Title

Novel tree shrew cytochrome P450 2Ds (CYP2D8a and CYP2D8b) are functional drug-metabolizing enzymes that metabolize bufuralol and dextromethorphan

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Running title: New functional tree shrew P450 2D8a 2D8b

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Abbreviations

RT, reverse transcription
P450, Cytochromes P450
PCR, polymerase chain reaction
SRS, substrate recognition sites
Abstract

Tree shrews are a non-primate species used in a range of biomedical studies. Recent genome analysis of tree shrews found that the sequence identities and the numbers of genes of cytochromes P450 (CYP or P450s), an important family of drug-metabolizing enzymes, are similar to those of humans. However, tree shrew P450s have not yet been sufficiently identified and analyzed. In this study, novel CYP2D8a and CYP2D8b cDNAs were isolated from tree shrew liver and were characterized, along with human CYP2D6, dog CYP2D15, and pig CYP2D25. The amino acid sequences of these tree shrew CYP2Ds were 75–78 % identical to human CYP2D6, and phylogenetic analysis showed that they were more closely related to human CYP2D6 than rat CYP2Ds, similar to dog and pig CYP2Ds. For tree shrew CYP2D8b, two additional transcripts were isolated that contained different patterns of deletion. The gene and genome structures of CYP2Ds are generally similar in dogs, humans, pigs, and tree shrews. Tree shrew CYP2D8a mRNA was most abundantly expressed in liver, among the tissue types analyzed, similar to dog CYP2D15 and pig CYP2D25 mRNAs. Tree shrew CYP2D8b mRNA was also expressed in liver, but at a level 7.3-fold lower than CYP2D8a mRNA. Liver microsomes and recombinant protein of both tree shrew CYP2Ds metabolized bufuralol and dextromethorphan, selective substrates of human CYP2D6, but the activity level of CYP2D8a greatly exceeded that of CYP2D8b. These results suggest that tree shrew CYP2D8a and CYP2D8b are functional drug-metabolizing enzymes, of which CYP2D8a is the major CYP2D in liver.

Keywords: bufuralol; CYP2D; dextromethorphan; dog; human; pig.
Significance Statement

Novel tree shrew CYP2D8a and CYP2D8b cDNAs were isolated from liver. Their amino acid sequences were 75–78% identical to human CYP2D6. For CYP2D8b, two additional transcripts contained different patterns of deletion. Tree shrew CYP2D8a mRNA was abundantly expressed in liver, similar to dog CYP2D15 and pig CYP2D25 mRNAs. Recombinant tree shrew CYP2Ds catalyzed the oxidation of bufuralol and dextromethorphan. Tree shrew CYP2D8a and CYP2D8b are functional drug-metabolizing enzymes, of which CYP2D8a is the major CYP2D in liver.
Introduction

Cytochrome P450 (CYP or P450) 2D6, one of the P450 drug-metabolizing enzymes in humans (Zanger and Schwab, 2013), metabolizes antipsychotics, β-blockers, and also the probe substrates of human CYP2D6, bufuralol and dextromethorphan (Rendic, 2002; Yuan et al., 2002). Genetic polymorphisms of CYP2D6 influence the pharmacokinetics of these drugs (Rendic, 2002; Yuan et al., 2002). Human CYP2D6 mRNA is abundantly expressed in liver (Bièche et al., 2007). Consequently, CYP2D6 is an important drug-metabolizing enzyme in humans.

Tree shrews (Tupaia belangeri), a non-primate and non-rodent animal species, are utilized in diverse fields of biomedical research, especially viral infections (Cao et al., 2003; Fan et al., 2013; Tsukiyama-Kohara and Kohara, 2014). Indeed, tree shrews are essential for the study of hepatitis B and C viruses because they are the only species susceptible to these viruses other than humans and chimpanzees (Cao et al., 2003; Tsukiyama-Kohara and Kohara, 2014). Tree shrews have potential as model animals for preclinical studies, including drug metabolism studies, considering their manageable size (50–270 g) and short reproductive cycle (Tsukiyama-Kohara and Kohara, 2014). Moreover, tree shrew genome sequences are more closely related to humans than are those of rodents, including the sequences of P450s (Fan et al., 2013).

In tree shrews, we have identified and analyzed CYP1, CYP2A, CYP2B, and CYP3A forms (Uno et al., 2022b; Uno et al., 2023a; Uno et al., 2023b; Ushirozako et al., 2023), but the CYP2D subfamily remains to be investigated. In the current study, we isolated two CYP2D cDNAs in tree shrews, which were named as tree shrew CYP2D8a and CYP2D8b by the P450 nomenclature committee (https://drnelson.uthsc.edu/nomenclature/) based on sequence identity and genomic arrangement as compared to P450s of primates (including humans) (Dr. David Nelson, personal communication). These tree shrew CYP2Ds were characterized by sequence analysis, phylogeny, genome organization, gene structure, tissue expression patterns, and drug-metabolizing assays, in comparison to dog, human, and pig CYP2Ds.
Materials and Methods

Materials

Integrated DNA Technologies (Coralville, IA) synthesized oligonucleotides. Bufuralol hydrochloride and 1’-hydroxybufuralol were acquired from TRC (North York, Ontario, Canada). Dextromethorphan and dextrorphan were obtained from Sigma-Aldrich (St. Louis, MO). Corning Life Sciences (Woburn, MA) provided pooled liver microsomes of dogs (males and females), humans, and minipigs. Recombinant protein of human CYP2D6 was prepared as described elsewhere (Uehara et al., 2015). All other reagents were bought from Fujifilm Wako Pure Chemicals (Osaka, Japan) or Sigma-Aldrich, unless otherwise stated.

Tissue samples and preparation of total RNA, genomic DNA, and microsomes

Tissue samples were obtained from three beagle dogs (males, 2 years of age, weight ~10 kg), one pig (female Microminipig, 10 years of age, weight ~13 kg), two tree shrews (one male, 3 months of age, #1; one female, 5 years of age #2) as explained previously (Uno et al., 2022a; Ushirozako et al., 2023); these tissue samples were used for molecular cloning. The dog and tree shrew tissues were also used for quantitative polymerase chain reaction (PCR). Oriental Yeast Co., Ltd. (Tokyo, Japan) provided pig adrenal gland, duodenum, ileum, jejunum, kidney, liver, lung, and testis (three male and three female Göttingen minipigs, 7–9 months of age, 12–17 kg), which were used for quantitative PCR. From these dog, pig, and tree shrew samples, total RNAs were extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) or a NucleoSpin RNA Plus kit (Macherey-Nagel, Düren, Germany) following the manufacturers’ protocols. Additional liver samples were obtained from two tree shrews (a male, 5 years of age, #C1; and a female, 4 years of age #C20) at Kagoshima University, and liver microsomes were generated as described previously (Uno et al., 2022b; Uno et al., 2023b). Genomic DNAs were extracted from liver of two tree shrews (#1 and #C1) using the NucleoSpin Tissue kit (Macherey-Nagel) by following the manufacturer’s protocols. This study was reviewed and
authorized by the Institutional Animal Care and Use Committee at Kagoshima University.

**Isolation and analysis of tree shrew CYP2D cDNAs**

Reverse transcription (RT)-PCR was conducted as explained elsewhere (Uno et al., 2006) to isolate CYP2D cDNAs. Briefly, RT reactions were carried out using total RNA (1 µg), oligo (dT), and ReverTra Ace (TOYOBO, Osaka, Japan), following the manufacturer’s instructions; subsequently, PCR was carried out using Q5 Hot start High-Fidelity Polymerase (New England BioLabs, Ipswich, MA) and CYP2D-specific primers (Table 1) in line with the manufacturers’ procedures. The thermal cycler protocol was an initial denaturation at 98°C (30 s) and 35 cycles of 98°C (10 s), 65°C (20 s), and 72°C (40 s), followed by a final extension at 72°C (2 min). A PCR Cloning Kit (New England BioLabs) was used to clone the amplified DNAs into pMiniT2.0 vectors as per the manufacturer’s protocol. Sequencing the inserts of the cloned cDNAs and the amplicons of the CYP2D genes were conducted using an ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), followed by electrophoresis with an ABI PRISM 3500xl Genetic Analyzer (Applied Biosystems). The Genetyx system (Software Development, Tokyo, Japan) was used to analyze the sequence data obtained; the ClustalW program and the neighbor-joining method, respectively, were applied for multiple alignment and generating a phylogenetic tree. Homology searches were carried out using BLAST (National Center for Biotechnology Information, NCBI) for sequence identities.

**Analysis of the genome and gene structure**

Genome data were analyzed using Sequence Viewer (NCBI) to establish the gene structure and genomic arrangement. The arrangement of human, dog, pig, and tree shrew CYP2D genes in the genome was referred to Sequence Viewer. To determine the gene structure, gene sequences of human CYP2D6 (NC_000022), dog CYP2D15 (NC_051814), pig CYP2D25 (NC_010447), and tree shrew CYP2Ds (NW_006159782) retrieved by Sequence Viewer were aligned with the corresponding cDNA using the Genetyx system. The sizes of all exons and introns were determined for each CYP2D gene.
To analyze the gene sequences of CYP2D8a (exons 6–8) and CYP2D8b (exons 6–9), located in a gap in the genome assembly of GenBank (NCBI), PCR was performed with tree shrew genomic DNA (#C1 for CYP2D8a or #1 for CYP2D8b) using TaKaRa Ex Premier DNA Polymerase (TaKaRa Bio, Kusatsu, Japan) and the primers shown in Table 1 according to the manufacturer’s protocol. The thermal cycler was set to deliver initial denaturation at 94°C (1 min) and 35 cycles of 98°C (10 s) and 60°C (15 s), followed by 68°C (1–5 min). For CYP2D8a, the initial PCR was performed with the primers tsCYP2D17 (5qrt1) and tsCYP2Ds(3ex8a), followed by a second PCR with the primers tsCYP2Ds (5ex6a) and tsCYP2Ds (3ex8a). For CYP2D8b, PCR was performed with the primers tsCYP2Ds (5ex6a) and tsCYP2Ds (3ex9a). The amplified DNAs of both CYP2D8a and CYP2D8b were sequenced using the primer tsCYP2Ds (5ex6a) and the data were analyzed as described earlier.

**Quantification of mRNA expression**

Expression levels of CYP2D mRNAs were quantified using real-time RT-PCR, as described elsewhere (Uno et al., 2006), in dog adrenal gland, brain, ileum, jejunum, kidney, liver, and lung; pig adrenal gland, duodenum, ileum, jejunum, kidney, liver, lung, and testis; and tree shrew adrenal gland, heart, kidney, liver, lung, small intestine, and uterus. Briefly, a ReverTra Ace qPCR RT kit (TOYOBO) was used for RT reactions according to the manufacturer’s protocol. The RT product generated was used as the template for real-time PCR, which was carried out with THUNDERBIRD Next SYBR qPCR Mix (TOYOBO) with a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories) in accordance with the manufacturers’ procedures. The final concentration of the primers (Table 1) was 300 nM. Quantitations were performed in triplicate. Cycling conditions were 95°C (30 s), followed by 40 cycles of 95°C (5 s) and 60°C (10 s). The 18S ribosomal RNA levels were evaluated using THUNDERBIRD Probe qPCR Mix (TOYOBO) with the primers and probe (Table 1) at final concentrations of 300 and 100 nM, respectively, and were used for normalization of the raw data.
Preparation of recombinant CYP2D proteins

Proteins of tree shrew CYP2D8a and CYP2D8b, along with dog CYP2D15 and pig CYP2D25, were expressed in *Escherichia coli* as explained previously (Iwata et al., 1998; Uno et al., 2006). PCR was done using Q5 Hot Start High-Fidelity Polymerase (New England BioLabs) with CYP2D cDNA as the template to alter the N-terminus for enhanced protein expression. PCR conditions comprised initial denaturation at 98°C (30 s) and 35 cycles of 98°C (10 s), 65–66°C (20 s), and 72°C (40 s), followed by a final extension at 72°C (2 min.) To assist subcloning into the pCW vector that contained human NADPH-P450 reductase cDNA, the NdeI and XbaI sites of the forward and reverse primers (Table 1), respectively, were utilized. Membrane preparation and concentration measurements of P450 protein and reductase of each preparation were performed as described previously (Iwata et al., 1998; Uno et al., 2006).

Enzymatic characterization of CYP2D

Bufuralol 1'-hydroxylation and dextromethorphan *O*-demethylation activities, as mediated by liver microsomes and recombinant P450 2D enzymes, were evaluated as expounded previously (Uehara et al., 2015). In brief, each 0.2-mL reaction mixture contained liver microsomes (0.50 mg/mL) or recombinant protein (25 pmol equivalent/mL), 100 mM potassium phosphate buffer (pH 7.4), an NADPH generating system, and bufuralol or dextromethorphan (10–100 µM). After incubation at 37°C for 10 min, reactions were halted by adding 25 µL of 60% (v/v) perchloric acid; reaction mixtures were then centrifuged at 10,000 × g for 10 min. The supernatant was analyzed using liquid chromatography with an analytical column (C18) and a fluorescence detector as explained previously (Uehara et al., 2015). Maximum velocities (*V*max) and Michaelis constants (*K*m) for dextromethorphan *O*-demethylation by recombinant P450 2D enzymes were computed from *v* versus [S] curves using the Michaelis–Menten equation fitted by nonlinear regression (mean ± standard error, 14 substrate concentrations from 0.63 to 300 µM) using Prism (GraphPad Software, La Jolla, CA).
Results

Isolation of CYP2D cDNAs

Novel tree shrew CYP2D8a and CYP2D8b cDNAs were isolated from tree shrew liver by RT-PCR. Tree shrew CYP2D8a and CYP2D8b cDNAs included open reading frames of 500 and 499 amino acid residues, respectively (Fig. 1), and their amino acid sequences shared high sequence identities (75–78%) with human CYP2D6 (Table 2). Tree shrew CYP2D8a and CYP2D8b amino acid sequences had one heme-binding region and six substrate recognition sites (SRSs) (Gotoh, 1992), the typical sequence motifs characteristic of P450s (Fig. 1). Phylogenetic analysis showed that tree shrew CYP2D was more closely related to human CYP2D than rat CYP2Ds are, similar to dog and pig CYP2Ds (Fig. 2). For tree shrew CYP2D8b, two additional transcripts were isolated and found to contain different patterns of in-frame deletions in the open reading frame over exons 6–7 or exons 5–8, resulting in the loss of 108 and 152 amino acid residues, respectively; both deletions contained SRS4 and SRS5. The cDNA sequences identified were registered in GenBank, including dog CYP2D15 (OR502968), pig CYP2D25 (KJ922570), and tree shrew CYP2D8a (OR502969) and CYP2D8b (OR502970).

Genomic organization and exon–intron structure of CYP2D genes

The genomic arrangement of the human, dog, pig, and tree shrew CYP2D genes were analyzed with Sequence Viewer using the genome data (Fig. 3). In the human, dog, pig, and tree shrew genomes, CYP2D genes were situated between TCF20 and NDUFA6, but the numbers of CYP2D genes were different among these species, resulting in the formation of a gene cluster in tree shrews and humans, but not in dogs and pigs (Fig. 3).

The gene structure of the human, dog, pig, and tree shrew CYP2D genes were determined by aligning the gene sequences with the corresponding cDNAs. The gene sequences of CYP2D8a and CYP2D8b located in a gap of the genome assembly (GenBank) were determined by PCR and sequencing, and were registered in GenBank under accession nos. OR982649 and OR982650,
respectively. Tree shrew CYP2D8a and CYP2D8b included nine exons, just as human, dog, and pig CYP2Ds do (Fig. 4). In tree shrew CYP2D8a and CYP2D8b, the sizes of exons 1–9 (in the coding region) were 189, 172, 153, 161, 177, 142, 188, 142, 179 bp, respectively, except that CYP2D8b exon 5 was 174 bp. Tree shrew CYP2D8a exons 2–9 had the same number of bases as those of human CYP2D6. All the introns of human, dog, pig, and tree shrew CYP2D genes began with GU and ended with AG, which follows the rules of eukaryotic gene splicing: the exception was intron 2, which begins with GC in human, dog, pig, and tree shrew CYP2D genes.

**Tissue distribution of mRNA expression**

The expression levels of tree shrew CYP2D8a and CYP2D8b mRNAs were determined using real-time RT-PCR, along with those of dog CYP2D15 and pig CYP2D25 mRNAs. Tree shrew CYP2D8a mRNA was most abundantly expressed in liver (just as dog CYP2D15 and pig CYP2D25 mRNAs are) and was also abundant in kidney and small intestine (Fig. 5). Tree shrew CYP2D8b mRNA showed a more ubiquitous expression pattern, with the most abundant expression in liver (Fig. 5). In liver, the expression level of CYP2D8b mRNA was approximately 7.3-fold lower than that of CYP2D8a (Fig. 5).

**Drug-metabolizing activities of CYP2D proteins**

The activities of bufuralol 1′-hydroxylation and dextromethorphan O-demethylation, typical probe reactions of human CYP2D6, were measured for liver microsomes and for recombinant CYP2D proteins. Liver microsomes and CYP2D proteins of humans, dogs, pigs, and tree shrews showed oxidation activities for both these substrates (Fig. 6). Bufuralol 1′-hydroxylation activity in tree shrew liver microsomes was greater than those of humans and dogs, but lower than that of pigs. Dextromethorphan O-demethylation activity in tree shrew liver microsomes was similar to that of humans, but higher than that of dogs and lower than that of pigs (Fig. 6A). For recombinant CYP2D proteins, tree shrew CYP2D8a showed substantial bufuralol 1′-hydroxylation activity, similar to those of human CYP2D6 and dog CYP2D15, but much higher than that of pig CYP2D25 (Fig. 6B).
contrast, tree shrew CYP2D8b showed only minimal activities for both bufuralol 1'-hydroxylation and dextromethorphan O-demethylation. Tree shrew CYP2D8a showed substantial dextromethorphan O-demethylation activity, similar to those of human CYP2D6, dog CYP2D15, and pig CYP2D25 (Fig. 6B). Kinetic analysis of dextromethorphan O-demethylation by recombinant tree shrew CYP2D8a revealed a $V_{\text{max}}$ of $26 \pm 1$ nmol/min/nmol, comparable to that of human CYP2D6 ($15 \pm 1$ nmol/min/nmol); however, tree shrew CYP2D8a had a high $K_m$ value of $8.0 \pm 1.0$ µM compared with that of human CYP2D6 ($0.8 \pm 0.1$ µM). Dog CYP2D15 and pig CYP2D25 had apparent $K_m$ values of 2.6 and 2.6 µM for dextromethorphan O-demethylation and $V_{\text{max}}$ values of 6.8 and 5.6 nmol/min/nmol, respectively. The catalytic function of the animal CYP2D enzymes tested in this study had similar $V_{\text{max}}/K_m$ values of 2.2-3.3 (1/min/µM). These results suggested that, in tree shrews, CYP2D8a plays a major role as a drug-metabolizing enzyme in the liver.
Discussion

In the current study, we discovered and characterized novel tree shrew CYP2D8a and CYP2D8b and established that they were 75–78% identical to human CYP2D6 (Table 2). Tree shrew CYP2D8a and CYP2D8b included the sequence structures characteristic of P450s (Fig. 1) and were more closely related to human CYP2D6 than rat CYP2Ds are in a phylogenetic tree, similar to dog and pig CYP2Ds (Fig. 2). Interestingly, two of the three CYP2D8b transcripts contained different patterns of deletion, resulting in both lacking SRS4 and SRS5, possibly indicating that tree shrew CYP2D8b is evolving toward becoming a pseudogene (pseudogenization). The genomic locations were similar for human and tree shrew CYP2D genes (Fig. 3). Therefore, there are similarities in the molecular characteristics of human and tree shrew CYP2Ds.

Genomic organization is important to assess the orthologous relationships of P450s among species. Tree shrew CYP2D8a and CYP2D8b constituted a gene cluster located between NDUFA6 and TDF20 in the genome, similar to human CYP2D genes, whereas a single CYP2D gene was found in a similar location in the dog and pig genomes (Fig. 3). In mice, the Cyp2d gene cluster is expanded, possibly due to selective pressure from their diet, and duplicated Cyp2d enzymes might play roles in the metabolism of exogenous substrates such as plants and dietary components (Nelson et al., 2004). Therefore, environmental factors could have influenced the expansion of CYP2D genes in humans and tree shrews. In tree shrews, CYP2D8b appears to be undergoing pseudogenization, whereas CYP2D8a is abundantly expressed in liver and encodes a functional drug-metabolizing enzyme. In humans, CYP2D6 is the only functional CYPD2 gene, and CYP2D7P and CYP2D8P are pseudogenes (Fig. 3) (Zanger and Schwab, 2013). Tree shrew CYP2D8a and CYP2D8b genes include nine exons, just as human, dog, and pig CYP2D genes do (Fig. 4). Therefore, a single CYP2D enzyme plays major roles in humans, dogs, pigs, and tree shrews.

Tree shrew CYP2D8a mRNA was most copiously expressed in liver, among the tissue types analyzed, followed by kidney, similar to dog CYP2D15 and pig CYP2D25 mRNAs; however, the level of dog CYP2D15 mRNA was very much lower in kidney than in liver, whereas pig CYP2D25
and tree shrew CYP2D8a mRNAs had relatively higher expressions in kidney (Fig. 5). Previous studies have also shown the abundant expression of human, dog, and pig CYP2D mRNAs in liver (Roussel et al., 1998; Hosseinpour and Wikvall, 2000; Bièche et al., 2007). In contrast to tree shrew CYP2D8a mRNA, CYP2D8b mRNA was expressed more ubiquitously (Fig. 5). The difference in expression patterns of tree shrew CYP2D8a and CYP2D8b mRNAs might indicate divergent transcriptional mechanism(s) between these two CYP2D genes. In humans, transcription factors such as hepatocyte nuclear factor-4α (HNF4α) participate in the regulation of CYP2D gene expression (Zanger and Schwab, 2013). Further investigation of the transcriptional mechanisms of tree shrew CYP2D genes is warranted.

Tree shrew CYP2D8a and CYP2D8b metabolized bufuralol and dextromethorphan, two typical substrates of human CYP2D6, and thus were functional drug-metabolizing enzymes; however, CYP2D8b exhibited only minimal activity toward both these substrates (Fig. 6). In marmosets, CYP2D8 (expressed in liver and extrahepatic tissues) appears to have substrate specificities different from those of marmoset CYP2D6 (expressed predominantly in liver), which is not unexpected, given that only minor amino acid changes can alter the substrate specificity of human CYP2D6 (Uehara et al., 2015). In dogs, the Ile109Val substitution decreases the functionality of CYP2D15 (Jimenez et al., 2023), and this residue (Ile109) is conserved in tree shrew CYP2D8a but not in CYP2D8b (Fig. 1). In humans, the substitutions of Ile106 and Glu216 influence human CYP2D6 activities toward bufuralol and dextromethorphan (de Graaf et al., 2007), and the corresponding residues (109th and 219th residues, respectively) are conserved in tree shrew CYP2D8a but not in CYP2D8b (Fig. 1). Differences in these amino acid residues, important for enzyme function, might account for the different metabolic activities of tree shrew CYP2D8a and CYP2D8b.

Pig CYP2D25 activity toward bufuralol 1′-hydroxylation was much lower than the activities of human CYP2D6, dog CYP2D15, and tree shrew CYP2D8a (Fig. 6). However, pig liver microsomes had a much higher activity for bufuralol 1′-hydroxylation than human or dog liver microsomes, similar to the findings of earlier studies (Anzenbacher et al., 1998; Bogaards et al., 2000), and also
higher than that of tree shrew liver microsomes (Fig. 6). This discrepancy could result from the possible involvement of pig CYP2B in bufuralol 1'-hydroxylation (Skaanild and Friis, 2002). Similarly, cynomolgus macaque liver microsomes catalyze bufuralol 1'-hydroxylation much more efficiently than human liver microsomes do (Shimada et al., 1997; Bogaards et al., 2000). Although cynomolgus CYP2D6 (formerly CYP2D17) and CYP2D8 (formerly CYP2D44) enzymes catalyze bufuralol 1'-hydroxylation (Mankowski et al., 1999; Uno et al., 2010), involvement of other P450 enzymes such as CYP2C76 and CYP3A4 (Iwasaki et al., 2010; Uno et al., 2011b) may partly explain the greater activity of cynomolgus macaque liver microsomes.

Unlike human CYP2D6, pig CYP2D25 catalyzes vitamin D₃ 25-hydroxylation, the initial step in the metabolic activation of vitamin D₃ (Hosseinpour and Wikvall, 2000). Site-directed mutagenesis found that the substitution of five pig amino acid residues in SRS3 with those of human CYP2D6, namely, Ala241Gly, Lys243Val, Phe244Lys, Pro245Arg, and Arg246Phe, diminished vitamin D₃ 25-hydroxylation but did not affect tolterodine hydroxylase activity (Hosseinpour et al., 2001), indicating the importance of SRS3 for substrate selectivity, depending on the substrate. Moreover, pig liver microsomes do not metabolize debrisoquine, a typical substrate of human CYP2D6 (Skaanild and Friis, 2002). Therefore, pigs are likely not to be a suitable animal species for modeling human CYP2D6-dependent drug metabolism. In contrast, dog CYP2D15 metabolizes bufuralol and dextromethorphan efficiently in an isoform-specific manner (Shou et al., 2003); consequently, dogs might be a more appropriate species for drug metabolism studies, similar to cynomolgus macaques (Uno et al., 2011a; Uno et al., 2016). It will be of great importance to explore the substrate selectivity of tree shrew CYP2D enzymes using various CYP2D substrates.

In the present study, we discovered and characterized novel tree shrew CYP2D8a and CYP2D8b and established that they are 75–78% identical to human CYP2D6. Tree shrew CYP2D8a mRNA was abundantly expressed in liver, as are human, dog, and pig CYP2D mRNAs; moreover, the hepatic expression level of tree shrew CYP2D8a mRNAs greatly exceeded that of tree shrew CYP2D8b mRNA. Tree shrew CYP2D8a metabolized bufuralol and dextromethorphan, typical substrates of
human CYP2D6, more efficiently than tree shrew CYP2D8b did. These findings suggest that tree shrew CYP2D8a is a major functional drug-metabolizing enzyme in liver with properties similar to those of human CYP2D6.
Acknowledgments

The authors thank Makiko Shimizu, Koichiro Adachi, Yukina Detto, and Chihiro Kato for their support of this work. We also thank David Smallbones for copyediting a draft of this article.

Data Availability Statement

The authors declare that all the data supporting the findings of this study are contained within the published article.

Authorship Contributions

Participated in research design: Yamazaki, Uno

Conducted experiments: Ushirozako, Murayama, Uno

Contributed new reagents or analytic tools: Tsukiyama-Kohara, Uno

Performed data analysis: Ushirozako, Murayama, Yamazaki, Uno

Wrote or contributed to the writing of the manuscript: Ushirozako, Yamazaki, Uno
References


Footnotes

Funding

This work was supported in part by the Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research numbers 23K05538 and 23K06217.

Declaration of Interest

The authors have no competing interests to declare.
Figure legends

**Fig. 1.** Multiple alignment of CYP2D amino acid sequences. Amino acid sequences of human (h), dog (d), pig (p), and tree shrew (ts) CYP2Ds were aligned using ClustalW. Solid and broken lines above the amino acid sequence indicate the putative substrate recognition sites (SRSs) and heme-binding region, respectively. Asterisks and dots under sequences indicate identical and conservatively changed amino acids, respectively.

**Fig. 2.** Phylogeny of tree shrew CYP2Ds. The phylogenetic tree was created by the neighbor-joining method using CYP2D amino acid sequences of humans (h), cynomolgus macaques (mf), common marmosets (cj), dogs (d), pigs (p), rats (r), and tree shrews (ts). Cynomolgus CYP2D6 and CYP2D8 were formerly called CYP2D17 and CYP2D44, respectively. Rabbit (rab) CYP2D23 was used as the outgroup. The scale bar represents 0.1 amino acid substitutions per site.

**Fig. 3.** Genomic organization of tree shrew *CYP2D* genes. Sequence Viewer was used to analyze the human, dog, pig, and tree shrew genomes. Tree shrew *CYP2D8a* and *CYP2D8b* were located in a genomic region that corresponded well with those of human, dog, and pig *CYP2D* genes.

**Fig. 4.** Gene structure of tree shrew *CYP2D* genes. The coding region of each CYP2D cDNA sequence was aligned with the genome using Sequence Viewer for humans (h), dogs (d), pigs (p), and tree shrews (ts). Asterisks indicate introns containing short gaps of unsequenced regions in the genome data.

**Fig. 5.** Tissue expression patterns of tree shrew CYP2D mRNAs. Expression levels of CYP2D mRNAs were quantified using real-time RT-PCR in dog (d), pig (p), and tree shrew (ts) tissues. The expression level of each mRNA was normalized to the corresponding 18S rRNA level, and values represent the average ± SD from three independent amplifications. The most abundant expression level was arbitrarily set to 1, and all other expression levels were adjusted accordingly.

**Fig. 6.** Drug oxidation activities by liver microsomes and recombinant CYP2D protein in humans,
dogs, pigs, and tree shrews. Drug oxidation activities were determined for liver microsomes (A) and recombinant protein (B) using bufuralol and dextromethorphan at substrate concentrations of 10 μM (open bars) and 100 μM (shaded bars). Rates of dextromethorphan O-demethylation are illustrated in pink.
Table 1.

Primer sequences

<table>
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<tr>
<th>RT-PCR</th>
<th>Sequence (5′ → 3′)</th>
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<tbody>
<tr>
<td>Dog CYP2D15</td>
<td>ATGGGGCTGCTGACCGGGGACAC</td>
</tr>
<tr>
<td>Pig CYP2D25</td>
<td>AGCCATGGCTGCTGACTG</td>
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<tr>
<td>Tree shrew CYP2D8a</td>
<td>ATGGGGCTGCTGACCGGGGACAC</td>
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<tr>
<td>Tree shrew CYP2D8b</td>
<td>GGGCTGCACTGTCGCTACAGAGAG</td>
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<tr>
<td>Amplification of exon–intron boundary</td>
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<tr>
<td>Tree shrew CYP2D8a</td>
<td>ATGAAGGACTGAAGGAGGAATCTG</td>
</tr>
<tr>
<td>Tree shrew CYP2D8b</td>
<td>AGAGCAGCTCTAATGAGAGAG</td>
</tr>
<tr>
<td>Preparation of expression plasmid</td>
<td></td>
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<tr>
<td>Dog CYP2D15</td>
<td>GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTCTGGGGCCCCTGGCCGTA</td>
</tr>
<tr>
<td>Pig CYP2D25</td>
<td>GCTCTAGACTAGCGAGGCTCCACACAGAGCTGGAA</td>
</tr>
<tr>
<td>Tree shrew CYP2D8a/b</td>
<td>GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTCTGGGGATCCTGGCCTTGG</td>
</tr>
<tr>
<td>Quantitative PCR</td>
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<tr>
<td>Dog CYP2D15</td>
<td>GATCCAGGAGCACAGGAAGAC</td>
</tr>
<tr>
<td>Pig CYP2D25</td>
<td>CCCTAACAACCTTCTGAATAAAGC</td>
</tr>
<tr>
<td>Tree shrew CYP2D8a</td>
<td>ATGAAGGACTGAAGGAGGAATCTG</td>
</tr>
<tr>
<td>Tree shrew CYP2D8b</td>
<td>GACGACTCTAGCTTCTGGCAC</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>FAM-TGCTGCGAC-ZEN-CAGACTTGCCCTC-IBFQ</td>
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F, forward primer; R, reverse primer; S, sequence; P, probe.
Table 2.

CYP2D amino acid sequence identities for tree shrews and other animal species relative to humans

<table>
<thead>
<tr>
<th>Species</th>
<th>CYP2D Gene</th>
<th>Human CYP2D6 cDNA %</th>
<th>Amino acid %</th>
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<tbody>
<tr>
<td>Dog</td>
<td>CYP2D15</td>
<td>82</td>
<td>75</td>
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<tr>
<td>Cynomolgus</td>
<td>CYP2D6(17)</td>
<td>94</td>
<td>93</td>
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<tr>
<td></td>
<td>CYP2D8(44)</td>
<td>94</td>
<td>91</td>
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<tr>
<td>Pig</td>
<td>CYP2D25</td>
<td>84</td>
<td>79</td>
</tr>
<tr>
<td>Rat</td>
<td>CYP2D1</td>
<td>79</td>
<td>72</td>
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<tr>
<td></td>
<td>CYP2D2</td>
<td>79</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>CYP2D3</td>
<td>78</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>CYP2D4</td>
<td>81</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>CYP2D5</td>
<td>79</td>
<td>72</td>
</tr>
<tr>
<td>Tree shrew</td>
<td>CYP2D8a</td>
<td>84</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>CYP2D8b</td>
<td>83</td>
<td>75</td>
</tr>
</tbody>
</table>

For cynomolgus, dog, pig, rat, and tree shrew CYP2Ds, amino acid sequences were compared with human CYP2D6 sequences using BLAST. Cynomolgus CYP2D6 and CYP2D8 were formerly called CYP2D17 and CYP2D44, respectively.
Fig. 1

hCYP2D6  1:MGL---EALV PLAVIVAIFL LLVDLMHRRQ RMARYPFPGE LRDPGLGNNL HDQFQNTFPC FDQLRBBFPG VPSLQLAWTP VVVLNLAAV REALVTGHED 97
dCYP2D15  1:MGLLIQITLG PLAVAIFVFL LLVDLMHRRQ RMARYPFPGE TPYVPPQGNN QMDFQEFPSI FDQLQORFON VPSLQLAWTP VVVLNLAAV REALVTGHED 100
pCYP2D25  1:MGLLIQITLG TLALAMVIFL LLVDLMHRRQ RMARYPFPGE MPQDPGLGNN QYQFOQPPGL FDIQDQFRGD VPSLQLQWRP VVVLNLAAV REALVTGHED 100
tsCYP2D8a 1:MGLLIQITLG TLALQGAVFL LLVDLMHRRQ RMARYPFPGE TPPLPGLGNN QLQFOQPPGL FDIQDQFRGD VPSLQLQWRP VVVLNLAAV REALVTGHED 100
tsCYP2D8b 1:MGLLIQITLG TLALQGAVFL LLVDLMHRRQ RMARYPFPGE TPPLPGLGNN QLQFOQPPGL FDIQDQFRGD VPSLQLQWRP VVVLNLAAV REALVTGHED 100

hCYP2D6  98:TADRPPVPIT QILGFGPRSQ GVFLARYGPA WREQRRFSVS TLRNLGLGKK SLEQWVTEEA ACLCAAFANH SGRPPFRPG LSKAVSNVIA SLTQRGFVEY 197
dCYP2D15  101:TADRPPMPIY THGGLGPESQ GLFLARYGRA WREQRRFSLS TLRNFGLGKK SLEQWVTEEA SCLCAAFAQ AGRPPFGGL LSKAVSNVIA SLTQRGFVEY 200
pCYP2D25  101:TSDRPPVFIL ELGGYGPGRK GLFLARYGPA WQEQRRFCLS TLRNFGLGKK SLEQWVTEEA ACLCAAFADQ AGRPPFRPDL LSKAVSNVIA LLTQRGFVEY 200
tsCYP2D8a 101:TSDRPPSPIY EHLGFGPRSQ GVILARYGPA WQEQRRFCLS TLRNFGLGKK SLEQWVTEEA ACLCAAFADQ AGRPPFRPDL LSKAVSNVIA LLTQRGFVEY 200
tsCYP2D8b 101:TSDRPPSHMH EHLGFGPRSQ GVILAPYGPT WREQRRFCLS TLRSFRLGKK SLEQWVTEEA ACLCAAFADQ AGRPPFRPDL LSKAVSNVIA LLTQRGFVEY 200

H CYP2D6  198:DDPRFLRLLD LAQEGLKEES GFLREVLNAV PVLLHIPALA GKVLRFQKAF LTQLDELLTE HRMTWDPAQP PRDLTEAFLA EMEKAKGNPE SSFNDENLRI 297
dCYP2D15  201:DDPRLLQLLE LTQQALKQDS GFLREALINSI PVLLHIPGLA SKVFSAQKAF ITLTNEMIQE HRKTRDPTQP PRHLIDAFVD EIEKAKGNPK TSFNEENLCM 300
pCYP2D25  201:NDPRMLKLLD LVLEGLKEEV GLMRQVLEAM PVLRHIPGLC AKLFPRQKAF LMAMDELVNE HKMTRDVLAP TKPRDLAFTE EKEQAKGNFL SSKENENLLL 300
tsCYP2D8a 201:DDERFFALMR MLDEGLKEES GFLRQVLNEV PVLLRIPGVA KKVFSGQKAF MAMMEELVNE HRGTRDLPDQ PRDLTDAFLA EVEKQAKGNFL SSKENENLLL 300
tsCYP2D8b 201:DDERFFSLMR LLERALKDDS SFWHQVLHKV PALLRIPGLA DKLFPGQKAF MAMDDLDVNE HK-TRAPDQP PRDFTDAFLA EVEKQAKGNFL SSKENENLLL 299

hCYP2D6  398:VVADLFSAGM VTTSTTLAWG LLLMILHPDV QRRVQQEIDD VIGQVRRPEM GDQAHMPYTH AVIHEVQRFG DIVPLGVTHM TSRLVQVQGF RPIPKGTTLIT 397
dCYP2D15  401:VTSDLFVAGM VSTSITLTWA LLLMILHPDV QRRVQQEIDE VIGHVRQPEI KDQALMPFTN AVLHEVQRFG DIVPLGVVHM TSRLVQVQGF LIPKGTTLIT 400
pCYP2D25  401:VVADLFSAGM ITTSTTLAWA LLLMILHPDV QRRVQQEIDE VIGHVRQPEI KDQALMPFTN AVLHEVQRFG DIVPLGVVHM TSRLVQVQGF LIPKGTTLIT 400
tsCYP2D8a 401:VVADLFSAGM VTTSTTLAWA LLLMILHPDV QRRVAQQEIDE VIGHVRQPEI KDQALMPFTN AVLHEVQRFG DIVPLGVVHM TSRLVQVQGF LIPKGTTLIT 400
tsCYP2D8b 400:VVADLFSAGM VTTSTTLAWA LLLMILHPNV QCQAQQEIDE VIGHVRQPEI KDQALMPFTN AVLHEVQRFG DIVPLGVVHM TSRLVQVQGF LIPKGTTLIT 399

hCYP2D6  398:NLSSVLKDEA VWEKPFRFHP EHFLDAQGHF VQGVRFPBEM GQAHMFVVTF AVIHEVQPPF DIVPLGVTPR THSLNFVFQS RPIPKGTTLIT 397
dCYP2D15  401:VTDLFLVAGM VSTSITLTA LLLMILHPDV QRPQDEVIE DVOQVFVFTF AVIHEVQPPF DIVPLGVTPR THSLNFVFQS LIPKGTTLIT 400
pCYP2D25  401:VVADLFSAGM VTTSTTLAWA LLLMILHPDV QRPQDEVIE DVOQVFVFTF AVIHEVQPPF DIVPLGVTPR THSLNFVFQS LIPKGTTLIT 400
tsCYP2D8a 401:VVADLFSAGM VTTSTTLAWA LLLMILHPDV QRPQDEVIE DVOQVFVFTF AVIHEVQPPF DIVPLGVTPR THSLNFVFQS LIPKGTTLIT 400
tsCYP2D8b 400:VVADLFSAGM VTTSTTLAWA LLLMILHPDV QCQAEDEVIE DVOQVFVFTF AVIHEVQPPF DIVPLGVTPR THSLNFVFQS LIPKGTTLIT 399

hCYP2D6  398:NLSVLSVDEK WKNKPFFPHF EHLADOGQFP VPQFPFPRPF DFSVFJPQVS DVLQVRGTVSP RPSVLFQVSP ELVGSVFQuad 497
dCYP2D15  401:NLSVLSVDEK WKNKPFFPHF EHLADOGQFP VPQFPFPRPF DFSVFJPQVS DVLQVRGTVSP RPSVLFQVSP ELVGSVFQuad 500
pCYP2D25  401:NLSVLSVDEK WKNKPFFPHF EHLADOGQFP VPQFPFPRPF DFSVFJPQVS DVLQVRGTVSP RPSVLFQVSP ELVGSVFQuad 500
tsCYP2D8a 401:NLSVLSVDEK WKNKPFFPHF EHLADOGQFP VPQFPFPRPF DFSVFJPQVS DVLQVRGTVSP RPSVLFQVSP ELVGSVFQuad 500
tsCYP2D8b 400:NLSVLSVDEK WKNKPFFPHF EHLADOGQFP VPQFPFPRPF DFSVFJPQVS DVLQVRGTVSP RPSVLFQVSP ELVGSVFQuad 499

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DMD Fast Forward. Published on January 23, 2024 as DOI: 10.1124/dmd.123.001603

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Fig. 3

Human chromosome 22
TCF20  CYP2D8P  CYP2D7P  CYP2D6  NDFUA6

Dog chromosome 10
TCF20  CYP2D15  NDFUA6

Pig chromosome 5
TCF20  CYP2D25  NDFUA6

Tree shrew (NW_006159782.1)
TCF20  CYP2D8a  CYP2D8b  NDFUA6
Fig. 4

0 1 2 3 4 5 (kb)

hCYP2D6

dCYP2D15

pCYP2D25

tsCYP2D8a

tsCYP2D8b

*
Fig. 5

Expression level (CYP2D mRNA / 18S rRNA)

**dCYP2D15**

- Adrenal gland
- Brain
- Ileum
- Jejunum
- Kidney
- Liver
- Lung

**pCYP2D25**

- Adrenal gland
- Duodenum
- Ileum
- Jejunum
- Kidney
- Liver
- Lung
- Testis

**tsCYP2Ds**

- Adrenal gland
- Heart
- Kidney
- Liver
- Lung
- Small intestine
- Uterus

- tsCYP2D8a
- tsCYP2D8b
Fig. 6

(A) Liver microsomes

(B) Recombinant P450

<table>
<thead>
<tr>
<th></th>
<th>Bufuralol 1'-hydroxylation and dextromethorphan O-demethylation, nmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Bufuralol 1’-hydroxylation and dextromethorphan O-demethylation, [S], 10 and 100 μM</td>
</tr>
<tr>
<td>Dog</td>
<td>Bufuralol 1’-hydroxylation and dextromethorphan O-demethylation, [S], 10 and 100 μM</td>
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<tr>
<td>Pig</td>
<td>Bufuralol 1’-hydroxylation and dextromethorphan O-demethylation, [S], 10 and 100 μM</td>
</tr>
<tr>
<td>Tree shrew, male</td>
<td>Bufuralol 1’-hydroxylation and dextromethorphan O-demethylation, [S], 10 and 100 μM</td>
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<tr>
<td>Tree shrew, female</td>
<td>Bufuralol 1’-hydroxylation and dextromethorphan O-demethylation, [S], 10 and 100 μM</td>
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<table>
<thead>
<tr>
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<th>Bufuralol 1'-hydroxylation and dextromethorphan O-demethylation, nmol/min/nmol P450 2D</th>
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<tbody>
<tr>
<td>Human 2D6</td>
<td>Bufuralol 1’-hydroxylation and dextromethorphan O-demethylation, [S], 10 and 100 μM</td>
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<tr>
<td>Dog 2D15</td>
<td>Bufuralol 1’-hydroxylation and dextromethorphan O-demethylation, [S], 10 and 100 μM</td>
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<td>Pig 2D25</td>
<td>Bufuralol 1’-hydroxylation and dextromethorphan O-demethylation, [S], 10 and 100 μM</td>
</tr>
<tr>
<td>Tree shrew 2D8a</td>
<td>Bufuralol 1’-hydroxylation and dextromethorphan O-demethylation, [S], 10 and 100 μM</td>
</tr>
<tr>
<td>Tree shrew 2D8b</td>
<td>Bufuralol 1’-hydroxylation and dextromethorphan O-demethylation, [S], 10 and 100 μM</td>
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</tbody>
</table>

 Bufuralol  Dextromethorphan