<td>1.00</td>
<td>0.96; 1.0</td>
<td>ND</td>
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<td>1.46</td>
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<td>Midazolam (PO)</td>
<td>Ketoconazole</td>
<td>2.99</td>
<td>1.0</td>
<td>7.53</td>
<td>5.54</td>
<td>8.22</td>
</tr>
<tr>
<td>Midazolam (IV)</td>
<td>Ketoconazole</td>
<td>1.00</td>
<td>1.0</td>
<td>3.13</td>
<td>2.17</td>
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<td>1.82</td>
<td>1.56</td>
<td>1.58</td>
</tr>
</tbody>
</table>

\(^a\)Value for midazolam was estimated from intravenous and oral pharmacokinetics; The midazolam value was also evaluated for temazepam based on the similar drug class, rP450 clearance, and ketoconazole inhibition profiles.

\(^b\)\(f_{m,\text{in vivo}}\) values used in DDI extrapolation were obtained from the literature where available and compared to the most conservative value, 1.0.

\(^c\)AUC ratios were calculated by multiplying Eq. 1 by the estimated \(F_{g,i}/F_g\) ratio; Input parameters for Eq. 1 were \([I]_{\text{in,u}}(C_{avg})\) and the unbound \(K_i\) and \(f_m\) values for each P450 isoform that was found to clear the victim drug *in vitro*; Hepatic abundance and victim substrate ISEF-derived \(f_{m,P450}\) values from Table 5 were multiplied by \(f_{m,\text{in vivo}}\) to generate \(f_m\) values for each P450.

\(^d\)Not determined due to low substrate turnover.