Reevaluate In Vitro CYP3A Index Reactions of Benzodiazepines and Steroids between Humans and Dogs

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Abbreviations

CYP3A: cytochrome P450 3A
rCYPs: recombinant P450 enzymes
MDZ: midazolam
1'h-MDZ: 1'-hydroxymidazolam
4h-MDZ: 4-hydroxymidazolam
APZ: alprazolam
αh-APZ: α-hydroxyalprazolam
4h-APZ: 4-hydroxyalprazolam
TRZ: triazolam
αh-TRZ: α-hydroxytriazolam
4h-TRZ: 4-hydroxytriazolam
F: cortisol
T: testosterone
6βh-T: 6β-hydroxytestosterone
2αh-T: 2α-hydroxytestosterone
2βh-T: 2β-hydroxytestosterone
16αh-T: 16α-hydroxytestosterone
DCA: deoxycholate, 3α, 12α-dihydroxy-5β-cholan-24-oic acid
LCA: lithocholate, 3α-hydroxy-5β-cholan-24-oic acid
CA: cholate, 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid
CDCA: chenodeoxycholate, 3α, 7α-dihydroxy-5β-cholan-24-oic acid
HDCA: hyodeoxycholic acid, 3α, 6α-dihydroxy-5β-cholan-24-oic acid
1βh-DCA: 1β-hydroxydeoxycholic acid, 1β, 3α,12α-trihydroxy-5β-cholan-24-oic acid
5βh-DCA: 5β-hydroxydeoxycholic acid, 3α, 5β, 12α-trihydroxy-5β-cholan-24-oic acid
PROG: progesterone
CLZ: clozapine
HLM: human liver microsomes
DLM: beagle dog liver microsomes
MM: Michaelis-Menten
Hill: Allosteric sigmoidal
SI: Substrate inhibition
CL_{int}: intrinsic clearance
V_{max}: maximal velocity
K_m (S_{50}): substrate concentration occupying half of the binding sites
NADPH: nicotinamide adenine dinucleotide phosphate
PBS: phosphate buffered saline
DMSO: dimethylsulfoxide
LC-MS/MS: liquid chromatography with tandem mass spectrometry
QC: quality control
Abstract

Cytochrome P450 3A (CYP3A), the most important class of drug-metabolizing enzymes, participates the metabolism of half of clinically used drugs. The CYP3A index reactions of dogs, one of the most widely used preclinical nonrodent species, are still poorly understood. This work evaluated the activity and selectivity of ten CYP3A index reactions, including midazolam (MDZ) 1’- and 4-hydroxylation, alprazolam (APZ) and triazolam (TRZ) α- and 4-hydroxylation, testosterone (T) 6β-hydroxylation, lithocholate (LCA) 6α-hydroxylation, deoxycholate (DCA) 1β- and 5β-hydroxylation, with quantitative reaction phenotyping and kinetic analysis in human and canine recombinant CYP enzymes (rCYPs). In human studies, all reactions are reconfirmed as mixed index reactions of CYP3A with minor contributions from non-CYP3A isoforms. In canine studies, all reactions are also primarily catalyzed by CYP3A12 with lower contributions from CYP3A26. However, the canine CYP2B11 appreciably contributes to the hydroxylation of benzodiazepine except for APZ 4-hydroxylation. The canine CYP3A isoforms have lower activity than human isoforms toward T 6β-hydroxylation and LCA 6α-hydroxylation and both substrates undergo non-CYP3A catalyzed side reactions. DCA 1β- and 5β-hydroxylation are validated as the CYP3A index reactions in both humans and dogs with limited non-CYP3A contributions and side reactions. In conclusion, this work provides a comprehensive overview for the selectivity and activity of in vitro CYP3A index reactions in humans and dogs. The validated CYP3A index reactions between humans and dogs may benefit future practices in drug metabolism and drug interaction studies.

Keywords

Cytochrome P450 3A, Species difference, CYP3A12, CYP3A26, CYP2B11, Canine, Human
Significance Statement

Dogs are one of the most important non-rodent animals with limited studies of Cytochrome P450 enzymes than humans. This work provides the most comprehensive quantitative data to date for the selectivity and activity of CYP3A index reactions in humans and dogs. The canine CYP2B11 was found to appreciably contribute to hydroxylation of midazolam, alprazolam and triazolam, the well-known probes for human CYP3A. DCA 1β- and 5β-hydroxylation are validated as the CYP3A index reactions in both humans and dogs.
Introduction

Cytochrome P450 3A (CYP3A), one of the most important drug-metabolizing enzymes, participates the disposition of nearly 50% of prescribed drugs (Wienkers and Heath, 2005; Rendic and Guengerich, 2015). Among the four functional hepatic CYP3A isoforms in human genome, CYP3A4 and 3A5 are the primary drug-metabolizing isoforms with heavily overlapping substrates, CYP3A7 is a fetal-specific isoform playing critical roles in placental estriol synthesis (Kitada et al., 1987a; Kitada et al., 1987b) and pediatric pharmacology (Stevens, 2006), and CYP3A43 is expressed in adult liver, kidney and testis with unknown functions (Westlind et al., 2001). The expression and activity of CYP3A demonstrate highly inter- and intra-individual variability due to susceptibility of inhibition and induction (Zanger and Schwab, 2013). The substrate specificity (Ohmori et al., 1998), catalytic efficiency (Williams et al., 2002; Shen et al., 2004), and metabolic regioselectivity (Gorski et al., 1994; Ohmori et al., 1998; Wang et al., 1998) show elaborate differences among them. Drug metabolism studies are under insistent demands of validated CYP3A index reactions to assess and discriminate their activities.

A number of in vitro CYP3A index reactions were developed decades ago with exogenous benzodiazepines and endogenous steroids as substrates, such as 1'- and 4-hydroxylation of midazolam (MDZ), α- and 4-hydroxylation of alprazolam (APZ) and triazolam (TRZ), 6β-hydroxylation of testosterone (T) and cortisol (F) (Liu et al., 2007). Quantitative reaction phenotyping in the recombinant P450 enzymes (rCYPs) is pivotal to verify the selectivity of index reactions. The classic human CYP3A index reactions had been studied in rCYPs since the 2000’s. It is widely accepted that MDZ 1'-hydroxylation and T 6β-hydroxylation are the most accepted index reactions of CYP3A4 and 3A5. It’s generally accepted that T 6β-hydroxylation is a CYP3A4-preferring index reaction, while CYP3A4 and 3A5 contribute equivalently to MDZ 1'-hydroxylation (Williams et al., 2002). Compared to MDZ and T, APZ and TRZ are less extensively studied. Recently, deoxycholate (DCA) and lithocholate (LCA) are proposed as novel CYP3A substrates. As the endogenous secondary bile acids, DCA and LCA are “exogenous” to the host because they are continuously produced by gut bacteria from the host-synthesized primary bile acids, cholate (CA) and chenodeoxycholate (CDCA), respectively. Deo and colleagues showed that the 6α-hydroxylation of LCA into hyodeoxycholic acid (HDCA) is a CYP3A4-prefering reaction by reaction phenotyping in 12 human rCYPs without inclusion of CYP3A7 (Deo and Bandiera, 2009). Hayes and colleagues performed phenotyping analysis of DCA 1β-hydroxylation in 21
human rCYPs and concluded that it is a CYP3A4 and 3A7 preferring reaction (Hayes et al., 2016). It was later disclosed that CYP3A4 and 3A7 are exclusively responsible for the regioselective oxidations of DCA, in which 1β- and 5β-hydroxylation are the main reactions (Zhang et al., 2019). To date, the selectivity and activity of the above index reactions are not quantitatively compared in human rCYPs, not to mention taking the inter-species differences into consideration.

Drug metabolism differs between human and preclinical species, producing confound data during drug development. Rodent animals are not recommended as preferred models to predicting clinical drug interactions. Gene editing technologies in rodent species may provide useful models that to some extent circumvent this limitation (Muruganandan and Sinal, 2008; Lu et al., 2021). Nonrodent species such as dogs, pigs and monkeys are more human like. However, the drug metabolism in dogs, one of the most widely used nonrodent preclinical species, is still an understudied area with incomplete and inconsistent data of CYP3A index reactions (Mealey et al., 2019). Thus far, 12 canine CYPs have been identified including CYP1A1, 1A2, 1B1, 2A13, 2A15, 2B11, 2C21, 2C41, 2D15, 2E1, 3A12, and 3A26, in which 9 rCYPs were reported in literatures (Shou et al., 2003; Locuson et al., 2009b; Zhou et al., 2010) and 8 rCYPs (CYP1A1, 1A2, 2B11, 2C21, 2C41, 2D15, 3A12, and 3A26) were commercially available for studies. The canine CYP3A12 and 3A26 are the analogue of human CYP3A4 and 3A5, respectively (Fraser et al., 1997). The homologous genes of human CYP3A7 and 3A43 are not reported in the canine genome. Although MDZ was generally accepted as a substrate of CYP3A for both dogs and humans (Martinez et al., 2013), it was suggested that MDZ might not be a sensitive probe for canine CYP3A since MDZ was oxidized into 1'-hydroxymidazolam (1'h-MDZ) more by canine CYP2B11 than 3A12 (Locuson et al., 2009a). However, the semi-quantitative data of our team showed that the oxidative elimination of MDZ was ascribed primarily to CYP3A12, secondly to CYP2B11 and CYP3A26, and minorly to CYP2C21 (Zeng et al., 2021). Quantitative revaluation of the index reactions between humans and dogs is of necessity to clarify the inconsistent data.

In this work, we quantitatively evaluated the selectivity and activity of CYP3A index reactions of exogenous benzodiazepines (MDZ, APZ, TRZ) and endogenous steroids (T, LCA, DCA) in eighteen human rCYPs and eight canine rCYPs in comparison to the corresponding liver microsomes. To allow for direct comparison between the measured activities in rCYP and liver microsomes and highlight the selectivity, the rCYP activity was adjusted based on the absolute P450 abundance estimates in liver microsomes of humans and dogs (Couto et al., 2019; Martinez et al., 2019). The hydroxylation kinetics of MDZ, APZ,
TRZ, T and DCA was subsequently investigated in the reactive canine isoforms to ascertain the selectivity and activity of these index reactions in dogs. These results have provided the most comprehensive quantitative data to date for the selectivity and activity of \textit{in vitro} CYP3A index reactions between humans and dogs.

\textbf{Materials and Methods}

\textit{Materials and Reagents}

TRZ, APZ, \(\alpha\)-hydroxytriazolam (\(\alpha\h\)-TRZ), \(\alpha\)-hydroxyalprazolam (\(\alpha\h\)-APZ), 6\(\beta\)-hydroxytestosterone (6\(\beta\h\)-T), DCA, LCA and HDCA were purchased from Sigma-Aldrich (St. Louis, MO). MDZ, 1'h-MDZ, 4-hydroxymidazolam (4h-MDZ), 4-hydroxytriazolam (4h-TRZ) and 4-hydroxyalprazolam (4h-APZ) were obtained from Cerilliant (Austin, USA), MedChem Express (New jersey, USA), Carboxynth (Berkshire, UK), Abcam Biotechnology (Cambridge, MA, USA) and Cayman Chemical (Ann Arbor, MI, USA), respectively. T and progesterone (PROG, internal standard used for the quantification of T and 6\(\beta\h\)-T) were purchased from Nine-Dinn Chemistry (Shanghai, China). 1\(\beta\)-hydroxydeoxycholic acid (1\(\beta\h\)-DCA) was obtained from QCC Inc. (Newark De, USA). Clozapine (CLZ), CA-2,2,4,4-D\(_4\) and DCA-2,2,4,4-D\(_4\) used as internal standards in metabolites quantification were procured from Sigma-Aldrich. 5\(\beta\)-hydroxydeoxycholic acid (5\(\beta\h\)-DCA) was synthesized from DCA in our lab as previously described (Zhang et al., 2019). Stock solutions of all the above authentic standards were prepared in dimethylsulfoxide (DMSO).

Human liver microsomes (HLM) from 200 mixed-gender pooled donors (100 male and 100 female) and beagle dog liver microsomes (DLM) from 20 mixed-gender pooled donors (8 male and 12 female) were purchased from XenoTech, LLC (Lenexa, KS, USA). NADPH regenerating system solution A (NADPH-A, containing 26 mM NADP\(^+\), 66 mM glucose-6-phosphate and 66 mM MgCl\(_2\) in water), NADPH regenerating system solution B (NADPH-B, containing 40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate) and 0.5 M Phosphate buffered saline (PBS, pH 7.4) were procured from Meilun Bio (Dalian, China). Human rCYPs prepared from insect cells infected with baculovirus (Supersomes), including CYP1A2, 1B1, 2A6, 2B6, 2C8, 2C9*1, 2C18, 2C19, 2D6*1, 3A4, 3A5, 3A7, 2E1, 2J2, 4A11, 4F2, 4F3B, and 4F12, were acquired from Corning (Tewksbury, MA, USA). Another set of human rCYPs (CYP3A4, 3A5 and 3A7) and Canine rCYPs (CYP1A1, 1A2, 1B1, 2B11, 2C21, 2C41, 2D15, 3A12, and 3A26) prepared from plasmid-transfected \textit{Escherichia coli} (\textit{E.coli}) (Bactosomes) were obtained from Cypex Ltd. (Dundee, Scotland). Methanol, acetonitrile, and formic acid were from Sigma-Aldrich (St. Louis, MO). DMSO was obtained.
from Thermo Fisher Scientific (Waltham, MA). Ultrapure water was obtained by using a Milli-Q System (Millipore Sigma, Bedford, MA).

**In Vitro Metabolism Assay**

*In vitro* metabolism assay was conducted as previously described (Chen et al., 2019; Zhang et al., 2019). In the reaction phenotyping studies in human and canine rCYPs, the initial substrate concentrations were 10 μM for TRZ, APZ and T, 4 μM for MDZ, 50 μM for DCA, and 20 μM for LCA. In the kinetic analysis of canine rCYPs, the initial substrate concentrations were 1-300 μM for MDZ and T, 5-500 μM for TRZ and APZ, 1-400 μM for DCA, 1-150 μM for LCA across at least 7 substrate levels. Due to enzyme inhibition at high substrate levels, the kinetic studies of MDZ, TRZ, APZ were further compared across 6 substrate levels within the lower linear ranges (1-6.25 μM for TRZ and MDZ, 1-12.5 μM for APZ).

The 100 μL incubation contained 0.1 M PBS (pH 7.4), 5.0 μL NADPH-A, 1.0 μL NADPH-B, 0.5 μL working solution of substrate, 0.5 μL blank solvent, and 2.5 μL liver microsomes (protein concentration of 20 mg/mL) or rCYP enzymes (2.0 nmol protein/mL). The final protein level in the incubation media was 50 pmol/mL of rCYPs or 0.5 mg/mL of liver microsomes, except for MDZ, whose incubations had a protein concentration of 0.1 mg/mL in liver microsomes. All samples were processed in triplicate and the DMSO concentration in incubation system was 1% (v/v). Metabolism was initiated by the addition of NADPH-B after 5-min preincubation at 37°C in a shaking water bath. The reactions were quenched at 10 min (MDZ), 10 min (TRZ), 60 min (APZ), 15 min (T), 60 min (DCA and LCA) by adding ice-cold acetonitrile containing internal standards. The proteins were removed by centrifugation at 12000 rpm for 20 min at 4°C. The diluted supernatant was subjected to LC-MS/MS analysis.

**Quantitative Determination of Metabolites in Incubations**

Quantitative determinations were performed on ACQUITY UPLC coupled to Xevo TQS mass spectrometer (Waters, Milford, MA, USA). The bioanalytical methods for each substrate and its metabolites were described in the supporting information. The calibration and quality control (QC) samples were prepared in 0.1 M PBS (pH 7.4). The calibration ranges were 0.015 to 15 μM (TRZ), 0.1 to 10 μM (αh-TRZ and 4h-TRZ), 0.018 to 15 μM (APZ), 0.05 to 10 μM (αh-APZ and 4h-APZ), 0.006 to 6.0 μM (MDZ), 0.004 to 4.0 μM (1’h-MDZ and 4h-MDZ), 0.3 to 15 μM (T), 0.2 to 10 μM (6βh-T), 0.275 to 55 μM (DCA), 0.25 to 50 μM (1βh-DCA and 5βh-DCA), 0.125 to 25 μM (LCA), and 0.01 to 2.0 μM (HDCA) with
correlation coefficient $R^2 > 0.99$. The QC (0.03, 6.0, 12 μM for TRZ, 0.2, 4.0, 8.0 μM for αh-TRZ and 4h-TRZ, 0.036, 6.0, 12 μM for APZ, 0.1, 4.0, 8.0 μM for αh-APZ and 4h-APZ, 0.018, 3.0, 4.5 μM for MDZ, 0.012, 2.0, 3.0 μM for 1'h-MDZ and 4h-MDZ, 0.45, 7.5, 11.25 μM for T, 0.3, 5.0, 7.5 μM for 6βh-T, 0.825, 27.5, 41.25 μM for DCA, 0.75, 25, 37.5 μM for 1βh-DCA and 5βh-DCA, 0.375, 12.5, 18.75 μM for LCA, 0.03, 1.0, 1.5 μM for HDCA) was allocated into each bioanalytical run, in which the bias was within ±15%.

Data Processing

The LC-MS/MS raw data was processed using UNIFI (V1.8, Waters, Milford, MA, USA). Percentage remaining (one-way ANOVA statistics), metabolite formation rates, contributions of reactive P450 isoforms and kinetic parameters were processed and plotted using GraphPad Prism (V8.0.2, GraphPad Software, LaJolla, CA). Percentage remaining was calculated by comparing the amount of substrate remaining in samples to that in control group. The absolute metabolite formation rates were calculated in the unit of nmol/min/nmol P450 for incubations in rCYPs or pmol/min/mg protein for incubations in liver microsomes. As previous report (Martinez et al., 2020), the absolute metabolite formation rates in rCYPs were adjusted by multiplying the mean absolute P450 abundance estimates (pmole P450 per mg microsomal protein), which were 14.14, 25.62, 6.78, 29.78, 37.53, 1.60, 3.43, 6.05, 54.38, 0.64, 28.52, 8.63, 5.54, 12.22, 5.06 for human CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5, 3A7, 4F2, 4F11 in HLM (Couto et al., 2019), and 3.3, 123, 86, 64, 10, 139, 73, 121, 3.3 for canine CYP1A1, 1A2, 2B11, 2C21, 2C41, 2D15, 2E1, 3A12, 3A26 in DLM (Martinez et al., 2019). The kinetic parameters were determined by best fitting to models, including Michaelis-Menten (MM), Allosteric sigmoidal (Hill) or Substrate inhibition (SI). The intrinsic clearance ($CL_{int}$) was calculated by the ratio of $V_{max}/K_m (S_{50})$.

Results

Kinetics of Index Reactions in the Pooled Liver Microsomes

Table 1 and Table 2 listed the apparent kinetic data of HLM and DLM for the tested index reactions with the kinetic plots illustrated in Figure S1. Side reactions were negligible for benzodiazepines in either HLM or DLM. MDZ was metabolized primarily to 1'h-MDZ and secondly to 4h-MDZ; APZ was metabolized primarily to 4h-APZ and secondly to αh-APZ; TRZ was metabolized primarily to 4h-TRZ and secondly to αh-TRZ. The formation of 1βh-DCA and 5βh-DCA contributed largely to the degradation of DCA in HLM and DLM. In contrast, significant side reactions were observed for T and LCA in both HLM and DLM by
matching the degradation of substrate and the formation of probe metabolites (Figure S2).

Besides the interconversion between T and androstenedione, T was hydroxylated into several metabolites including 6βh-T, 2αh-T, 2βh-T and 16αh-T (Shou et al., 2003; Kandel et al., 2017), in which T 6β-hydroxylation contributed moderately in HLM and minorly in DLM to the degradation of substrate. LCA was also metabolized into a series of metabolites, in which LCA 6α-hydroxylation contributed minorly in HLM and rarely in DLM to the degradation of LCA (Figure S2).

In HLM, the apparent kinetics of most index reactions over the tested substrate ranges was best fit with MM or Hill models except for MDZ. The apparent kinetics of MDZ 1'- and 4-hydroxylation best fit SI model within the substrate levels of 1-300 μM. According to the CL_int data, HLM showed the strongest activity to MDZ 1'-hydroxylation (405 μl/min per mg protein) and the weakest activity to DCA 5β-hydroxylation (0.121 μl/min per mg protein). In DLM, the apparent kinetics of most index reactions over the same substrate ranges best fit MM or Hill models except that MDZ 1'-hydroxylation and TRZ α-hydroxylation were best explained by SI model within the substrate levels of 1-300 μM and 5-500 μM, respectively. In DLM, MDZ 1'-hydroxylation exhibited the strongest activity with CL_int of 1063 μl/min per mg protein, while LCA exhibited the weakest activity with CL_int of 0.0194 μl/min per mg protein. In summary, both HLM and DLM exhibited the strongest activity to MDZ 1'-hydroxylation, moderate activity to MDZ 4-hydroxylation, T 6β-hydroxylation and hydroxylation of APZ and TRZ, and the weakest activity to the oxidation of DCA and LCA.

**Phenotyping of Index Reactions in Human rCYPs**

Eighteen human rCYP enzymes were evaluated for their selectivity and activity toward the tested index reactions at appropriate substrate levels according to their apparent kinetics in liver microsomes. Figure 1A showed the percentage substrate remaining in human rCYPs and HLM. Figure 1B illustrated the absolute formation rate of probe metabolites in human rCYPs at the unit of per nmole protein. At the human rCYP level of 50 pmole/mL, CYP3A4 had the greatest activity toward TRZ 4-hydroxylation and the least activity toward MDZ 4-hydroxylation; CYP3A5 exhibited the strongest activity toward MDZ 1'-hydroxylation and the weakest activity toward LCA 6α-hydroxylation, and CYP3A7 showed the strongest activity toward DCA 1β-hydroxylation and the weakest activity toward APZ α-hydroxylation. The absolute activities of these index reactions in human rCYP enzymes were adjusted according to the absolute P450 abundance estimates in HLM. As shown in Figure 1C, the adjusted CYP3A4 activity increased, while those of CYP3A5 and 3A7 decreased compared
to their absolute activities due to the higher abundance of CYP3A4 in HLM. According to the adjusted data, the percentage contributions of the reactive human rCYPs were illustrated in Figure 1D for each index reaction. In this way, the non-CYP3A isoforms that contaminated these CYP3A index reactions in HLM were highlighted. Considering the selectivity among CYP3A isoforms, APZ 4-hydroxylation is the most selective for CYP3A4, APZ α-hydroxylation has the most contribution from CYP3A5, and MDZ 4-hydroxylation has the most contribution from CYP3A7. In all, it may be concluded that TRZ α- and 4-hydroxylation, APZ α- and 4-hydroxylation, MDZ 1'-hydroxylation and T 6β-hydroxylation are mixed probes of human CYP3A4 and 3A5, in which APZ 4-hydroxylation and T 6β-hydroxylation are the most CYP3A4-selective. In contrast, LCA 6α-hydroxylation, DCA 1β- and 5β-hydroxylation are mixed probes of human CYP3A4 and 3A7, in which DCA 5β-hydroxylation is the most CYP3A4-selective.

Phenotyping of Index Reactions in Canine rCYP Enzymes

The phenotyping data of tested maker reactions in eight canine rCYP enzymes was shown in Figure 2. Besides CYP3A, the canine CYP2B11 significantly contributed to almost all the tested index reactions of MDZ, TRZ and APZ except for APZ 4-hydroxylation at the canine rCYP level of 50 pmole/mL. In contrast, trace or minor CYP2B11 activity was observed for T 6β-hydroxylation and LCA 6α-hydroxylation, and no CYP2B11 activity was detected for DCA 1β- and 5β-hydroxylation. Consistent to previous report (Shou et al., 2003), T was significantly metabolized by CYP2C21 into 16α-T without detection of 6βh-T in the incubations. As shown in Figure 2B, CYP3A12 and 3A26 showed the maximum absolute activity toward MDZ 1'-hydroxylation and TRZ 4-hydroxylation, respectively, while both CYP3A12 and 3A26 exhibited the minimum activity to LCA 6α-hydroxylation. Similarly, the absolute activity data was adjusted according to the absolute P450 abundance estimates in DLM (Figure 2C). The other canine rCYPs that potentially contributed to these index reactions in DLM were shown in the pie chart (Figure 2D). The phenotyping data indicated that MDZ 1’- and 4-hydroxylation, TRZ α- and 4-hydroxylation, and APZ α-hydroxylation are mixed index reactions of canine CYP3A and 2B11, while APZ 4-hydroxylation, T 6β-hydroxylation, and DCA 1β- and 5β-hydroxylation are selective index reactions of canine CYP3A12 and 3A26.

Kinetics of Index Reactions in Reactive Canine rCYP Enzymes

According to the phenotyping data of canine rCYPs, the kinetic data was comparatively acquired in the reactive isoforms including CYP2B11, 3A12 and 3A26. LCA 6α-
hydroxylation was not studied because it has more side reactions, lower activity and poorer selectivity than DCA oxidations. Table 3 and Figure S3 listed the kinetic parameters obtained within a large range of substrate levels that were the same as those in the studies of DLM. In all three tested rCYPs, TRZ α- and 4-hydroxylation were best explained by SI model, while T 6β-hydroxylation and DCA hydroxylation best fit the MM model. The best model fitting the large substrate range varied with rCYPs for the hydroxylation of MDZ and APZ. According to the CL\text{int} data acquired over the large substrate range, the activity of CYP3A12 is 123-, 23-, 5.3-, 4.1-, 1.0-, and 0.28-fold of that of CYP2B11 for APZ 4-hydroxylation, TRZ 4-hydroxylation, MDZ 1'-hydroxylation, TRZ α-hydroxylation, MDZ 4-hydroxylation, and APZ α-hydroxylation, respectively. In contrast, T 6β-hydroxylation, DCA 1β- and 5β-hydroxylation are genuine probes of canine CYP3A without detection of any CYP2B11 activity.

The heterogeneity of the kinetic models for index reactions of MDZ, APZ and TRZ over a large substrate concentration range introduced bias to evaluate their relative selectivity. Table 4 and Figure S4 tabulated the kinetic parameters of MDZ, APZ and TRZ obtained within the lower substrate range, at which all reactions best fit the MM model except for MDZ 4-hydroxylation. Comparison of CL\text{int} data acquired over the lower substrate range is believed to be more concisely related to the in vivo circumstance. In this way, the activity of CYP3A12 is 134-, 24-, 3.7-, 2.8-, 2.1-, and 0.62-fold of that of CYP2B11 for APZ 4-hydroxylation, TRZ 4-hydroxylation, TRZ α-hydroxylation, MDZ 1'-hydroxylation, MDZ 4-hydroxylation, and APZ α-hydroxylation, respectively. Among the index reactions of benzodiazepine substrates, APZ 4-hydroxylation and APZ α-hydroxylation are the least and the most contaminated by CYP2B11, respectively. The relative activity of CYP3A12 and 3A26 may be reasonably evaluated based on the uniformed kinetic model for the tested index reactions. The activity of CYP3A12 is 56-, 16-, 9.5-, 8.0-, 7.8-, 6.6-, 6.0-, 1.1- and 1.0-fold of that of CYP3A26 for TRZ α-hydroxylation, MDZ 1'-hydroxylation, APZ α-hydroxylation, DCA 1β-hydroxylation, APZ 4-hydroxylation, TRZ 4-hydroxylation, T 6β-hydroxylation, MDZ 4-hydroxylation and DCA 5β-hydroxylation, respectively. In summary, according to the kinetic data, MDZ 1'- and 4-hydroxylation, TRZ α- and 4-hydroxylation, and APZ α-hydroxylation are mixed index reactions of canine CYP2B11, 3A12 and 3A26; T 6β-hydroxylation, DCA 5β-hydroxylation, APZ 4-hydroxylation and DCA 1β-hydroxylation are mixed index reactions of canine CYP3A12 and 3A26, and the latter two are the most selective index reactions of canine CYP3A12.
Discussion

The species difference of drug metabolism is a long-standing challenge in drug research and development. Rodents are not recommended as preferred models to predicting clinical drug interactions on account of a large gap in inducer (Lu and Li, 2001), inhibitor (Kobayashi et al., 2003), substrate specificity (Ishigami et al., 2001), and abundance (Guengerich et al., 1982) between human CYP3A4 and rat CYP3A1/2. Diverse metabolic patterns were also found in CYP3A selectivity of MDZ and TRZ hydroxylation between humans and mice (Perloff et al., 2000; Van Waterschoot et al., 2008; Grimsley et al., 2013). The more human like nonrodent species are therefore required in drug metabolism studies. For example, rhesus monkey CYP3A64 shares a highly overlapping substrate profile and similar activity with human CYP3A4 (Carr et al., 2006). In contrast, dogs are the most widely used non-rodent animals to bridge the efficacies and toxicities between rodents and humans. However, the studies of canine P450 enzymes heavily lag behind that of humans (Mealey et al., 2019). As a result, misleading comments and conclusions are commonly seen when it was taken for granted to extrapolate the substrates and index reactions of human P450 enzymes to those of dogs. This work addressed the selectivity and activity of *in vitro* CYP3A index reactions of classic substrates (MDZ, APZ, TRZ and T) and novel bile acid substrates (LCA and DCA). An estimate of the abundance of each CYP isoform in HLM and DLM was introduced to extrapolate the metabolic activities from rCYPs to that of the pooled HLM or DLM. In the human studies, all the tested reactions are reconfirmed as the mixed probes of human CYP3A4, 3A5 and 3A7 with limited or trace contributions from non-CYP3A isoforms that were not studied in previous reports. We therefore did not go further into their kinetic studies because these findings are consistent with the previous reports (Emoto and Iwasaki, 2006; Deo and Bandiera, 2009; Kandel et al., 2017; Zhang et al., 2019).

In the canine studies, however, it is revealed that CYP2B11 to varying degrees contributes to the metabolism of all benzodiazepines (MDZ, APZ, TRZ), minorly to the oxidation of LCA, but not to the oxidation of T and DCA. Collectively, based on the kinetic data in canine rCYPs, it was concluded that MDZ 1’- and 4-hydroxylation, TRZ α- and 4-hydroxylation, and APZ α-hydroxylation are mixed index reactions of canine CYP2B11, 3A12 and 3A26; T 6β-hydroxylation and DCA 5β-hydroxylation are mixed index reactions of canine CYP3A12 and 3A26, while APZ 4-hydroxylation and DCA 1β-hydroxylation are the most selective index reactions of canine CYP3A12. These results verified our preliminary finding that the oxidative elimination of MDZ in DLM was ascribed primarily to CYP3A12,
secondly to CYP2B11 and CYP3A26, and minorly to CYP2C21 (Zeng et al., 2021). Our data are different from Locuson’s single concentration phenotyping result that 1’h-MDZ was formed in higher quantities by CYP2B11 and 2C21 than by 3A12 (Locuson et al., 2009a).

We sought to enroll in this work another CYP3A maker reaction, F 6β-hydroxylation, because it was the first proposed endogenous probe for the in vivo CYP3A activity in humans (Ged et al., 1989). However, our previous work revealed that the degradation of F (10 µM, 120 min incubation) in all the tested canine rCYPs (50 pmole/mL protein) were below 6%, which is much lower than the degradation rate (18%) in DLM (0.5 mg/mL protein). The 6β-hydroxycortisol was also not detected in incubations of either DLM or any canine rCYPs (Zeng et al., 2021). The observation is consistent with that F 6β-hydroxylation was not detected after intravenous administration of [1,2-3H]-F in dogs (Miyabo et al., 1973). Due to the vast species difference in glucocorticoid metabolism, we concluded that F 6β-hydroxylation may not be used as canine CYP3A probes both in vitro and in vivo.

In conclusion, this work has provided the most comprehensive quantitative data to date for the selectivity and activity of in vitro CYP3A index reactions in humans and dogs. The detailed kinetic analysis revealed that the human CYP3A index reactions of benzodiazepine substrates (MDZ, APZ, TRZ) were contaminated by canine CYP2B11 in dogs except for APZ 4-hydroxylation. Although T 6β-hydroxylation is a selective CYP3A probe for both humans and dogs, it has lower activity and more side reactions in DLM when compared with HLM. DCA 1β- and 5β-hydroxylation are validated as the first CYP3A probe in both humans and dogs despite that they have relatively lower activity. Collectively based on the proof-of-concept evidence that the pre-dosing serum ratios of either 1βh-DCA/DCA or 5βh-DCA/DCA is predictive of the in vivo CYP3A activities in beagle dogs represented by oral MDZ clearance (Zeng et al., 2021), we believe that the DCA oxidations will become useful tools to assess the CYP3A activity in practices of drug development, clinical pharmacology and therapeutics.
Acknowledgments

We are grateful to Prof. Dr. Liang Xu (West China School of Pharmacy, Sichuan University) for his support of bile acids synthesis.

Authorship Contributions

Participated in research design: Lan, Miao, Liu, Jia
Conducted experiments: Wu, Hu, Wang, Wei, Zeng, Gui
Performed data analysis: Wu, Hu
Contributed new reagents or analytic tools: Wang, Miao
Wrote or contributed to the writing of the manuscript: Lan, Wu, Hu, Wang, Miao

References


Footnotes

a) This work was supported by the National Natural Science Foundation of China (82073921) and the Clinical Research Incubation Project of West China Hospital, Sichuan University (2019HXFH032).

b) *contributed equally to this work.

c) The authors declare no conflict of interest.
Figure Legends

**Figure 1.** Reaction phenotyping results of midazolam (MDZ, 4 µM), alprazolam (APZ, 10 µM), triazolam (TRZ, 10 µM), deoxycholate (DCA, 50 µM), lithocholate (LCA, 20 µM) and testosterone (T, 10 µM) in the recombinant human CYP enzymes (rCYP, 50 pmole/mL). The percentage substrate remaining in rCYP enzymes in comparison to that in human liver microsomes (HLM, 0.5 mg/mL, MDZ was incubated in HLM at 0.1 mg/mL) (A); the absolute metabolite formation rates of human rCYP enzymes (B); the adjusted metabolite formation rates based on the published absolute CYP abundance estimates in HLM (C); the pie chart for the relative contributions of active P450 isoforms to the tested reactions (D). Data was shown as mean ± SD of triplicate values. One-way ANOVA was adopted in significant difference analysis between experimental and control groups (p < 0.05: *, p < 0.01: **, p < 0.001: ***, p < 0.0001: ****).

**Figure 2.** Reaction phenotyping results of midazolam (MDZ, 4 µM), alprazolam (APZ, 10 µM), triazolam (TRZ, 10 µM), deoxycholate (DCA, 50 µM), lithocholate (LCA, 20 µM) and testosterone (T, 10 µM) in the recombinant canine CYP enzymes (rCYP, 50 pmole/mL). The percentage substrate remaining in rCYP enzymes in comparison to in dog liver microsomes (DLM, 0.5 mg/mL, MDZ was incubated in HLM at 0.1 mg/mL) (A); the absolute metabolite formation rates of canine rCYP enzymes (B); the adjusted metabolite formation rates based on the published absolute CYP abundance estimates in DLM (C); the pie chart for the relative contributions of active P450 isoforms to the tested reactions (D). Data was shown as mean ± SD of triplicate values. One-way ANOVA was adopted in significant difference analysis between experimental and control groups (p < 0.05: *, p < 0.01: **, p < 0.001: ***, p < 0.0001: ****).
Table 1. Kinetic parameters for the hydroxylation of midazolam (MDZ), alprazolam (APZ), triazolam (TRZ), deoxycholate (DCA), lithocholate (LCA) and testosterone (T) in the pooled human liver microsomes (HLM).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>MDZ</th>
<th>APZ</th>
<th>TRZ</th>
<th>DCA</th>
<th>LCA</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tested range (μM)</td>
<td>1-300</td>
<td>5-500</td>
<td>5-300</td>
<td>1-400</td>
<td>1-150</td>
<td>1-300</td>
</tr>
<tr>
<td>HLM protein level (mg/mL)</td>
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<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>10</td>
<td>60</td>
<td>10</td>
<td>60</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>Hydroxylation site</td>
<td>1'-4-</td>
<td>α-4-</td>
<td>α-4-</td>
<td>1β-</td>
<td>5β-</td>
<td>6α-</td>
</tr>
<tr>
<td>Best Fit to Model a</td>
<td>SI</td>
<td>SI</td>
<td>MM</td>
<td>MM</td>
<td>Hill</td>
<td>Hill</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (pmol/min/mg protein)</td>
<td>1422</td>
<td>431</td>
<td>156</td>
<td>4413</td>
<td>1413</td>
<td>8976</td>
</tr>
<tr>
<td>$S_{50}$ or $K_{m}$ (μM)</td>
<td>3.51</td>
<td>39.4</td>
<td>93.2</td>
<td>278</td>
<td>38.2</td>
<td>113</td>
</tr>
<tr>
<td>$\text{CL}_{\text{int}}$ b (μl/min per mg protein)</td>
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<td>11.0</td>
<td>1.67</td>
<td>15.9</td>
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<td>79.5</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.34</td>
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<td>Goodness of fit ($R^2$)</td>
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<td>0.999</td>
<td>0.993</td>
<td>0.995</td>
<td>0.995</td>
<td>0.994</td>
</tr>
</tbody>
</table>

a: The kinetic parameters were determined by best fitting to models, including Michaelis-Menten (MM), Allosteric sigmoidal (Hill) or Substrate inhibition (SI).
b: The intrinsic clearance ($\text{CL}_{\text{int}}$) was calculated by the ratio of $V_{\text{max}}/K_{m}$ ($S_{50}$).
Table 2. Kinetic parameters for the hydroxylation of midazolam (MDZ), alprazolam (APZ), triazolam (TRZ), deoxycholate (DCA), lithocholate (LCA) and testosterone (T) in the pooled dog liver microsomes (DLM).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>MDZ</th>
<th>APZ</th>
<th>TRZ</th>
<th>DCA</th>
<th>LCA</th>
<th>T</th>
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</thead>
<tbody>
<tr>
<td>Tested range (μM)</td>
<td>1-300</td>
<td>5-500</td>
<td>5-500</td>
<td>1-400</td>
<td>1-150</td>
<td>1-300</td>
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<tr>
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<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>10</td>
<td>60</td>
<td>10</td>
<td>60</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>Hydroxylation site</td>
<td>1'- 4-</td>
<td>α- 4-</td>
<td>α- 4-</td>
<td>1β- 5β-</td>
<td>6α-</td>
<td>6β-</td>
</tr>
<tr>
<td>Best Fit to Model *</td>
<td>SI</td>
<td>MM</td>
<td>Hill</td>
<td>MM</td>
<td>SI</td>
<td>Hill</td>
</tr>
<tr>
<td>Vmax (pmol/min/mg protein)</td>
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<td>871</td>
<td>447</td>
<td>3122</td>
<td>1888</td>
<td>5928</td>
</tr>
<tr>
<td>S50 or Km (μM)</td>
<td>2.95</td>
<td>57.4</td>
<td>123</td>
<td>439</td>
<td>108</td>
<td>85.9</td>
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<tr>
<td>CLint b (μl/min per mg protein)</td>
<td>1063</td>
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<td>3.63</td>
<td>7.11</td>
<td>17.4</td>
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<td>Hill coefficient</td>
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<td>NA</td>
<td>0.830</td>
<td>NA</td>
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<td>1.33</td>
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<td>Goodness of fit (R2)</td>
<td>0.923</td>
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<td>0.998</td>
<td>0.997</td>
<td>0.996</td>
<td>0.993</td>
</tr>
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</table>

* The kinetic parameters were determined by best fitting to models, including Michaelis-Menten (MM), Allosteric sigmoidal (Hill) or Substrate inhibition (SI).

b: The intrinsic clearance (CLint) was calculated by the ratio of Vmax/Km (S50).
Table 3. Kinetic parameters within a large substrate range for the hydroxylation of midazolam (MDZ), alprazolam (APZ), triazolam (TRZ), deoxycholate (DCA), and testosterone (T) in the recombinant canine CYP2B11, 3A12 and 3A26 at the protein levels of 50 pmole/mL.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>MDZ</th>
<th>APZ</th>
<th>TRZ</th>
<th>DCA</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tested range (μM)</td>
<td>1-300</td>
<td>5-500</td>
<td>5-500</td>
<td>1-400</td>
<td>1-300</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>10</td>
<td>60</td>
<td>10</td>
<td>60</td>
<td>15</td>
</tr>
<tr>
<td>Hydroxylation site</td>
<td>1'-4</td>
<td>α-4</td>
<td>α-4</td>
<td>α-4</td>
<td>1β-5β</td>
</tr>
<tr>
<td>Best Fit to Model</td>
<td>CYP2B11 SI SI MM MM SI SI / / /</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>CYP3A12 SI MM Hill SI SI MM MM MM</td>
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<td></td>
<td>CYP3A26 Hill Hill SI MM SI SI MM MM</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>V_max (nmol/min/nmol P450)</td>
<td>CYP2B11 5.05 0.665 1.11 0.0335 2.95 1.15 / / /</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CYP3A12 16.1 3.64 1.25 8.24 2.64 16.4 6.55 1.69 4.91</td>
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<td></td>
<td>CYP3A26 4.79 3.60 1.17 1.99 2.57 22.2 0.442 0.659 0.855</td>
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<tr>
<td></td>
<td>S_50 or K_m (μM)</td>
<td>CYP2B11 46.9 0.72 81.9 26.5 / / /</td>
<td></td>
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<tr>
<td></td>
<td>CYP3A12 189 93.4 7.84 27.1 260 478 36.4</td>
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<td></td>
<td>CYP3A26 863 224 439 189 140 181 38.1</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CL_int (μl/min per nmol P450)</td>
<td>CYP2B11 1594 96.3 23.6 0.72 81.9 26.5 / / /</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CYP3A12 8376 99.2 6.64 88.2 337 606 25.2 3.54 135</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>CYP3A26 25.1 5.42 8.86 5.85 117 3.15 3.64 22.5</td>
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<td></td>
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<td></td>
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<tr>
<td>Hill coefficient</td>
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<td></td>
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<td></td>
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<td>Goodness of fit</td>
<td>CYP2B11 0.992 0.985 0.995 0.991 0.998 0.987 / / /</td>
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</table>

*a: The kinetic parameters were determined by best fitting to models, including Michaelis-Menten (MM), Allosteric sigmoidal (Hill) or Substrate inhibition (SI).

*b: The intrinsic clearance (CL_int) was calculated by the ratio of V_max/K_m(S_50).
Table 4. Kinetic parameters within a lower substrate range for the hydroxylation of midazolam (MDZ), alprazolam (APZ) and triazolam (TRZ) in the recombinant canine CYP2B11, 3A12 and 3A26 at the protein levels of 50 pmole/mL.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>MDZ</th>
<th>APZ</th>
<th>TRZ</th>
</tr>
</thead>
<tbody>
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<td>1-12.5</td>
<td>1-6.25</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>10</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>Hydroxylation site</td>
<td>1'- 4-</td>
<td>α- 4-</td>
<td>α- 4-</td>
</tr>
<tr>
<td>Best Fit to Model a</td>
<td>CYP2B11</td>
<td>MM</td>
<td>MM</td>
</tr>
<tr>
<td></td>
<td>CYP3A12</td>
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<td>Hill</td>
</tr>
<tr>
<td></td>
<td>CYP3A26</td>
<td>MM</td>
<td>MM</td>
</tr>
<tr>
<td>V$_{max}$ (nmol/min/nmol P450)</td>
<td>CYP2B11</td>
<td>8.76</td>
<td>0.846</td>
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<td>CYP3A12</td>
<td>30.4</td>
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<td></td>
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<td>12.4</td>
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<td>CYP3A26</td>
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<td>8.12</td>
</tr>
<tr>
<td>CL$_{int}$ b (μl/min per nmol P450)</td>
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<td>1.64</td>
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a: The kinetic parameters were determined by best fitting to models, including Michaelis-Menten (MM), Allosteric sigmoidal (Hill) or Substrate inhibition (SI).

b: The intrinsic clearance (CL$_{int}$) was calculated by the ratio of V$_{max}$/K$_{m}$ (S$_{50}$).
Figure 2

Midazolam

Alprazolam

Triazolam

Deoxycholic acid

Lithocholic acid

Testosterone
Supplemental Material

Journal title: Drug Metab Dispos

Manuscript number: DMD-AR-2022-000864

Reevaluate In Vitro CYP3A Index Reactions of Benzodiazepines and Steroids between Humans and Dogs

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Quantitative Determination of Metabolites in Incubations

Quantitative determinations were performed on ACQUITY UPLC coupled to Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA). The detection was performed at the positive mode except that DCA, LCA and their metabolites were monitored at the negative mode. The capillary voltage, source temperature, desolvation temperature were set at 3.0 kV, 150°C and 550°C, respectively. The cone voltages and collision energies for analytes were set at 23 V and 24 eV (TRZ, m/z 343>308), 44 V and 26 eV (αh-TRZ, m/z 359>176), 26 V and 30 eV (4h-TRZ, m/z 359>273), 29 V and 25 eV (APZ, m/z 309>281), 40 V and 23 eV (αh-APZ, m/z 325>297), 20 V and 17 eV (4h-APZ, m/z 325>307), 31 V and 36 eV (MDZ, m/z 326>291), 35 V and 18 eV (1'h-MDZ, m/z 342>324), 20 V and 15 eV (4h-MDZ, m/z 342>325), 14 V and 44 eV (CLZ, m/z 327>192), 36 V and 20 eV (T, m/z 289>97), 27 V and 14 eV (6βh-T, 2αh-T, 2βh-T and 16αh-T, m/z 305>269), 49 V and 22 eV (PROG, m/z 315>109), 40 V and 27 eV (LCA, m/z 375>375), 80 V and 29 eV (DCA and HDCA, m/z 391>391), 80 V and 27 eV (1βh-DCA and 5βh-DCA, m/z 407>407), 80 V and 29 eV (DCA-2,2,4,4-D4, m/z 395>395), 80 V and 27 eV (CA-2,2,4,4-D4, m/z 411>411), respectively.

MDZ and its metabolites were separated on an ACQUITY BEH C18 column (1.7 μm, 50 mm × 2.1 mm) (Waters, Milford, MA) maintained at 40°C. The sample (1.0 μL) was eluted with the solvent conditions of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) delivered at a rate of 0.4 mL/min. A stepwise gradient profile was as follows: 0.0-1.1 min (70-5% A), 1.1-1.4 min (5% A), 1.4-1.6 min (5-70% A).

TRZ, APZ and their metabolites were separated on an ACQUITY HSS T3 column (1.8 μm, 50 mm × 2.1 mm) maintained at 40°C. The sample (1.0 μL) in the studies of TRZ and APZ was eluted at a flow rate of 0.45 mL/min with the mobile phase consisted of a mixture of 0.01% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) by the following gradient: 0.0-0.2 min (95% A), 0.2-0.5 min (95-87% A), 0.5-0.8 min (87-82% A), 0.8-1.5 min (82-77% A), 1.5-2.0 min (77-72% A), 2.0-3.0 min (72-67% A), 3.0-3.7 min (67-63% A), 3.7-4.5 min (63-5% A), 4.5-4.8 min (5% A), 4.8-5.0 min (5-95% A).

T and 6βh-T were separated on an ACQUITY HSS T3 column (1.8 μm, 50 mm × 2.1 mm) maintained at 40°C. The sample (1.0 μL) in the studies of T was eluted with the mobile phase consisted of 0.01% formic acid in water (mobile phase A) and 0.01% formic acid in methanol (mobile phase B) delivered at a rate of 0.45 mL/min. A stepwise gradient profile was as follows:
0.0-0.3 min (70% A), 0.3-1.2 min (70-40% A), 1.2-3.2 min (40-5% A), 3.2-3.6 min (5% A), 3.6-4.0 min (5-70% A). The separation of 6βh-T from 2αh-T, 2βh-T and 16αh-T was achieved by matching the relative retention time of T and 6βh-T with reference to the ion chromatograms in previous report (Shou et al., 2003; Kandel et al., 2017).

Quantitative analysis of DCA, LCA and their metabolites was conducted as previously described (Yin et al., 2017; Zhu et al., 2018). The sample (5.0 μL) was injected into an ACQUITY BEH C18 Column (1.7 mm, 100 mm × 2.1mm) maintained at 45°C and eluted with the mobile phase consisted of 0.01% formic acid in water (mobile phase A) and acetonitrile (mobile phase B) at a flow rate of 0.45 mL/min with the following gradient: 0.0-0.5 min (95% A), 0.5-1.0 min (95-64% A), 1.0-2.0 min (64-74% A), 2.0-4.0 min (74-70% A), 4.0-6.0 min (70% A), 6.0-7.0 min (70-62% A), 7.0-9.0 min (62-55% A), 9.0-12.5 min (55-30% A), 12.5-13.0 min (30-0% A), 13.0-14.0 min (0% A), 14.0-14.1 (0-95% A) and 14.1-15.0 min (95% A).

The retention times of analytes were 4.12 (TRZ), 3.51 (αh-TRZ), 3.59 (4h-TRZ), 3.99 (APZ), 3.51 (αh-APZ), 3.37 (4h-APZ), 0.69 (MDZ), 0.67 (1'h-MDZ), 0.61 (4h-MDZ), 0.62 (CLZ), 2.33 (T), 1.69 (6βh-T), 1.80 (16αh-T), 1.90 (2αh-T), 1.99 (2βh-T), 2.74 (PROG), 11.02 (DCA), 6.30 (1βh-DCA), 4.83 (5βh-DCA), 12.76 (LCA), 9.00 (HDCA) min, respectively.
**Figure S1.** Kinetic plots of 1'-hydroxymidazolam (1'h-MDZ), 4-hydroxymidazolam (4h-MDZ), α-hydroxyalprazolam (αh-APZ), 4-hydroxyalprazolam (4h-APZ), α-hydroxytriazolam (αh-TRZ), 4-hydroxytriazolam (4h-TRZ), 1β-hydroxydeoxycholic acid (1βh-DCA), 5β-hydroxydeoxycholic acid (5βh-DCA), hyodeoxycholic acid (HDCA) and 6β-hydroxytestosterone (6βh-T) formation by human liver microsomes (HLM, squares) and dog liver microsomes (DLM, circles). Formation rate was plotted as a function of substrate concentration for 1-300 μM midazolam (MDZ, 10 min), 5-500 μM alprazolam (APZ, 60 min), 5-300 (HLM) or 500 μM (DLM) triazolam (TRZ, 10 min), 1-300 (HLM) or 400 μM (DLM) deoxycholate (DCA, 60 min), 1-150 μM lithocholate (LCA, 60 min), 1-300 μM testosterone (T, 15 min) with HLM and DLM (0.5 mg/mL), except for MDZ (0.1 mg/mL). Lines represented nonlinear regression curves. Data was shown as mean ± SD of triplicates.
Figure S2. Substrate degradation and metabolites formation of testosterone (T, A) and lithocholic acid (LCA, B) in 0.5 mg/mL of human liver microsomes (HLM, a) and dog liver microsomes (DLM, b). T (10 μM) and LCA (20 μM) were incubated for 15 and 60 min, respectively. T, LCA, 6β-hydroxytestosterone (6βh-T), and hyodeoxycholic acid (HDCA) were quantitated with standards, and androstenedione (AD), 2β-hydroxytestosterone (2βh-T), 16α-hydroxytestosterone (16αh-T), 3-dehydrolithocholic acid (3-dehydroLCA), and unknown were assessed by semiquantitative methods.

Note: identification of 2βh-T, 16αh-T, and AD was achieved by matching the relative retention times reported previously (Usmani et al., 2003).
Figure S3. Enzyme kinetic plots of 1'-hydroxymidazolam (1'h-MDZ), 4-hydroxymidazolam (4h-MDZ), α-hydroxyalprazolam (αh-APZ), 4-hydroxyalprazolam (4h-APZ), α-hydroxytriazolam (αh-TRZ), 4-hydroxytriazolam (4h-TRZ), 1β-hydroxydeoxycholic acid (1βh-DCA), 5β-hydroxydeoxycholic acid (5βh-DCA), 6β-hydroxytestosterone (6βh-T) formation by canine recombinant CYP2B11 (triangles), 3A12 (circles) and 3A26 (squares) (rCYP) over a large range of substrate levels. Formation rate was plotted as a function of substrate concentration for 1-300 μM midazolam (MDZ, 10 min), 5-500 μM alprazolam (APZ, 60 min), 5-500 μM triazolam (TRZ, 10 min), 1-400 μM deoxycholate (DCA, 60 min) and 1-300 μM testosterone (T, 15 min) with rCYP (50 pmole/mL). Lines represented nonlinear regression curves. Data was shown as mean ± SD of triplicate determinations.
Figure S4. Enzyme kinetic plots of 1'-hydroxymidazolam (1'h-MDZ), 4-hydroxymidazolam (4h-MDZ), α-hydroxyalprazolam (αh-APZ), 4-hydroxyalprazolam (4h-APZ), α-hydroxytriazolam (αh-TRZ), 4-hydroxytriazolam (4h-TRZ) formation by canine recombinant CYP2B11 (triangles), 3A12 (circles) and 3A26 (squares) (rCYP) within a lower substrate range. Formation rate was plotted as a function of substrate concentration for 1-6.25 μM midazolam (MDZ, 10 min), 1-12.5 μM alprazolam (APZ, 60 min), 1-6.25 μM triazolam (TRZ, 10 min) with rCYP (50 pmole/mL), respectively. Lines represented nonlinear regression curves. Data points were shown as mean ± SD of triplicate values.


