Expression and Characterization of Dog Cytochrome P450 2A13 and 2A25 in Baculovirus Infected Insect Cells

Diansong Zhou, Alban J. Linnenbach, Ruifeng Liu, Rick A. Luzietti, Jennifer J. Harris, Catherine L. Booth-Genthe¹, Scott W. Grimm

Running title

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Corresponding author:
Diansong Zhou
Clinical Pharmacology & DMPK
AstraZeneca Pharmaceuticals
1800 Concord Pike
Wilmington, DE, USA 19803
Tel 1-302-8862007
Fax 1-302-8865345
Email Diansong.zhou@AstraZeneca.com

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List of abbreviations: OR, NADPH-cytochrome P450 reductase; 7EC, 7-ethoxycoumarin.
Abstract

Dog CYP2A13 and CYP2A25 were co-expressed with dog NADPH-cytochrome P450 reductase (OR) in baculovirus infected-Sf9 insect cells. CYP2A13 effectively catalyzed 7-ethoxycoumarin (7EC) deethylation and coumarin hydroxylation with apparent Km values of 4.8 and 2.1 µM, respectively, similar to those observed using dog liver microsomes (7.5 and 0.75 µM, respectively). CYP2A25 exhibited much lower affinity towards 7EC, with apparent Km value of 150 µM, indicating CYP2A13 plays a more significant role in the metabolism of these CYP2A substrates. Similar to the dog CYP1A2 enzyme, CYP2A13 efficiently catalyzed phenacetin deethylation with a Km value of 3.9 µM, suggesting phenacetin is not a selective probe for dog CYP1A2 activity. Both dog CYP2A13 and CYP2A25 exhibited little or no catalytic activity towards other common CYP probe substrates including bupropion, amodiaquine, diclofenac, S-mephenytoin, bufuralol, dextromethorphan, midazolam and testosterone. These results provided additional information about the selectivity of these commonly used probe substrates.
Introduction:

Dog is a commonly used species in preclinical development for metabolism, pharmacokinetic and toxicological testing of new drugs. At least twelve dog CYP genes have been identified to date (Martignoni et al., 2006). Knowledge of dog CYP enzymes and their differences from CYP enzymes in human and other species is of great importance in drug development. Shou et al. (2003) have expressed and characterized seven dog CYP enzymes (CYP1A1, 2B11, 2C21, 2C41, 2D15, 3A12 and 3A26) in baculovirus infected-Sf21 insect cells. Recently, the same 7 dog CYPs have also been expressed in *E. coli* with co-expression of dog OR (Locuson et al., 2009). Dog CYP2A enzymes have not been reported in any cDNA-directed expression system. Drug metabolizing capability and specificity of dog CYP2A enzymes have not been characterized as extensively as the human and rodent isoforms. Human CYP2A6 is mainly expressed in human liver and contributes ~4% of total hepatic CYP (Martignoni et al., 2006), while human CYP2A13 is primarily expressed in the respiratory tract (Ling et al., 2007). Both dog CYP2A13 and CYP2A25 are present predominantly in liver tissues (Martignoni et al., 2006). Human CYP2A6 and CYP2A13 differ at 32 amino acid positions and exhibit different metabolic specificity. Carcinogenic epoxide metabolites of aflatoxin B1 were only produced by human CYP2A13, but not CYP2A6 (He et al., 2006). Human CYP2A6 exhibited little activity towards phenacetin, while human CYP2A13 metabolized phenacetin more efficiently than human CYP1A2 (Fukami et al., 2007). CYP2A isoforms in rat and mouse have been reported to catalyze 7α- and 15α- testosterone hydroxylation, in addition to coumarin 7-hydroxylation (Martignoni *et al*., 2006). In contrast, dog liver microsomes catalyze coumarin 7-hydroxylation, but not 7α- and 15α- testosterone hydroxylation, which is similar to that observed in human liver.
microsomes (Pearce et al., 1992). However, the enzyme(s) responsible for coumarin metabolism have not been clearly demonstrated in dog.

In the present study, dog CYP2A13 and CYP2A25 were co-expressed with dog OR by using the baculovirus-insect cell system. The activities of these enzymes towards various CYP probe compounds were evaluated more broadly in this study.
Materials and Methods

Materials. Chemicals were obtained from Sigma-Aldrich (St. Louis, MO), Toronto Research Chemicals (Toronto, Canada) and Steraloids (Newport, RI). Polymerase Chain Reaction (PCR)-Ready First-Strand cDNA from female beagle dog normal liver was obtained from BioChain Institute, Inc. (Hayward, CA). Bac-to-Bac® Baculovirus Expression System and Sf9 (*Spodoptera frugiperda*) insect cells, protein mini-gels and XCell *SureLock™* Mini-Cell with XCell II™ Blot Module Kit were obtained from Invitrogen (Carlsbad, CA). BaculoELISA titer kit was purchased from Clontech (Mountain View, CA). CYP2A goat polyclonal antibody raised against a peptide mapping at the C-terminus of CYP2A of human origin and donkey anti-goat IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolation of cDNAs and Construction of Recombinant Baculoviruses. cDNAs for dog CYP2A13, CYP2A25 and OR were PCR-amplified from dog liver single-strand cDNA with *PfuUltra™* High-Fidelity DNA polymerase following the manufacturers’ protocols. Forward and reverse primers, respectively, were: 5’-ATGTTGGCCTCAGGGCTTCTTCTG and 5’-TCAGCGGGGCTGGAAGCTCATGGT (CYP2A13); 5’-ATGGTGGCCTCAGGGATCCTTCTG and 5’-TCAGCGGGGCTGGAAGCTCATGGT (CYP2A25); and 5’-ATGGAGGACTCCAGTATGGATGCCA and 5’-CTAGCTCCACACGTCCAGGGAGTAGCG (OR). The PCR product was ligated into pCR-Blunt and sub-cloned into restriction enzyme digested and dephosphorylated pFastBac1. Clones and subclones were propagated in TOP10 *E. coli* cells. The cDNA sequences were determined by using BigDye® Terminator v3.1 Cycle Sequencing Kits (ABI, Foster City, CA). The cDNA sequences of CYP2A13 and CYP2A25 cloned in the present study were identical to GenBank.
reference sequences NM_001037345 and NM_001048027.1, respectively. Dog OR was cloned and deposited GenBank (GU827421). Two synonymous nucleotide differences (T256C and C1694T) were identified in the cloned dog OR in the present study compared to the GenBank reference sequence for dog OR (XM_844050.1).

Baculovirus recombinants were obtained by using the Invitrogen Bac-to-Bac baculovirus expression system, according to manufacturer’s protocols. Briefly, *E. coli* DH10Bac competent cells harboring bacmid vector DNA were transformed with pFastBacI plasmid recombinant DNAs via heat-shock. Recombinant bacmid DNA was isolated and used to transform Sf9 insect cells via Cellfectin® II -mediated gene transfer to generate recombinant baculovirus. Recombinant baculovirus-containing culture supernatants were harvested, amplified and titered by using BaculoELISA kits. High-titer passage 3 stock was used for protein expression.

**Expression of Dog CYPs with OR in Sf9 Insect Cells.** Sf9 insect cell liquid cultures were grown at 27°C in Sf-900 II SFM medium to a cell density of ~1-1.5 x 10^6 cells/mL. Cells were co-infected with virus encoding CYP and OR at multiplicity of infection (MOI) of 0.02 and 0.002, respectively. At 24 hr post-infection, a hemin-albumin complex was added to achieve 1 μg/mL hemin (Shou et al., 2003). Cells were harvested by centrifugation after 72 hr infection.

Microsomes were prepared by homogenization and 2-speed centrifugation (10,000 and 105,000g) and were reconstituted in phosphate buffered saline (pH 7.4). Microsomal protein concentrations were measured by using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). P450 content of dog recombinant CYP2A13 and CYP2A25 were measured by CO-difference spectrum (Omura and Sato, 1964). OR activities were measured by
cytochrome c reduction assay (Yasukochi and Masters, 1976). Microsomes were stored at -70°C until use.

**Western Blots.** The expression of recombinant dog CYP2A13 and CYP2A25 in microsomes isolated from baculovirus-infected insect cells was determined by electrophoresis under denaturing/reducing conditions and immunoblot analysis, according to manufacturer’s specifications. Briefly, protein (0.2 pmol CYP) was treated with NuPAGE® Sample Reducing Agent, separated on a 4 – 12% NuPAGE Bis-Tris mini-gel in NuPAGE® MOPS SDS Running Buffer and then electrotransferred to polyvinylidene difluoride membrane. Both upper gel running and transfer buffers contained NuPAGE® Antioxidant. The blot was blocked with phosphate buffered saline containing 0.05% Tween-20 and nonfat dry milk and sequentially incubated with goat polyclonal anti-CYP2A (1:200; Santa Cruz Biotechnology) and horseradish peroxidase-conjugated donkey anti-goat IgG (1:10,000; Santa Cruz Biotechnology). CYP2A protein was visualized by using Luminol chemiluminescent reagent and Amersham Hyperfilm ECL. Pooled dog liver microsomes (10 µg) and human recombinant CYP2A6 (0.2 pmol) were included as references. Microsomes (10 µg) from uninfected Sf9 insect cells were used as a negative control.

**Enzyme Activity and Kinetics.** Phenacetin, 7EC, coumarin, bupropion, amodiaquine, diclofenac, S-mephenytoin, bufuralol, dextromethorphan, midazolam and testosterone were incubated at 50 µM in the presence of 25 pmol/mL of dog recombinant CYP2A13 or CYP2A25 for 30 min at 37°C in a final volume of 0.2 mL in duplicate. Nicotine was incubated under the same conditions as other compounds with addition of 1 mg/mL of dog liver cytosol as an excess aldehyde oxidase source (Messina et al., 1997). The reaction mixtures contained 100 mM
potassium phosphate (pH 7.4) and 5 mM MgCl₂. Reactions were initiated with NADPH (1 mM), and stopped with 0.2 mL of acetonitrile containing 200 nM α-hydroxytriazolam (internal standard). Supernatants were analyzed using Waters UPLC (Milford, MA) and Applied Biosystems Sciex API-4000 Q-Trap mass spectrometer (Foster City, CA) (Wang and Zhang, 2007; Zhou et al., 2009).

To further evaluate dog recombinant CYP2A13 and CYP2A25 kinetics, reaction rates were measured under linear conditions using a number of substrates. Phenacetin (0.3~100 µM), 7EC (0.01~100 µM) and coumarin (0.3~100 µM) were incubated with CYP2A13 (10 pmol/mL) for 10 min. 7EC (1~500 µM) was incubated in the presence of CYP2A25 (25 pmol/mL) for 30 min. Phenacetin, 7EC and coumarin were also incubated with 0.2 mg/mL dog liver microsomes for 15 min. Incubations with 7EC were performed in triplicates while other substrates were performed in duplicates. Sample preparation and analysis were the same as described above.

**Kinetic Analysis.** Enzyme kinetics for each metabolite was obtained by fitting Michaelis-Menten equation \( v = \frac{V_{max} [S]}{K_m + [S]} \) to experimental data using nonlinear regression (Prism 4, GraphPad Software, La Jolla, CA). Kinetic constants were reported as the mean ± standard error of the parameter estimated.
Results and Discussion

The cDNA-directed expression of individual CYP enzymes has provided valuable tools for the assessment of enzyme selectivity towards certain compounds (Bjornsson et al., 2003). Baculovirus has been successfully used to express full-length human CYP cDNAs (Crespi and Penman, 1997; Gonzalez and Korzekwa, 1995). In the current study, dog CYP2A13 and CYP2A25 were cloned and co-expressed with dog OR in baculovirus-Sf9 insect cells and enzyme activities towards commonly used human CYP probe compounds were evaluated. To our knowledge, this is the first report of the expression and substrate selectivity for dog CYP2A13 and CYP2A25.

Protein Expression. Dog CYP2A13 and CYP2A25 were co-expressed with dog OR in Sf9 insect cells. Dog CYP2A13 and CYP2A25 exhibited P450 content of 40 and 48 pmol/mg microsomal protein, and OR activity of 46 and 30 nmol/min/mg protein, respectively. Expression was also confirmed by immunoblot analysis using anti-CYP2A antibody (Figure 1). Human CYP2A6 and dog liver microsomes were used as control test systems. Microsomes prepared from uninfected Sf9 cells exhibited no cross reactivity to anti-CYP2A antibody.

Activity Characterization and Kinetic Analysis. Initially, a set of known human CYP probe substrates were incubated with dog recombinant CYP2A13 and CYP2A25 to evaluate substrate selectivity. CYP2A13 catalyzed 7EC deethylation, phenacetin deethylation, coumarin hydroxylation and nicotine oxidation. Under the same conditions, CYP2A25 catalyzed 7EC deethylation (2.1 pmol/min/pmol) and showed relatively low activities towards phenacetin, coumarin and nicotine metabolism with 0.01, 0.025 and 0.17 pmol/min/pmol, respectively (Figure 2A). CYP2A13 exhibited high affinity (K_m 4.8 µM) and high capacity (V_max, 47
pmol/min/pmol) while CYP2A25 demonstrated low affinity (K_m 150 µM) and low capacity (V_max 8.4 pmol/min/pmol) towards 7EC deethylation (Table 1). The 7EC deethylation catalyzed by CYP2A13 was found to be ~170 fold more efficient than that by CYP2A25. Dog liver microsomes showed an apparent K_m of 7.5 µM, similar to that calculated for CYP2A13. Coumarin 7-hydroxylation by dog liver microsomes and dog recombinant CYP2A13 both exhibited one-enzyme Michaelis-Menten kinetics, with apparent K_m values of 0.75 and 2.1 µM, respectively. These results suggest dog CYP2A13 plays a more significant role in the metabolism of these CYP2A substrates in dog liver.

CYP2A13 exhibited high affinity (K_m 3.9 µM) and low capacity (V_max 3.6 pmol/min/pmol) for phenacetin deethylation (Table 1). In our previous report (Zhou et al., 2009) dog CYP1A2 exhibited relative low affinity (K_m 12.3 µM) and high capacity (V_max 13.9 pmol/min/pmol). The intrinsic clearance (V_max/K_m) of phenacetin deethylation from this study was comparable for dog CYP2A13 (0.92 µL/min/pmol) and dog CYP1A2 (1.1 µL/min/pmol). Similar results were reported for human CYP2A13 enzyme, which catalyzed phenacetin more efficiently than human CYP1A2 (Fukami et al., 2007). Human CYP2A13 is mainly expressed in the respiratory tract (Ling et al., 2007), while dog CYP2A13 is predominantly present in liver tissue (Martignoni et al., 2006). For this reason, phenacetin should not be used as a selective probe drug for dog CYP1A2 enzyme activity in vitro or in vivo.

There was no measurable dextrophan or 1’-hydroxybufuralol metabolite formation in CYP2A13 or CYP2A25 incubations (Figure 2A). Both bufuralol and dextromethorphan are well known human CYP2D6 substrates (Bjornsson et al., 2003) and were reported as dog CYP2D15 selective substrates (Shou et al., 2003). The present study provides supplemental information to
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confirm their selectivity. Both CYP2A13 and CYP2A25 catalyzed metabolism of bupropion, amodiaquine, diclofenac and S-mephenytoin at very low rate (< 0.13 pmol/min/pmol) at 50 µM substrate concentration. Diclofenac has been reported to be metabolized by dog CYP2B11 and CYP2C21 efficiently (Shou et al., 2003). CYP2A13 and CYP2A25 would be minor contributors for the metabolism of diclofenac in dog liver microsomes.

CYP2A13 metabolized midazolam to 1’-hydroxylation and 4-hydroxylation metabolite at rates of 0.11 and 0.28 pmol/min/pmol, respectively (Figure 2A), while CYP2A25 exhibited much lower catalytic capability towards midazolam. Both enzymes would be minor contributors for the metabolism of midazolam in dog liver microsomes compared to other dog CYPs, such as CYP3A12 (Locuson et al., 2009). Dog CYP2A13 and CYP2A25 exhibited low catalytic activities in hydroxylating testosterone at multiple sites (Figure 2B). No measurable 7α-hydroxylation was observed in either CYP2A13 or CYP2A25 incubations, which is consistent with previous observations (Pearce et al., 1992). The overall contribution of CYP2A13 and CYP2A25 to the metabolism of testosterone is likely to be insignificant in dog liver microsomes.

Dog CYP2A13, dog CYP2A25, human CYP2A6 and human CYP2A13 retain more than 85% amino acid sequence identity. Dog CYP2A13 and CYP2A25 differ only at 34 out of 494 amino acids and exhibit significant catalytic differences towards several common CYP2A substrates. Inspection of the amino acid sequences of human and dog CYP2A enzymes revealed an interesting observation. All 4 enzymes have conserved Asn297, which is believed to play a key role in the ligand orientation by hydrogen bonding with substrate. Asn297 is the only polar residue accessible to substrate in the active sites of human CYP2A6, CYP2A13 and dog CYP2A13 enzymes (Table 2). However, CYP2A25 has an additional polar residue Thr117 while
other CYP2A enzymes have either Ala or Val at this position. Residue 117 is located at the base of the active site near the heme and has been demonstrated to substantially impact the catalytic efficiency of human CYP2A13 by changing $V_{\text{max}}$ of several substrates (Smith et al., 2007). It has been suggested substrate orientation may be related to catalytic activity changes. In the case of CYP2A25, Thr$^{117}$ may compete with Asn$^{297}$ in aligning substrate by providing different orientation of hydrogen bonding, which could be one of the reasons for the significant catalytic difference between dog CYP2A13 and CYP2A25.

In summary, dog CYP2A13 and CYP2A25 have been expressed and characterized in Sf9 insect cells. This work also revealed that dog CYP2A13 plays a significant role in the metabolism of several typical CYP2A substrates. Dog CYP2A13 exhibited catalytic activity towards phenacetin comparable to dog CYP1A2. The contributions of dog CYP2A13 and CYP2A25 to the metabolism of probe substrates of other major CYP isoforms is likely to be insignificant, providing additional information for the selectivity of these probe substrates.
References


Locuson CW, Ethell BT, Voice M, Lee D, and Feenstra KL (2009) Evaluation of Escherichia coli membrane preparations of canine CYP1A1, 2B11, 2C21, 2C41, 2D15, 3A12, and
3A26 with coexpressed canine cytochrome P450 reductase. Drug Metab Dispos 37: 457-461.


Footnote

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1 current affiliation: Respiratory Centre of Excellence in Drug Discovery, GlaxoSmithKline, King of Prussia, PA, USA.
Legends for figures

**Figure 1.** Western blot analysis of CYP2A13 and CYP2A25 expression in microsomes prepared from baculovirus-infected Sf9 insect cells. Dog liver microsomes (DLM) and human CYP2A6 were used as positive controls. Microsomes from uninfected insect cells were used as a negative control. Lanes were loaded with 0.2 pmol of CYP2A13, CYP2A25 or CYP2A6 or 10 µg of DLM or microsomes from uninfected insect cells.

**Figure 2.** Metabolite formation of phenacetin, 7-ethoxycoumarin, coumarin, nicotine, bupropion, amodiaquine, diclofenac, S-mephenytoin, bufuralol, dextromethorphan and midazolam (A) or testosterone (B) at 50 µM substrate concentration in the presence of 25 pmol/mL of dog recombinant CYP2A13 or CYP2A25. Data is presented as average of duplicate incubations, and the variation between duplicate incubations was within 10% of each other in all cases.
Table 1. Apparent kinetic parameters for metabolism of phenacetin, 7-ethoxycoumarin (7EC) and coumarin in dog recombinant CYP2A13, CYP2A25 and pooled dog liver microsomes. Incubations with 7EC were performed in triplicates while other substrates were performed in duplicates. Data are presented as mean ± standard error of parameter fit using one-enzyme Michaelis-Menten model.

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<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pmol/min/pmol)</th>
<th>$CL_{int}$ ($V_{max}/K_m$) (µL/min/pmol)</th>
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<td>CYP2A13</td>
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<td>7EC</td>
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<td>9.8</td>
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<td>coumarin</td>
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<td>19 ± 1</td>
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<td>CYP2A25</td>
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<th>Substrate</th>
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<td></td>
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<td></td>
<td>coumarin</td>
<td>0.75 ± 0.42</td>
<td>310 ± 10</td>
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Table 2. Comparison of key active site residues in CYP2A enzymes.

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Figure 1