DMD Fast Forward. Published on December 12, 2008 as doi:10.1124/dmd.108.025312

DMD/2008/025312

Evaluation of *Escherchia coli* membrane preparations of canine CYP1A1, 2B11, 2C21, 2C41, 2D15, 3A12, and 3A26 with co-expressed canine P450 reductase

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c) Text pages:	11 (13 with references)
Tables:	2
Figures:	1
References:	22
Words in Abstract:	220
Words in Introduction:	327
Words in Discussion:	1282

d) Non-standard abbreviations: cytochrome c, cyt c; cytochrome P450, CYP; escherichia coli, *E. coli*; NADPH cytochrome P450 reductase, CYP reductase; recombinant cytochrome P450, rCYP; ultra-performance liquid chromatography, UPLC

Abstract

The preparation of bacterial membranes ("Bactosomes") containing expressed canine (beagle) hepatic cytochromes P450 (CYPs) is described. cDNAs from seven canine CYPs were subcloned into inducible expression plasmids, and for the first time, co-transformed and expressed with a canine P450 oxidoreductase in *E. coli* to produce active, full-length, native sequence CYPs. Enzyme expression levels, while variable, were generally sufficient to enable short incubation times and to limit the total protein present in enzyme incubations. Steady-state kinetics of CYP1A1, 2C21, and 2D15 Bactosomes demonstrated similarities with dog liver microsomes or baculosomes. However, 3A12 lacked substrate inhibition in the formation of 1'-OH midazolam, and 2B11 displayed non-Michaelis-Menten kinetics suggesting possible differences in protein interaction effects. In monitoring the metabolites of common CYP substrates, phenacetin deethylation, temazepam demethylation, and bufuralol 1'-hydoxylation were shown to be relatively selective reactions catalyzed by CYPs 1A1, 2B11, and 2D15, respectively. 1'-OH midazolam was formed in higher quantities by CYPs 2B11 and 2C21 than by 3A12 raising questions about the use of midazolam as a CYP3A12 probe *in vivo*. In summary, a panel of recombinant CYPs was produced to make up for the lack of commercially available canine CYP isoforms. The Bactosomes are expected to facilitate reaction phenotyping and metabolic drug-drug interaction assessment in canine drug development, and to enable the study of inter-species differences in CYP-mediated drug metabolism.

Introduction

The cytochromes P450 (CYPs) are well known for their role in the metabolism of drugs and other xenobiotics. While hepatocytes and other liver tissue preparations are a rich source of the CYP isoforms that tend to be involved in xenobiotic metabolism, use of recombinant, expressed P450s (rCYPs) has grown over the last decade. Several recombinant human P450 isoforms are expressed from cDNA and partially purified as membrane preparations, and are commercially available. Common uses of rCYPs in pharmaceutical industry include the assessment of drug-drug interactions (Crespi and Stresser, 2000), reaction "phenotyping", metabolite generation, and estimating the *in vivo* clearance of drugs (Venkatakrishnan et al., 2001).

Currently, non-human rCYPs are not widely available. Yet, pre-clinical species used in human pharmaceutical research, as well as veterinary species, could arguably benefit from the availability of an expanded panel of rCYPs. For instance, dogs are an important non-rodent species used in scaling pharmacokinetics and evaluating toxicology in drug development research. Yet, several notable differences have been described in the ligand selectivity and expression levels of human CYPs and their canine homologs (Eguchi et al., 1996; Tibbitts, 2003). As a patient population, dogs are also increasingly being considered by pharmaceutical companies (Petkewich, 2007). For all of these reasons, canine and other non-human rCYPs would prove a valuable tool to the pharmaceutical scientist.

In order to expand the number of canine rCYPs available for research, we describe the plasmid constructs and bacterial expression methods for seven of the major beagle hepatic drug-metabolizing CYPs. Shou *et al* previously described the insect cell expression of the canine CYPs with human CYP reductase (Shou et al., 2003). In the current work, the authors have cloned the hepatic beagle CYP reductase, co-expressed it with the dog CYPs, and evaluated the activity of common substrates with a completely dog-specific enzyme system for the first time.

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Materials and Methods

Materials. Chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK, or Milwaukee, WI, USA). except for some metabolite standards obtained from BD Gentest (Franklin Lakes, NJ), and modified terrific broth powder (Merck KgA, Darmstadt, Germany), IPTG (Melford Laboratories, Ipswich, UK) and δ-aminolevulinic acid (Molekula, Gillingham, UK). Solvents were purchased from Burdick and Jackson (Morristown, NJ).

Isolation of the cDNAs coding for 7 canine CYP enzymes and canine NADPH P450 reductase. The cDNAs coding for the 7 canine CYPs were isolated by RTPCR from mRNA made from isolated cultured beagle hepatocytes. Prior to the isolation of the mRNA, the hepatocytes were treated in culture with phenobarbital (1 mM), 3-methylcholanthrene (25 mM) and rifampicin (25 mM) for 48 h. Reverse transcription was carried out using random primers and Superscript Reverse Transcriptase III (Invitrogen, Paisley, UK) following the manufacturer's instructions. The PCR was carried out using Phusion DNA polymerase (NEB, Ipswich, MA, USA) using conditions recommended by the manufacturer.

The primer pairs used for the PCR reactions to isolate the cDNAs coding for the CYPs and P450 reductase are as follows: CYP1A1 – GCTCCGATGATGTCCATGTTCAGACTCTC and GCGAATTCTAGGCTGCAGGGCTCTCAG, CYP2B11 - ATGGAGCTCAGCGTCCTTCTCC and GTGAATTCAGCACCCTCCACGAGACAG, CYP2C21 - ATGGATCTCTTCATAGTTCTGGTGAT and GCTCTAGATCAGACTGGAACAAAACAGAGCTTATAG, CYP2C41 – ATGGATCCAGTTGTGGTTCTGGTG and CATCTAGATCACACGGGAATGAAGCAGAGC, CYP3A12 – ATGGACTTGATCCCAAGCTTTTCCAC and AGTCTAGATCAGGCTCCATTTACACTCCCATC, CYP3A26 - ATGGACTTGATCCCAAGCTTTTCCAC and CATCTAGATCAGGCTCCACTTACACTCCCA, CYP2D15 – ATGGGGCTGCTGACCGGGGACAC and

TGTCTAGACTAGCGAGGCTCCACACAGAGCTGG, and Reductase – ATGAAGAAGACGGGCAGGAACATC and CTGAATTCTAGCTCCACACGTCCAGGG.

The accession numbers for the sequences are as follows: CYP1A1 - L77459 (full length sequence supplied by Dr Magang Shou, Amgen, Thousand Oaks, CA 91320), CYP2B11 - M92447, CYP2C21 - XM543973, CYP2C41 - AF016248, CYP3A12 - X54915, CYP3A26 - AF547269, CYP2D15 - AB004268, Reductase - XM852956.

The full-length cDNAs coding for the canine P450s were cloned into a pCW vector under the control of a *tac tac* promoter, fused in frame with the sequence coding for the leader peptide of the bacterial ompA protein. This system has been used previously to express native mammalian CYPs in *E. coli* (Pritchard et al., 1998). An Ala-Pro insertion was made between ompA and the CYP1A1 N-terminus to enable more efficient cleavage in the bacterial membrane by the signal peptidase. The full-length cDNA coding for canine NADPH P450 reductase was cloned into a derivative of pACYC184 in frame with a *tac tac* promoter element (designated pCY070). A CYP1A2 isoform is known to be expressed in dog liver (Uchida et al., 1990), but it has not been cloned nor its expression relative to CYP1A1 characterized.

Co-expression of canine CYPs with canine NADPH P450 reductase. The expression plasmid for each canine CYP was co-transformed into *E. coli* JM109 (Promega, Southampton, UK) with the canine P450 reductase plasmid, pCY070. Next, a 3 ml starter culture was grown overnight at 37°C and 2 ml was used to inoculate 200 ml modified terrific broth in a 2 litre conical flask. The culture was grown at 30°C with shaking at 150 rpm until the OD_{600} reached 0.8 – 1.0 when expression of the recombinant proteins was induced by the addition of IPTG to 0.5 mM and δ-aminolevulinic acid to 1 mM. The induced cultures were incubated for a further 48 hours at 30°C with shaking at 150 rpm. The cultures were harvested by centrifugation and the harvested cells were resuspended in 10 ml 100 mM Tris acetate pH 7.6, 500 mM sucrose, 0.5 mM EDTA.

A 0.5 ml sample was taken and diluted 1:1 with deionised water, 1.25 ml 20 mg/ml lysozyme was added to the remainder of the resuspended cells. 10 ml deionised water was added to the resuspended cells and the suspension was incubated at 4°C for 10 minutes. The lysozyme treated cells (sphaeroplasts) were harvested by centrifugation and the pellet was resuspended in 4 ml 100 mM potassium phosphate pH 7.5, 20% glycerol, 1 mM PMSF, 1 µg/ml leupeptin. The sphaeroplasts were disrupted by sonication and the cell debris was removed by centrifugation at 12000 x g for 20 min. The bacterial membrane fraction was collected by centrifugation at 100,000 x g for 1 h and resuspended in 2 ml 500 mM Tris acetate pH 7.6, 250 mM sucrose, 0.25 mM EDTA. Large-scale cultures were grown in 10 litre fermenters (Brighton Systems, Brighton, UK) under standard conditions and subsequently treated the same as the shake flask cultures above with volumes were scaled appropriately. After the final resuspension in 500 mM Tris acetate pH 7.6, 250 mM sucrose, 0.25 mM EDTA the membrane suspension was centrifuged at least once at 12,000 x g for 20 minutes and filtered through a 0.22 µm syringe filter to remove any intact cells.

The P450 content of the whole cells after harvesting and the membrane preparations was determined using Fe^{2+} - CO vs. Fe^{2+} difference spectra (Omura and Sato, 1964). CYP reductase content was estimated using a spectrophotometric assay to determine the rate of NADPH dependent reduction of cytochrome *c* (Strobel and Dignam, 1978). The total protein content of the membrane preparations was determined using a BioRad DC protein assay following the manufacturer's instructions (BioRad, Hemel Hempstead, UK).

Kinetic assays. Incubations of CYP drug substrates with the Bactosomes prepared from fermenter cultures were conducted to assess their activity. Reactions (0.1 mL) were conducted in 20 mm-tall 96-well polypropylene plates with 1 mM NADPH in 0.1 M potassium phosphate buffer (pH 7.4) containing 2.5 - 5 pmol of CYP (0.5 pmol/mL) across a range of substrate concentrations encompassing K_m (phenacetin: 1-1000, temazepam: 1-500, diclofenac: 1 – 1000, bufuralol: 0.1-100, midazolam: 0.2-200 µM). After 5 min at 37 °C, reactions were quenched by

the addition of 0.1 mL of 25% formic acid containing an internal standard. Acetonitrile and DMSO used to solubilize and dilute substrate stock solutions did not exceed 1.0 and 0.1 % (v/v) of the final incubation volume, respectively. All experiments were controlled by using the same proportion of solvent in all reactions. Reactions were conducted in triplicate, except those containing CYP3A26 due to lower expression and need for higher levels of Bactosomes. Linearity of metabolite formation was established with time up to 20 min and with CYP concentrations up to 50 - 100 pmol/mL.

Quantitation of metabolite formation was carried out with UPLC tandem mass spectrometry methods. Briefly, an Acquity UPLC system (Waters, Milford, MA) with a Waters BEH Shield RP18 column (1.7 µ, 2.1 × 50 mm) or a Phenomenex Synergi Polar RP column (4 µ, 2 × 50 mm) was used for separation. Typical chromatography conditions began at 20 – 40 % methanol in water containing 0.1% formic acid and used a gradient from 0.2 – 0.4 min that reached 60 – 95 % methanol with flow rates between 0.5 – 0.7 mL/min. Retention times of analytes ranged from 0.39 to 0.97 min. Detection was accomplished with a Sciex 4000 triple quadrupole mass spectrometer (Foster City, CA) operating in positive mode. The transitions used for quantitating metabolites were reported previously (Aidasani et al., 2008) except for 4-OH midazolam (342 → 297). All methods were developed to ensure substrate did not co-elute with the monitored metabolites to reduce suppression because co-elution of midazolam suppressed the signal of 1'-OH midazolam and gave the impression of substrate inhibition. In addition, the two hydroxy midazolam metabolites required separation since both are detected with the m/z 297 transition.

The 4'-OH diclofenac peak, as verified with the synthetic 4'-OH diclofenac standard, had a retention time of 0.80 min in diclofenac incubations. However, this metabolite peak appeared in chromatograms as an early eluting shoulder to a larger peak at 0.82 min using UPLC separation (not shown). This larger peak was dependent on the presence of NADPH and was present under varying liquid chromatography conditions so that it did not appear to be an artifact or a matrix effect. The unknown peak detected with the *m/z* 312 → 231 transition may suggest that

CYP2C21/41 forms more 3'-OH diclofenac than human CYP2C enzymes. Quantitation was carried out with the merged peaks.

CYP phenotyping. Reactions containing phenacetin (50 µM), temazepam (5 µM), diclofenac (30 µM), bufuralol (10 µM), and midazolam (4 µM) were conducted near K_m with all seven constructed canine Bactosomes in quadruplicate using the same conditions, volumes, and sample preparation as described above.

Kinetic analysis. Enzyme kinetics for each substrate was defined using a single site (Michaelis-Menten) or two site saturation model in SigmaPlot v9.0 (Systat Software, Inc., San Jose, CA). Appropriateness of the kinetic model was assessed by the R^2 resulting from non-linear regression and the linearity of Eadie-Hofstee replots (V/[S] v V). Velocity of CYP2C21 slightly decreased at saturating diclofenac concentrations, but fitting with a substrate inhibition model greatly overestimated V. CYP2C41 demonstrated some biphasic nature, but regression failed with the two site model. Therefore, diclofenac hydroxylation by both CYP2C enzymes was fit with the Michaelis-Menten model.

Results and Discussion

Expressed recombinant CYP preparations have proven to be a useful research tool in the drug discovery environment for the *in vitro* assessment of inhibition, reaction phenotyping, or to generate metabolites for identification purposes. However, species differences in the regioselectivity and turnover rate of drugs (Fink-Gremmels, 2008; Turpeinen et al., 2007) by CYPs may complicate the scaling of pre-clinical pharmacokinetics and metabolite exposures from animals to humans. Therefore, a method that could be used to produce more rCYPs from pre-clinical species was desired. Common expression hosts for mammalian CYPs include insect ovary cells (Supersomes® and Baculosomes®), and human lymphoblastoid cells, but *E. coli* expression of CYPs (Barnes et al., 1991; Parikh et al., 1997) and reductase (Blake et al., 1996; Shen et al., 1989) have also seen progress. Bacterial CYP preparations have even

demonstrated surprisingly similar results to hepatocytes in the assessment of drug-drug interactions (McGinnity et al., 2008). Since beagle dogs are an important pre-clinical and veterinary species, a method used to express the major hepatic canine rCYPs in *E. coli* (Pritchard et al., 1998), along with the canine CYP reductase, was shown to be a reliable approach to produce multiple CYP isoforms using the same conditions.

Expression of canine rCYPs. All but one of the tested canine hepatic CYPs (CYP3A26) was produced well in *E. coli* by the addition of a bacterial sequence leader (Table 1). Moreover, this modification has been shown to be cleaved off in the bacterial membrane to yield the native CYP, and no modification of the native canine CYP sequences was necessary. An important step in the present work is that a hepatic canine CYP reductase transcript was identified, cloned, and co-expressed with the rCYPs from a separate plasmid. Initial *E. coli* expression experiments carried out in shake flasks did, however, give a large spread in the expression levels achieved for each CYP (Table 1). The levels of co-expressed CYP reductase as measured by NADPH dependent cytochrome c reduction were less variable than CYP content between preparations.

All of the CYPs were next scaled-up to expression in 10 litre fermenters but, again, there was a wide variation in the yield of P450 between the CYPs with the yield of CYP1A1 being the highest and CYP3A26 being the lowest (Table 1). The protein concentration tended to be lower (8.4 – 11.9 mg/mL) in membrane suspensions made from the 10 litre fermenter cultures than shake flask cultures (8.3 – 19 mg/mL) because of the extra centrifugation and filtration steps. CYP3A26 and CYP2D15 preparations were difficult to filter through the 0.22 µm syringe filters so were centrifuged more than once at 12,000 x g after resuspension of the membrane fraction. The reason for the poorer expression of CYP3A26 is not yet known and it is likely that further optimization of expression conditions will be needed.

Activity characterization. The activity level of the Bactosomes prepared from fermenter cultures was suitable for typical rCYP applications, requiring relatively low CYP/protein levels and short incubation times. Catalytic efficiencies (V/K_m) calculated from Table 2 were similar to the diclofenac (CYP2C21), bufuralol (CYP2D15), and midazolam (CYP3A12) values described previously in dog liver microsomes (Aidasani et al., 2008) and were also similar to those of rCYPs expressed in insect cells in the cases of phenacetin, temazepam, and bufuralol (Lu et al., 2005; Shou et al., 2003). The apparent affinities (K_m) and maximum velocities (V) for three substrates (diclofenac, bufuralol, and midazolam) determined with the beagle rCYP/reductase Bactosomes were comparable to those described for both canine liver microsomes and beagle rCYPs with human P450 reductase (Aidasani et al., 2008; Shou et al., 2003) (Table 2). Using the same three studies above as a comparison, velocities for phenacetin metabolism were higher with Bactosome rCYP1A1 than with microsomes or rCYP1A1 with human CYP reductase, and the K_m for temazepam was lower with Bactosomes. The higher catalytic efficiency of CYP2C21 over CYP2C41 and of CYP3A12 over CYP3A26 was consistent with previous findings (Carr et al., 2006; Shou et al., 2003).

Some other notable findings were the non-hyperbolic kinetics of temazepam demethylation by CYP2B11 and midazolam 1'-hydroxylation and 4-hydroxylation by CYP3A26. In both cases, kinetics followed a biphasic response with substrate concentration. Also, there was no indication of substrate inhibition on 1'-OH midazolam formation at up to 200 µM midazolam, which was surprising given that substrate inhibition is observed with canine liver microsomes (Aidasani et al., 2008) and CYP3A12 baculosomes (Carr et al., 2006). This is the first observation of atypical kinetics displayed by rCYP2B11. Differences in protein-protein interactions caused by the presence of multiple CYPs in microsomes, the species of CYP reductase, or the CYP:CYP reductase ratio may explain these findings.

The CYP:CYP reductase ratio will certainly impact velocities, and conceivably, might subtly modulate drug affinity, regioselectivity, and kinetic profiles via changes in CYP conformation. As

mentioned, the canine rCYP content:cyt c reductase activity ratios were variable, but on average, they were the same as the ratios characterized for human Bactosomes (CYPex, data not shown). The CYP1A1, 2C21, 2D15 and 3A12 levels exceed CYP reductase by two- to nine-fold (Table 1). It is interesting to note that this is the case with the physiological ratios where total CYPs outnumber CYP reductase ~10:1 in purchased human liver microsomes and ~27:1 in beagle liver microsomes according to cyt c reductase activities (Xenotech, Lenexa, KS). While increased CYP reductase levels may enhance rCYP activity, what gives optimal reaction velocities *in vitro* may have fewer physiological characteristics of CYP catalysis *in vivo* where CYP oligomerization is likely important.

Phenotyping of CYP substrates. The seven rCYP panel was screened to characterize the turnover of phenacetin, temazepam, diclofenac, bufuralol, and midazolam (Fig. 1). Such phenotyping experiments used to define the selectivity of substrates and inhibitors for specific CYPs are now commonly conducted with panels of rCYPs (Zhang et al., 2007). Phenotyping experiments with four of the studied substrates demonstrated the CYP isoforms with the highest turnover were identical to that previously described with baculovirus expressed CYPs (Lu et al., 2005; Shou et al., 2003). The monitored metabolites of CYP1A1, 2B11, 2C21, and 2D15 were produced at levels 9-, 5.3-, 1.9-, and 3.8-fold greater than the enzymes producing the second highest levels of the metabolites.

1'-OH midazolam formation by the major hepatic canine rCYPs is presented for the first time. A common CYP3A probe, midazolam was not selective for canine CYP3A12 or 3A26 and instead, CYP2B11 (56-fold) and CYP2C21 (16-fold) were larger contributors to 1'-OH midazolam formation. Considering that CYP2B11, 2C21, and 3A12 constitute ~10, 34, and 15 % of canine liver total CYP content (Eguchi et al., 1996), the relative *in vitro* turnover rate for midazolam by CYP3A12 would be estimated to be responsible for ~ 2 % of 1'-OH midazolam formed in the liver in the absence of any intersystem normalization methods (Proctor et al., 2004). Again, the Bactosome CYP reductase levels could be important, though the sheer magnitude in isoform

differences might argue against this as the sole explanation. Previously the authors found that common CYP3A ligands did tend to inhibit of midazolam hydryoxylation in dog liver microsomes (Aidasani et al., 2008), but the number of inhibitors identified for canine CYP3A was low considering the documented promiscuity of CYP3A enzymes. Together, the findings suggest midazolam may not be the most sensitive *in vivo* canine CYP3A probe.

In summary, increasing the number of canine rCYPs should give more opportunities to answer specific questions related to drug metabolism in dogs. Additionally, the novel use of canine CYP reductase is expected to be an advantage over other commercial enzyme preparations that do not match CYPs with CYP reductase from the same species. Based on the findings, the authors are working to make all seven canine CYPs available for research purposes.

Acknowledgments

We thank Matthew Zaya for helpful discussions and the manuscript reviewers for their comments.

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Footnotes

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Figure Legend

Figure 1. Reaction phenotyping with phenacetin (A), temazepam (B), diclofenac (C), bufuralol (D), and midazolam (E) to determine the specificity of metabolite formation by seven hepatic canine rCYPs. The monitored metabolites were 4-acetamidophenol, oxazepam, 4'-OH diclofenac, 1'-OH bufuralol, and 1'-OH midazolam, respectively. Experiments were performed in quadruplicate and normalized to the isoform with the highest specific activity (± standard deviation).

DMD Fast Forward. Published on December 12, 2008 as DOI: 10.1124/dmd.108.025312
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Table 1. Canine CYP and Reductase expression yields using shake flask and fermenter cultures.

	Total P450 yield (nmol/litre culture)		[a]Specific P450 content (pmol/mg)		[a,b]CYP reductase content (pmol/mg)	
Enzyme	Flask	Fermenter	Flask	Fermenter	Flask	Fermenter
CYP1A1	108	74	545	1416	95	158
CYP2B11	52	12	309	307	126	309
CYP2C21	44	17	198	303	83	124
CYP2C41	59	6	316	133	141	105
CYP2D15	55	7	242	381	82	171
CYP3A12	14	7	74	198	73	53
CYP3A26	4.8	0.16	48	29	30	45

[a]Specific enzyme content and activity of *E. coli* membranes from fermenter cultures were determined after additional centrifugation and filtering steps as described in the Methods.

[b]CYP reductase content was estimated from cytochrome c reductase activity (Parikh et al., 1997).

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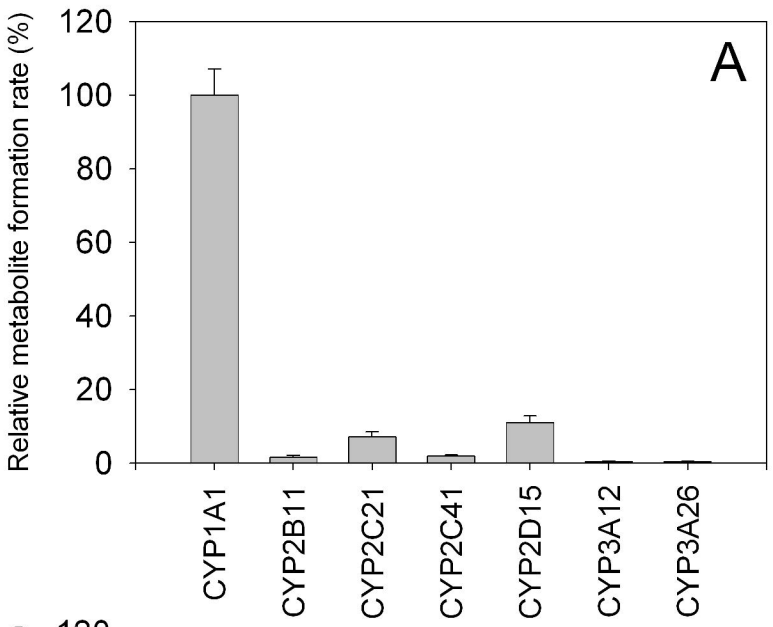
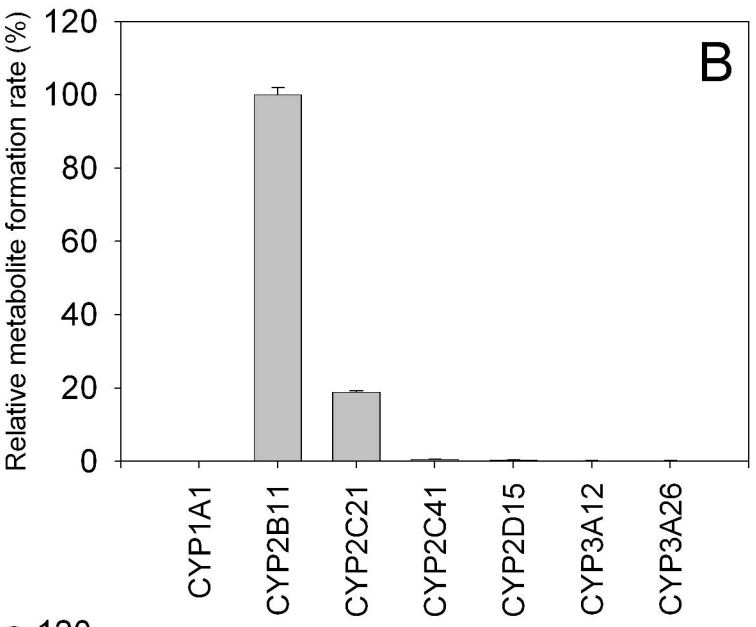
Table 2. Canine rCYP Bactosome kinetic parameters.

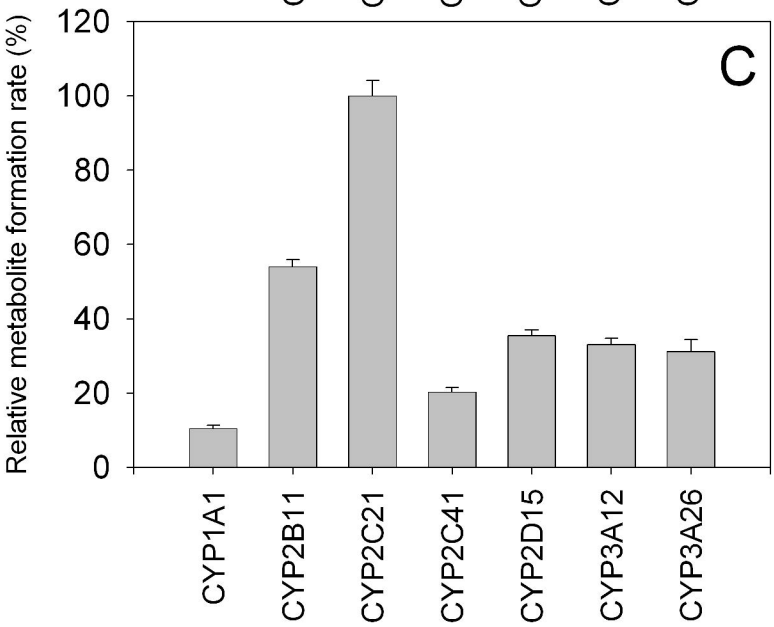
Isoform	Substrate/ Metabolite	[a]V_1 *(nmol/min/nmole)*	K_{m1} *(μM)*	V_2 *(nmol/min/nmole)*	K_{m2} *(μM)*
CYP1A1	phenacetin/ 4-acetamidophenol	10.5 ± 0.1	49.3 ± 2.0	[b]NA	NA
CYP2B11	temazepam/ oxazepam	2.1 ± 0.9	4.7 ± 4.4	20.6 ± 2.8	374 ± 147
CYP2C21	diclofenac/ 4'-OH	12.9 ± 0.93	50.1 ± 14.0	NA	NA
CYP2C41	diclofenac/ 4'-OH	4.6 ± 0.7	569 ± 165	NA	NA
CYP2D15	bufuralol/ 1'-OH	11.3 ± 0.2	9.4 ± 0.6	NA	NA
CYP3A12	midazolam/ 1'-OH	0.84 ± 0.02	3.8 ± 0.4	NA	NA
CYP3A26	midazolam/ 1'-OH	0.074 ± 0.021	2.0 ± 1.3	0.30 ± 0.03	120 ± 53
CYP3A12	midazolam/ 4-OH	0.56 ± 0.02	37 ± 3.2	NA	NA
CYP3A26	midazolam/ 4-OH	0.073 ± 0.040	0.83 ± 1.4	0.50 ± 0.04	62.3 ± 23.7

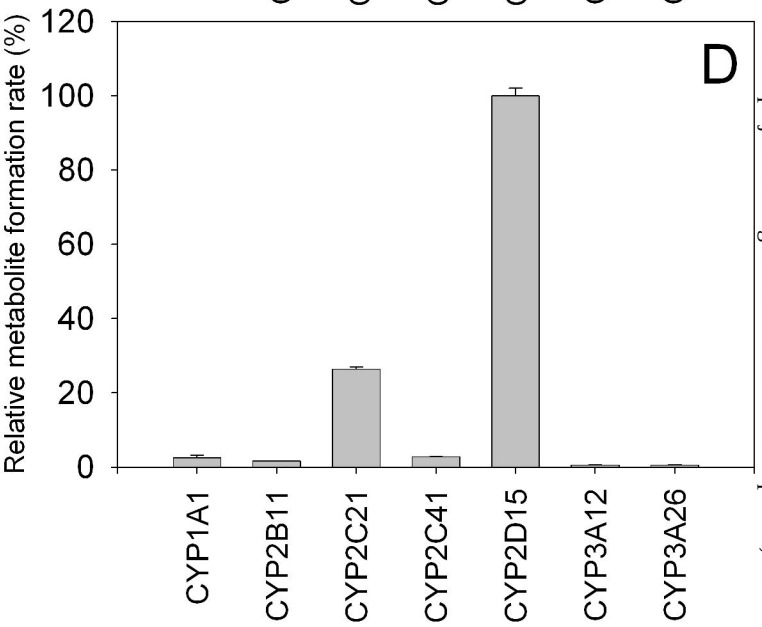
[a]See Materials and Methods for description of experiments and kinetic models.

[b]Not applicable.









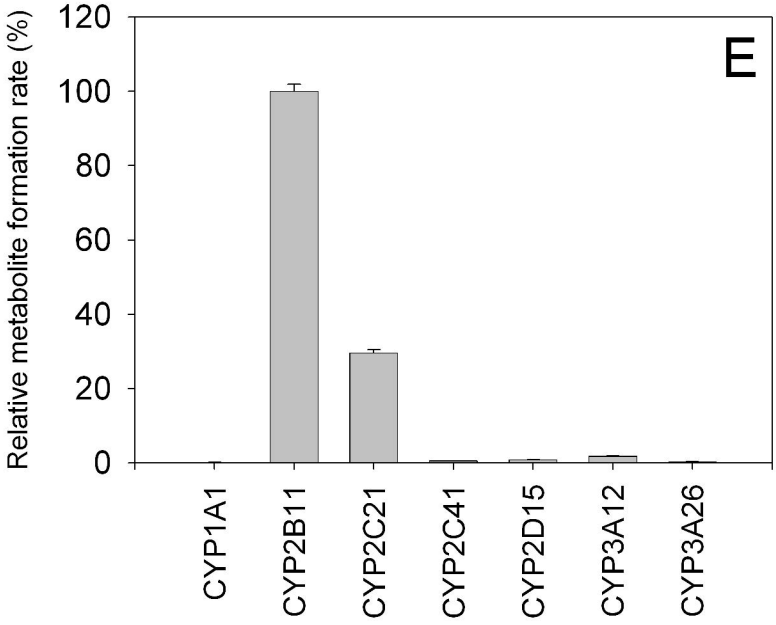


Figure 1