

**Title page**

**Newly Identified Tree Shrew *Cytochrome P450 2A13 (CYP2A13)* is Expressed in Liver and Lung and Encodes a Functional Drug-Metabolizing Enzyme Similar to Dog CYP2A13 and Pig CYP2A19**

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**Running title:** New Tupaia, Dog, and Pig P450 2A Enzymes

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### **Abbreviations**

CYP2A, cytochromes P450 2A; P450, cytochrome P450; PCR, polymerase chain reaction; RT,  
reverse transcription; SRS, substrate recognition site.

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## Abstract

The tree shrew, a non-rodent primate-like species, is used in various fields of biomedical research, including hepatitis virus infection, myopia, depression, and toxicology. Recent genome analysis found that the numbers of cytochromes P450 (P450 or *CYP*) genes are similar in tree shrews and humans and their sequence identities are high. Although the P450s are a family of important drug-metabolizing enzymes, they have not yet been fully investigated in tree shrews. In the current study, tree shrew CYP2A13 cDNA was isolated from liver, and its characteristics were compared with those of pig, dog, and human CYP2As. Tree shrew CYP2A13 amino acid sequences were highly identical (87–92%) to the human CYP2As and contained sequence motifs characteristic of P450s. Phylogenetic analysis revealed that tree shrew CYP2A13 was more closely related to human CYP2As than to rat CYP2As, similar to dog and pig CYP2As. Among the tissue types analyzed, tree shrew CYP2A13 mRNA was preferentially expressed in liver and lung, similar to dog CYP2A13 mRNA, whereas dog CYP2A25 and pig CYP2A19 mRNAs were predominantly expressed in liver. Tree shrew liver microsomes and tree shrew CYP2A13 proteins heterologously expressed in *Escherichia coli* catalyzed coumarin 7-hydroxylation and phenacetin *O*-deethylation, just as human, dog, and pig CYP2A proteins and liver microsomes do. These results demonstrate that tree shrew *CYP2A13* is expressed in liver and lung and encodes a functional drug-metabolizing enzyme.

### **Significance Statement**

Novel tree shrew cytochrome P450 2A13 (CYP2A13) was identified and characterized in comparison with human, dog, and pig CYP2As. Tree shrew CYP2A13 isolated from liver had high sequence identities and close phylogenetic relationships to its human homologs and was abundantly expressed in liver and lung at the mRNA level. Tree shrew CYP2A13 metabolized coumarin and phenacetin, human selective CYP2A6 and CYP2A13 substrates, respectively, similar to dog and pig CYP2As, and is a functional drug-metabolizing enzyme likely responsible for drug clearances.

## Introduction

Tree shrews (*Tupaia belangeri*) are members of the genus *Tupaia* that inhabit South and Southeast Asia. Tree shrews are important model animals for hepatitis B virus and hepatitis C virus research because, other than chimpanzees and humans, the tree shrew is the only animal species susceptible to these viruses (Cao et al., 2003; Tsukiyama-Kohara and Kohara, 2014). Because of their phylogenetic closeness to primates, short reproductive cycle, small body size, ease of handling, low maintenance cost, tree shrews are also used in biomedical research in fields such as depression, myopia, and learning behavior (Cao et al., 2003; Fan et al., 2013; Tsukiyama-Kohara and Kohara, 2014).

The cytochrome P450 (P450 or CYP) family comprises heme–thiolate enzymes that catalyze the oxidation or reduction reactions of various endogenous and exogenous substrates. In humans, the *CYP* family consists of 57 functional genes and 58 pseudogenes (Nelson et al., 2004), with the *CYP2A* subfamily being composed of *CYP2A6/7/13* (Nelson et al., 2004). Human *CYP2A6* is expressed in liver (Koskela et al., 1999), whereas human *CYP2A13* is expressed in the respiratory tract (Su et al., 2000). Although human *CYP2A7* mRNA is expressed in liver, no resulting activity has been observed (Yamano et al., 1990; Ding et al., 1995). Human *CYP2A6* and *CYP2A13* mediate coumarin 7-hydroxylation, and *CYP2A6* is largely responsible for this reaction in liver.

Recent genome analysis revealed a relatively closer affinity of tree shrews to primate species as compared with mice or rats, including similarities in the numbers of P450s (Fan et al., 2013); however, P450s have not yet been characterized in tree shrews. We recently identified tree shrews *CYP1* and *CYP2B* enzymes (Uno et al., 2022a; Uno et al., 2022c). The purpose of this study was to investigate if *CYP2A* enzyme(s) would play functional roles as drug-metabolizing enzymes in tree shrews. In this study, to elucidate the properties of tree shrew *CYP2A* in comparison with dog, pig, and human *CYP2As*, tree shrew *CYP2A13* cDNA was identified and characterized by sequence analysis, phylogeny, tissue expression patterns, genome organization, gene structure, protein expression, and drug-metabolizing functional assays. Herein, we report that novel *CYP2A13* of tree shrews is highly identical to human *CYP2A6/13* and plays a functional role as a drug-metabolizing enzyme.

## **Materials and Methods**

### **Materials**

Synthesized oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Coumarin, phenacetin, 7-hydroxycoumarin, and acetaminophen were obtained from Fujifilm Wako Pure Chemicals (Osaka, Japan). Pooled liver microsomes of humans (75 males and 75 females), 3 male and 3 female dogs, and 3 male and 3 female minipigs were purchased from Corning Life Sciences (Woburn, MA). Liver microsomes were prepared from two tree shrews as described previously (Yamazaki et al., 2014; Uno et al., 2022a; Uno et al., 2022c). Recombinant human CYP2A6 and CYP2A13 in bacterial membranes were prepared using the bicistronic protein expression system with human NADPH-P450 reductase as described previously (Shimada et al., 1997; Uehara et al., 2015; Uno et al., 2006; Uno et al., 2022a; Uno et al., 2022c). All other reagents were purchased from Sigma-Aldrich or Fujifilm Wako Pure Chemicals, unless otherwise specified.

### **Tissue Samples and RNA Preparation**

A variety of tissue samples was collected from a beagle dog (male, 2 years of age, weight ~10 kg) at Shin Nippon Biomedical Laboratories (Kainan, Japan) and from a pig (Microminipig, female, 10 years of age, weight ~13 kg) at Kagoshima University (Kagoshima, Japan). Similarly, tissue samples were collected from, one of two tree shrews (a male, 3 months of age, #2; and a female, 5 years of age, #1) at Kagoshima University. This study was reviewed and approved by the Institutional Animal Care and Use Committee at Kagoshima University. From the collected samples, total RNAs were extracted for molecular cloning and real-time polymerase chain reaction (PCR) using an RNeasy Mini Kit (Qiagen, Valencia, CA) as described previously (Uno et al., 2022a; Uno et al., 2022b; Uno et al., 2022c). The liver total RNA previously prepared (Yamazaki et al., 2014) was used for isolation of pig CYP2A19 cDNA.

### **Isolation of CYP2A cDNAs**

Reverse transcription (RT)-PCR was performed using liver total RNAs as described previously (Uno et al., 2006; Uno et al., 2022a; Uno et al., 2022c) with the following modifications. Briefly, RT reaction was incubated at 42°C for 1 h with total RNA (1 µg), oligo (dT), and ReverTra Ace (TOYOBO, Osaka, Japan), followed by PCR performed using Q5 Hot Start High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA) in a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA). The primers used are listed in **Table 1**. PCR conditions were an initial denaturation at 98°C for 30 s; 35 cycles at 98°C for 10 s, 65°C for 20 s, and 72°C for 40 s; followed by a final extension at 72°C for 2 min. The amplicons were cloned into pMiniT2.0 vectors using a PCR Cloning Kit (New England BioLabs). The inserts were verified by sequencing using an ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), followed by electrophoresis with an ABI PRISM 3500xl Genetic Analyzer (Applied Biosystems).

### **Bioinformatic Analysis of Gene, cDNA, and Amino Acid Sequences**

The Genetyx system (Software Development, Tokyo, Japan) was used for multiple alignment with the ClustalW program and for creating a phylogenetic tree with the neighbor-joining method. Sequence similarity was determined using the BLAST program (National Center for Biotechnology Information). Analysis of the genome data was done by BLAT (UCSC Genome Bioinformatics) and Sequence Viewer (National Center for Biotechnology Information). The amino acid and cDNA sequences used were from GenBank or the present study.

### **Quantification of mRNA Expression**

Expression level of each mRNA was measured by real-time RT-PCR performed as described previously (Uno et al., 2006; Uno et al., 2022a; Uno et al., 2022c) in dogs (adrenal gland, brain, heart, ileum, jejunum, kidney, liver, lung, and testis), pigs (adrenal gland, brain, heart, ileum, jejunum, kidney, liver, lung, ovary, and uterus), and tree shrews (heart, small intestine, kidney, liver, lung, spleen, and uterus). Briefly, RT reactions were performed using a ReverTra Ace qPCR RT kit

(TOYOBO), followed by PCR that was conducted using a THUNDERBIRD SYBR qPCR Mix Kit (TOYOBO) with a StepOnePlus Real-Time PCR System (Applied Biosystems). The primers (**Table 1**) were used at final concentrations of 300 nM. The thermal cycler conditions were 30 s at 95°C, followed by 40 cycles at 95°C for 5 s and at 60°C for 10 s. Standard curves were generated by serial tenfold dilutions of a plasmid for the corresponding cDNA. Based on three independent amplifications, relative expression levels were determined by normalizing the raw data to 18S ribosomal RNA levels, for which TaqMan Gene Expression Assays (Assay ID: Hs99999901\_s1, Applied Biosystems) were used for measurement with THUNDERBIRD Probe qPCR Mix (TOYOBO).

### **Preparation of CYP2A Proteins**

Expression plasmids of CYP2As were prepared for dogs, pigs, and tree shrews, and proteins were expressed in *Escherichia coli* membranes as described previously (Iwata et al., 1998; Uno et al., 2006; Uno et al., 2022a; Uno et al., 2022c). Modification of the *N*-terminus was carried out by PCR; the coding region of each CYP2A cDNA was amplified using Phusion Hot Start Flex DNA Polymerase (New England BioLabs) and the primers (**Table 1**) with an initial denaturation at 98°C for 30 s; 35 cycles of denaturation at 98°C for 10 s, annealing at 65°C for 20 s, and elongation at 72°C for 40 s; and a final extension was at 72°C for 5 min. To facilitate subcloning into the pCW vector, the forward and reverse primers respectively, contained the *Nde*I and *Xba*I sites. This vector contained human NADPH-P450 reductase cDNA. *E. coli* membranes were prepared, and the P450 protein and reductase contents in bacterial membrane preparations were measured as described previously (Iwata et al., 1998; Uno et al., 2006).

### **Enzymatic Characterization of CYP2As**

The recombinant human, dog, pig, and tree shrew CYP2A proteins were analyzed for coumarin 7-hydroxylation and phenacetin *O*-deethylation, as described previously (Uehara et al., 2015). Briefly, typical reaction mixtures (0.20 mL) contained recombinant P450 protein (5.0–10 pmol equivalents) or

liver microsomes (0.25–0.50 mg protein/mL), an NADPH-generating system (0.25 mM NADP<sup>+</sup>, 2.5 mM glucose 6-phosphate, and 0.25 unit/ml glucose 6-phosphate dehydrogenase), and substrate (10 μM or 100 μM coumarin or phenacetin, unless otherwise specified) in 100 mM potassium phosphate buffer (pH 7.4). Reactions were incubated at 37°C for 10 min (coumarin) and 20 min (phenacetin) in triplicate and then terminated by adding 10 μL of 60% HClO<sub>4</sub> (w/v). Incubates were centrifuged at 900 × *g* for 5 min, and supernatants were analyzed by high-performance liquid chromatography with a fluorescence (at the excitation wavelength of 338 nm and the emission wavelength of 458 nm for coumarin) or UV (at 245 nm for phenacetin) detector and a reverse phase analytical column (C<sub>18</sub>; 5 μm, 4.6 x 250 mm) as described previously (Uehara et al., 2015). The eluents consisted of 45% acetonitrile (v/v) containing 20 mM NaClO<sub>4</sub> (pH 2.5) for coumarin and 20% acetonitrile (v/v) containing 50 mM K<sub>3</sub>PO<sub>4</sub> (pH 4.9). Metabolite formation intensities increased linearly up to 15 min (coumarin) or 30 min (phenacetine) incubation and to 20 pmol P450 equivalents in our preliminary experiments. Kinetic parameters were calculated by nonlinear regression (mean ± standard error, n = 13 substrate concentrations, in triplicate) based on the Michaelis–Menten equation using Prism 9 (GraphPad Software, La Jolla, CA). The goodness of fit of the empirical model was appropriately evaluated using the coefficient (*r*) squared of the determination magnitude.

## Results

### Cloning of CYP2A cDNAs

CYP2A13 cDNA was newly identified in tree shrews and the cDNAs previously identified in dogs (CYP2A13 and CYP2A25) and pigs (CYP2A19) were successfully isolated by RT-PCR. Amino acid sequences translated from these CYP2A cDNAs contained 495 amino acid residues with sequence motifs characteristic of P450s, i.e., putative substrate recognition sites (Gotoh, 1992) and a heme-binding region (**Fig. 1**). In a phylogenetic tree of CYP2A amino acid sequences, tree shrews were more closely related to humans than to rats, just as dog and pig CYP2As are (**Fig. 2**). The CYP2A cDNAs identified in the present study were deposited in GenBank, i.e., dog CYP2A13 (ON164789) and CYP2A25 (ON164788), pig CYP2A19 (ON164797), and tree shrew CYP2A13 (ON164809).

### Genomic and Exon–Intron Structure of CYP2A Genes

The genomic locations of the *CYP2A* genes were analyzed in the human, dog, pig, and tree shrew genomes using BLAT and Sequence Viewer. The *CYP2A* gene(s) were found in the large *CYP2ABFGST* gene cluster formed with *CYP2B*, *CYP2F*, *CYP2G*, *CYP2S*, and *CYP2T* genes, and this gene cluster was located at the corresponding region of the genome in humans, dogs, pigs, and tree shrews; however, the number, direction, and location of the genes within each gene cluster did not correspond perfectly (**Fig. 3**). The analysis also showed that *CYP2A* genes contain nine exons in dogs, pigs, tree shrews, and humans (**Fig. 4**). Among these species, the sizes of the coding exons were well conserved among all the *CYP2A* genes: in particular, exons 2–8 contained 163, 150, 161, 177, 142, 188, and 142 bp, respectively. All the *CYP2A* introns analyzed began with GU and ended with AG, conserved with the consensus intron border sequences in eukaryotic genes. Therefore, *CYP2A* gene structures were highly conserved among humans, dogs, pigs, and tree shrews.

### Tissue Distribution of mRNA Expression

The expression levels of tree shrew CYP2A13 mRNA and dog and pig CYP2A mRNAs were quantified by real-time RT-PCR in adrenal gland, brain, heart, ileum, jejunum, kidney, liver, lung, ovary, spleen, testis, and/or uterus. Tree shrew CYP2A13 mRNA was predominantly expressed in lung and liver, similar to dog CYP2A13 mRNA (**Fig. 5**). Dog CYP2A25 mRNA was also expressed in liver at a comparable level to that of CYP2A13 mRNA (**Fig. 5**). Pig CYP2A19 mRNA was most abundantly expressed in liver, followed by kidney (**Fig. 5**).

### Drug-Metabolizing Activities of CYP2As

Coumarin 7-hydroxylation activities by human liver microsomes were high (**Fig. 6A**) under the present conditions. Pooled liver microsomes from male and female dogs, female pigs, and one tree shrew also exhibited coumarin 7-hydroxylation activities but at lower levels than for pooled human liver microsomes. In contrast, high phenacetin *O*-deethylation activities were seen in liver microsomes from male and female dogs, male and female pigs, and tree shrews. The mean yield of new recombinant tree shrew CYP2A13 was 8.7 nmols per litter cultures; the ratios of NADPH-P450 reductase/P450 was and 1.6. Tree shrew CYP2A13 metabolized coumarin and phenacetin, typical substrates of human CYP2A6 and CYP2A13, respectively, indicating that tree shrew CYP2A13 is a functional drug-metabolizing enzyme. Dog CYP2A13, pig CYP2A19, and tree shrew CYP2A13 had similar patterns of coumarin 7-hydroxylation and phenacetin *O*-deethylation activities, whereas recombinant human CYP2A6 and CYP2A13 more specifically mediated coumarin 7-hydroxylation and phenacetin *O*-deethylation, respectively (**Fig. 6B**). Dog CYP2A25 exhibited no activity toward coumarin 7-hydroxylation or phenacetin *O*-deethylation (data not shown).

*In vitro* kinetic analyses found that the apparent  $K_m$  values for coumarin 7-hydroxylation by recombinant P450 2A proteins were in the range of ~0.5–12  $\mu\text{M}$  (**Table 3**), whereas the estimated intrinsic clearance ( $V_{\max}/K_m$ ) values were in the range of ~0.4–3.2  $\mu\text{M}^{-1}\text{min}^{-1}$  (**Fig. 7**). In contrast, the apparent  $K_m$  values for phenacetin *O*-deethylation by recombinant P450 2A proteins were in the range

of ~7–54  $\mu\text{M}$  (except for >300 for human 2A6) (**Table 3**), whereas the estimated intrinsic clearance ( $V_{\text{max}}/K_m$ ) values were in the range of ~0.7–3.8  $\mu\text{M}^{-1}\text{min}^{-1}$  (**Fig. 7**). The goodness of fit for both coumarin 7-hydroxylation and phenacetin *O*-deethylation activities by five recombinant CYP2A enzymes tested under the current conditions was confirmed by the coefficient squared. Overall, these results suggest that dog CYP2A13 exhibited similar catalytic activities toward typical human CYP2A6 probe substrate coumarin and human CYP2A13 substrate phenacetin (**Fig. 7**); in contrast, pig CYP2A19 and tree shrew CYP2A13 had higher catalytic function toward phenacetin than toward coumarin. The  $V_{\text{max}}/K_m$  values for coumarin 7-hydroxylation and phenacetin *O*-deethylation by the recombinant CYP2A proteins tested were apparently inversely correlated (**Fig. 7**), with the exception of the data point of human CYP2A13.

## Discussion

Tree shrews are new experimental animal models in biomedical research; however, their usefulness in drug metabolism studies has not been fully investigated. In this study, we identified and characterized tree shrew CYP2A13 and found it to be highly identical to human CYP2A6, CYP2A7, and CYP2A13 (**Table 2**); moreover, it contained sequence structures characteristic of P450s (**Fig. 1**). In phylogenetic tree, tree shrew CYP2A13 was found to be more closely related to human CYP2As than to rat CYP2As, just as dog and pig CYP2As are (**Fig. 2**).

Similar to the human, dog, and pig *CYP2A* genes, tree shrew *CYP2A13* contained nine coding exons and formed a large *CYP2ABFGST* gene cluster with *CYP2B*, *CYP2F*, *CYP2G*, *CYP2S*, and *CYP2T* genes, located between *EGLN2* and *AXL* in the genome, (**Figs. 3 and 4**). The location of the *CYP2ABFGST* gene cluster corresponded well among humans, dogs, pigs, and tree shrews, but the numbers of the subfamily genes and their locations within the gene cluster differed. During evolution, the *CYP2ABFGST* gene cluster evidently diverged among species through gene duplication, inversion, and insertion of genomic segments (Hu et al., 2008). Consequently, it is sometimes difficult to determine orthologous relationships to humans even for related primate species such as cynomolgus macaques (Uno et al., 2011).

Tree shrew CYP2A13 mRNA showed abundant expression in liver and lung, similar to the expression pattern in dog CYP2A13 mRNA (**Fig. 5**). Dog CYP2A25 mRNA was also abundantly expressed in liver at a comparable level to that of CYP2A13 mRNA (**Fig. 5**); however, dog CYP2A25 generally shows minimal metabolic activity toward typical P450 substrates (Zhou et al., 2010), indicating that CYP2A13 is likely the major CYP2A in dog liver. In humans, CYP2A6 mRNA is abundantly expressed liver for (Koskela et al., 1999), whereas CYP2A13 mRNA is expressed in the respiratory tract including lung (Su et al., 2000). Therefore, tree shrew and dog CYP2A13 might play roles in liver and lung, similar to those of human CYP2A6 and CYP2A13, respectively. In contrast, pig CYP2A19 mRNA was preferentially expressed in liver and kidney (**Fig. 5**). These different tissue expression patterns are accounted for by their divergent transcriptional mechanism(s). Constitutive

androstane receptor is involved in transactivation of human *CYP2A6* and pig *CYP2A19* (Maglich et al., 2003; Gillberg et al., 2006). It would be of interest to investigate the transcriptional mechanism(s) of tree shrew *CYP2A13*.

Tree shrew *CYP2A13* showed coumarin 7-hydroxylation and phenacetin *O*-deethylation activities, similar to dog *CYP2A13* and pig *CYP2A19*, and liver microsomes of humans, dogs, pigs, and tree shrews (**Fig. 6**). Coumarin and phenacetin were metabolized by dog *CYP2A13* (**Fig. 6**), but not by dog *CYP2A25*, similar to the results of a previous study (Zhou et al., 2010). In dogs, *CYP1A2* catalyzes phenacetin *O*-deethylation with comparably activity to *CYP2A13* (Zhou et al., 2010), but the abundance of *CYP1A2* protein is much higher than that of *CYP2A13* (Martinez et al., 2013), suggesting a contribution of *CYP1A2* to phenacetin *O*-deethylation in dogs, similar to the situation in humans. In tree shrews, *CYP1A2* mRNA is expressed and is a functional enzyme (Uno et al., 2022a). It would be of interest to assess the contribution of tree shrew *CYP1A2* to phenacetin *O*-deethylation to see if phenacetin is a typical substrate of *CYP1A2* in tree shrews, just as it is in humans.

Pig *CYP2A19* also metabolized coumarin (**Fig. 6**), which is consistent with the results of previous studies (Skaanild and Friis, 1999; Matal et al., 2009). We previously reported occasional differences in terms of undetected low *CYP2A*-dependent coumarin 7-hydroxylation in minipigs at 8-months-old (Murayama et al., 2009). Interestingly, coumarin 7-hydroxylation activity in liver is different between males and females in Göttingen minipigs, but not in conventional pigs (Skaanild and Friis, 1999). Indeed, the present study also found sex differences in coumarin 7-hydroxylation in minipig liver (**Fig. 6**). Similarly, the abundance of dog *CYP2A13* and *CYP2A25* proteins in liver varies significantly between males and females, albeit to a relatively small extent, i.e., 35% and 22%, respectively (Martinez et al., 2013). Tree shrews showed substantial differences in coumarin 7-hydroxylation between two individuals, a male and a female (**Fig. 6**). It would be of interest to investigate sex differences of *CYP2A* activity in tree shrews, as was suggested to exist in a preliminary *in vitro* study (Litterst et al., 1976).

A single substituted amino acid is reportedly sufficient to convert the specificity of P450 2A

enzymes to different substrates (Lindberg and Negishi, 1989). There was an apparent inverse relationship between  $V_{\max}/K_m$  values for coumarin 7-hydroxylation and phenacetin *O*-deethylation (**Table 3**) by the currently studied recombinant CYP2A proteins (**Fig. 7**), implying similar but different substrate specificities of these structurally related CYP2A proteins. In human CYP2A13, the amino acid substitutions Ser208Ile, Ala213Ser, Phe300Ile, Ala301Gly, Met365Val, and Gly369Ser account for decreased phenacetin affinity, resulting in different phenacetin metabolism between human CYP2A6 and 2A13 (DeVore et al., 2008). Tree shrew CYP2A13, along with dog CYP2A13 and pig CYP2A19, contained the same amino acid residues in these positions as human CYP2A13 (**Fig. 1**), thereby explaining their metabolic activities toward phenacetin *O*-deethylation. In contrast, tree shrew CYP2A13, along with pig CYP2A19 and dog CYP2A13, showed lower coumarin 7-hydroxylation activities than human CYP2A6 (**Fig. 6**). In human CYP2A6, substitutions of the 117th and 372nd residues to those of CYP2A13, i.e., V117A and R372H, reduce coumarin 7-hydroxylation activity, and the equivalent reciprocal substitutions, A117V and H372R in CYP2A13, increase the enzyme activity (He et al., 2004), indicating the importance of 117V and 372R for coumarin 7-hydroxylation. Tree shrew CYP2A13, pig CYP2A19, and dog CYP2A13 contain 117A (**Fig. 1**), partly explaining their lower coumarin 7-hydroxylation activities, although they contained the same 372R as human CYP2A6.

Human *P450* genes are known to be polymorphic; for example, in some individuals, the entire *CYP2A6* and *CYP2D6* genes are lost, or their protein functions are inactivated, by nonsense mutations (see [https://www.pharmvar.org/htdocs/archive/index\\_original.htm](https://www.pharmvar.org/htdocs/archive/index_original.htm)). In humans, defective *CYP2A6* alleles, such as 2A6\*2 and 2A6\*4 found in smokers of Caucasian and Asian populations are associated with a reduced risk of lung cancer (Fujieda et al., 2004; Rotunno et al., 2009). In minipig *CYP2A19*, three different polymorphisms have been identified, but did not account for the differences of *CYP2A19* activity, for which a different transcriptional regulation was suggested to be involved (Skaanild and Friis, 2005). The differences found in coumarin and phenacetin metabolism in the two tree shrews (**Fig. 6**) could be interindividual differences caused by genetic polymorphisms, similar to those in humans. It would be of use to investigate genetic polymorphisms of tree shrew *CYP2A13*.

In conclusion, we identified novel tree shrew CYP2A13 highly identical to functional human CYP2A6 and CYP2A13. Tree shrew CYP2A13 mRNA was predominantly expressed in liver and lung, similar to human CYP2A6 and CYP2A13, respectively. Tree shrew CYP2A13 metabolized coumarin and phenacetin, which are typical substrates of human CYP2A6 and CYP2A13, respectively. These results indicate that tree shrew CYP2A13 is a functional drug-metabolizing enzyme and possibly plays roles similar to those of human CYP2As in liver and lung.

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### **Authorship Contributions**

*Participated in research design:* Uno, Yamazaki.

*Conducted experiments:* Ushirozako, Noda, Murayama, Uno.

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

*Performed data analysis:* Uno, Yamazaki.

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## **Footnotes**

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## **Declaration of Interest**

The authors have no competing interests to declare.

## Figure legends

**Fig. 1.** Multiple alignment of CYP2A amino acid sequences. Amino acid sequences of dog (d), pig (p), tree shrew (ts), and human (h) CYP2A cDNAs were aligned using ClustalW. Solid and broken lines, respectively, indicate the putative substrate recognition sites (SRSs) and heme-binding region. Asterisks and dots under the sequences, respectively, indicate identical and conservatively changed amino acids.

**Fig. 2.** Phylogeny of tree shrew CYP2A13. The phylogenetic tree was created by the neighbor-joining method using CYP2A amino acid sequences of humans (h), cynomolgus macaques (mf), dogs (d), pigs (p), tree shrews (ts), and rats (r). Cynomolgus CYP2G2 was used as the outgroup. The scale bar represents 0.1 amino acid substitutions per site.

**Fig. 3.** Genomic organization of *CYP2A* genes. BLAT and Sequence Viewer were used to analyze the human, dog, pig, and tree shrew genomes. *CYP2A* genes were located in the *CYP2ABFGST* gene cluster in the human, dog, pig, and tree shrew genomes. The gene sizes and the distances between them are not proportional to actual values.

**Fig. 4.** Gene structures of *CYP2A* genes. The coding region of each cDNA sequence was aligned with the genome using BLAT or Sequence Viewer for human (h), dog (d), pig (p), and tree shrew (ts) *CYP2A* genes.

**Fig. 5.** Tissue expression of CYP2A mRNAs. Expression of CYP2A mRNAs was quantified by real-time RT-PCR in dog, pig, and tree shrew tissues. The expression level of each mRNA was normalized to the 18S rRNA level, and values represent the average  $\pm$  SD from three independent amplifications. The most abundant expression was arbitrarily set to 1, and all other expression levels were adjusted accordingly.

**Fig. 6.** Oxidation activities by liver microsomes and recombinant CYP2A proteins in humans, dogs, pigs, and tree shrews. Drug oxidation activities were determined in liver microsomes (A) and

recombinant proteins (B) using coumarin (pink bars) and phenacetin (gray bars) at substrate concentrations of 10  $\mu\text{M}$  (pale bars) and 100  $\mu\text{M}$  (normal bars) in triplicate. Data with bars represent mean and standard deviation values, respectively. Dog CYP2A25 activity was under detection limit ( $< 0.001$  nmol/min/nmol P450) and is not shown.

**Fig. 7.** Apparent inverse relationship between  $V_{\text{max}}/K_m$  values for coumarin 7-hydroxylation and phenacetin *O*-deethylation by recombinant CYP2A proteins. The numbers in parentheses are the results after excluding the data point for human CYP2A13. Solid and dashed lines represent the regression line and 95% confidential intervals, respectively.

**Table 1**

Primer sequences.

				Sequence (5' → 3')
<b>RT-PCR</b>				
Dog	CYP2A13	F	dCYP2A13 (5rt1)	CTGACTGTCTCTCTCAGTGCCACCATGT
		R	dCYP2A13 (3rt1)	CTCTCCTATTCACCCCTGCCCTATAATTCTC
	CYP2A25	F	dCYP2A7 (5rt1)	CAGCCAGCACCATCTGACCGTCTTG
		R	dCYP2A7 (3rt1)	CTATTTCATTAATCCACCTCCCTCTGCTA
Pig	CYP2A19	F	pCYP2A19 (5rt1)	CCATGCTGGCCTCAGGCTTGCTTCTC
		R	pCYP2A19 (3rt1)	TCaGgCGGGGCTGGAAGCTCATGGT
Tree shrew	CYP2A13	F	tsCYP2A13 (5rt1)	CTCAGCCATCACCATTGGACTGTAACCTC
		R	tsCYP2A13 (3rt1)	CTCAGCGGGGAAGGAACTCATGGTATAG
<b>Exp</b>				
Dog	CYP2A13	F	dCYP2A13 (5exp1)	GGAATTCATATGGCTCTGTTATTAGCAGTTTTT-GTGGCTTTGC
		R	dCYP2A7/13_pCYP2A19 (3exp1b)	TGGCCTGCCTGA GCTCTAGA-CTCAGCGGGGCTGGAAGCTCAT
	CYP2A25	F	dCYP2A7 (5exp1)	GGAATTCATATGGCTCTGTTATTAGCAGTTTTT-GTGGCTTTGC
		R	dCYP2A7/13_pCYP2A19 (3exp1b)	TCACCTGCTTGAC GCTCTAGA-CTCAGCGGGGCTGGAAGCTCAT
Pig	CYP2A19	F	dCYP2A7 (5exp1)	GGAATTCATATGGCTCTGTTATTAGCAGTTTTT-GTGGCTTTGC
		R	dCYP2A7/13_pCYP2A19 (3exp1b)	TCACCTGCTTGAC GCTCTAGA-CTCAGCGGGGCTGGAAGCTCAT
Tree shrew	CYP2A13	F	tsCYP2A13 (5exp1)	GGAATTCATATGGCTCTGTTATTAGCAGTTTTT-GTGGCTCTG
		R	tsCYP2A13 (3exp1b)	CTGGCCACCCTGACT GCTCTAGA-CCTGGCTCAGCGGGGAAGGAACTCATGGTATAG
<b>qPCR</b>				
Dog	CYP2A13	F	dCYP2A7/13 (5qrt1)	GCTTTGACTATGAGGACAAAGAGT
		R	dCYP2A7 (3qrt1)	TTATCACTGAATGGAACATTCAC
	CYP2A25	F	dCYP2A7/13 (5qrt1)	GCTTTGACTATGAGGACAAAGAGT
		R	dCYP2A13 (3qrt1)	TTTCATCACTGAGTAAAACATCTCAT
Pig	CYP2A19	F	p&tsCYP2A (5qrt1)	CAGATGTACAACCTCCCTCATGAAGAT
		R	p&tsCYP2A (3qrt1)	CAGGTGGACGGTGAACACA
Tree shrew	CYP2A13	F	p&tsCYP2A (5qrt1)	CAGATGTACAACCTCCCTCATGAAGAT
		R	p&tsCYP2A (3qrt1)	CAGGTGGACGGTGAACACA

F, forward primer; R, reverse primer; RT-PCR, reverse transcription polymerase chain reaction; Exp, expression; qPCR, quantitative polymerase chain reaction.

**Table 2**

Sequence identities of dog, pig, and tree shrew CYP2As compared with human CYP2As in amino acids.

		Human		
		CYP2A6	CYP2A7	CYP2A13
Dog	CYP2A13	89	87	92
	CYP2A25	86	84	88
Pig	CYP2A19	88	85	90
Tree shrew	CYP2A13	88	87	92

Each CYP2A amino acid sequence was compared with human CYP2A sequences using BLAST.

Data show sequence identities as percentages.

**Table 3**

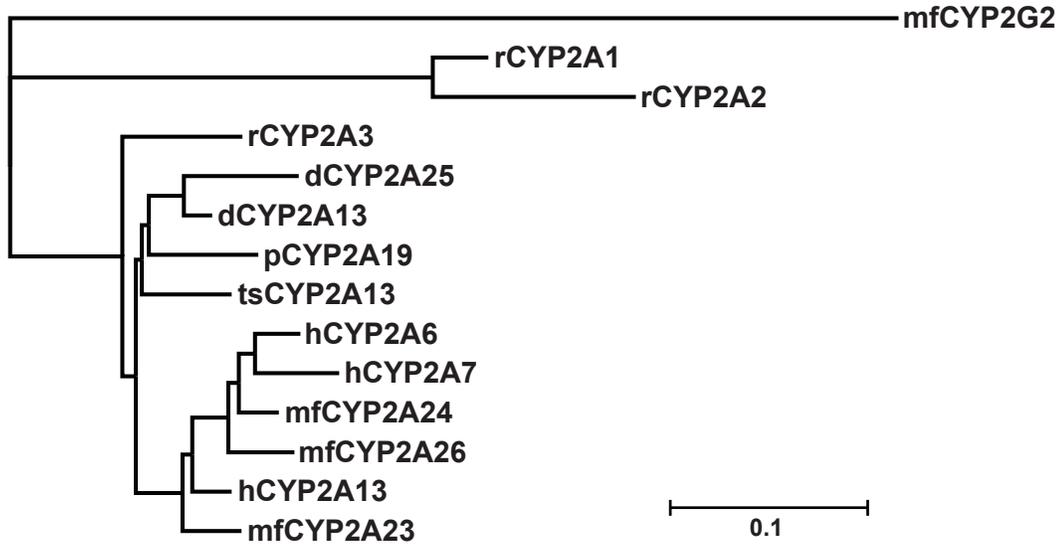
*In vitro* kinetic parameters for coumarin 7-hydroxylation and phenacetin *O*-deethylation by recombinant CYP2A enzymes

P450	Coumarin 7-hydroxylation			Phenacetin <i>O</i> -deethylation		
	$K_m$ , $\mu\text{M}$	$V_{\max}$ , $\text{min}^{-1}$	$V_{\max}/K_m$ , $\mu\text{M}^{-1}\text{min}^{-1}$	$K_m$ , $\mu\text{M}$	$V_{\max}$ , $\text{min}^{-1}$	$V_{\max}/K_m$ , $\mu\text{M}^{-1}\text{min}^{-1}$
Human 2A6	$3.1 \pm 0.3$	$10 \pm 1$	3.2	> 300	$3.9 \pm 0.6$	< 0.01
Human 2A13	$0.47 \pm 0.02$	$0.29 \pm 0.01$	0.62	$54 \pm 14$	$36 \pm 3$	0.67
Dog 2A13	$1.1 \pm 0.1$	$1.1 \pm 0.1$	1.0	$6.7 \pm 0.9$	$12 \pm 1$	1.8
Pig 2A19	$11 \pm 2.0$	$6.0 \pm 0.4$	0.55	$25 \pm 2$	$90 \pm 3$	3.6
Tree shrew 2A13	$12 \pm 3.0$	$5.0 \pm 0.3$	0.42	$18 \pm 2$	$68 \pm 2$	3.8

Coumarin and phenacetin (0.36–300  $\mu\text{M}$ ) were incubated with recombinant CYP2A enzymes (25–50 pmol equivalent/mL) at 37°C for 10–20 min. Kinetic parameters were calculated by nonlinear regression (mean  $\pm$  standard error,  $n = 13$  substrate concentrations, in triplicate). The goodness of fit for coumarin 7-hydroxylation and phenacetin *O*-deethylation activities by five recombinant CYP2A enzymes was confirmed by coefficient squared ( $r^2$ , 0.96-0.99).



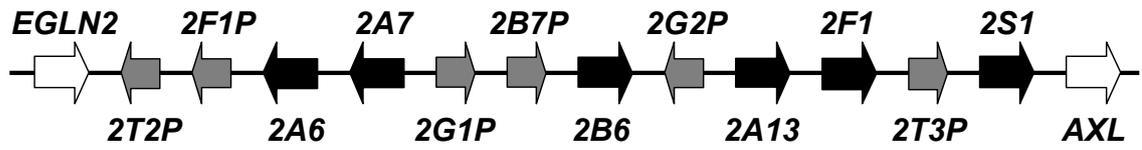
Fig. 2



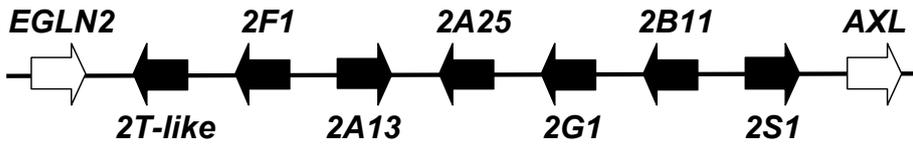
**Fig. 3**

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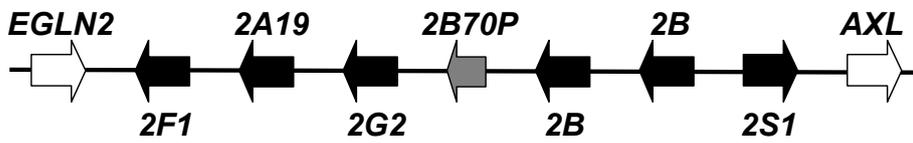
**Human chromosome 6**



**Dog chromosome 1**



**Pig chromosome 6**



**Tree shrew (NW\_006159476, NW\_006203712)**

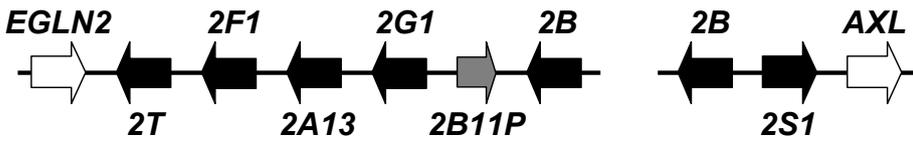
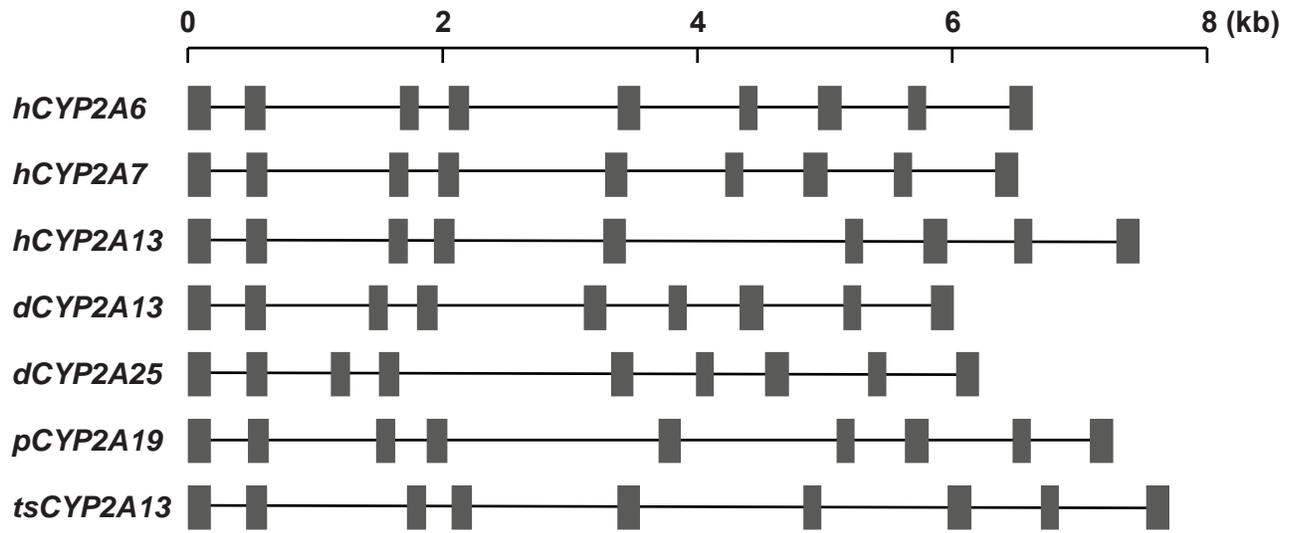
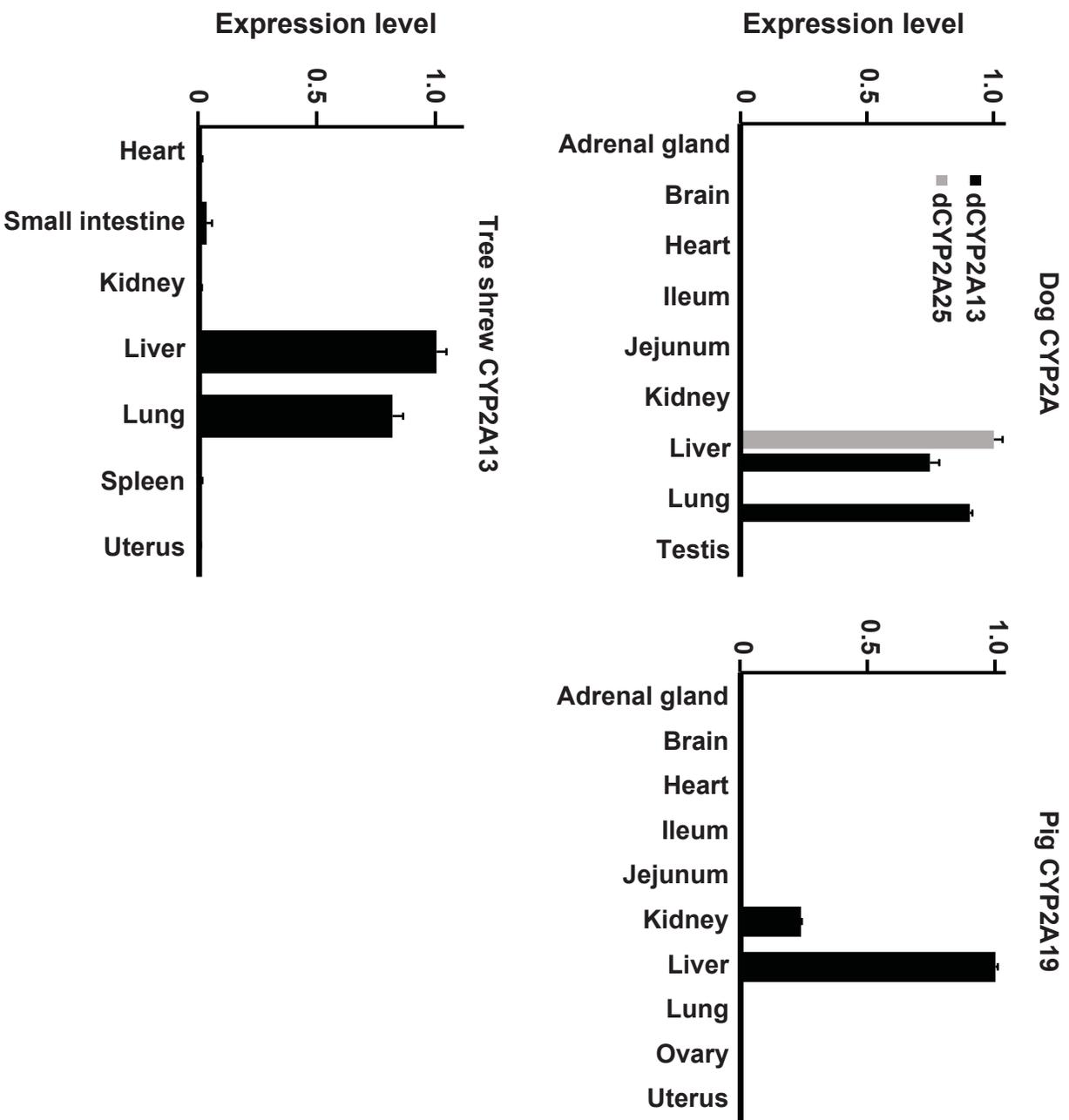


Fig. 4

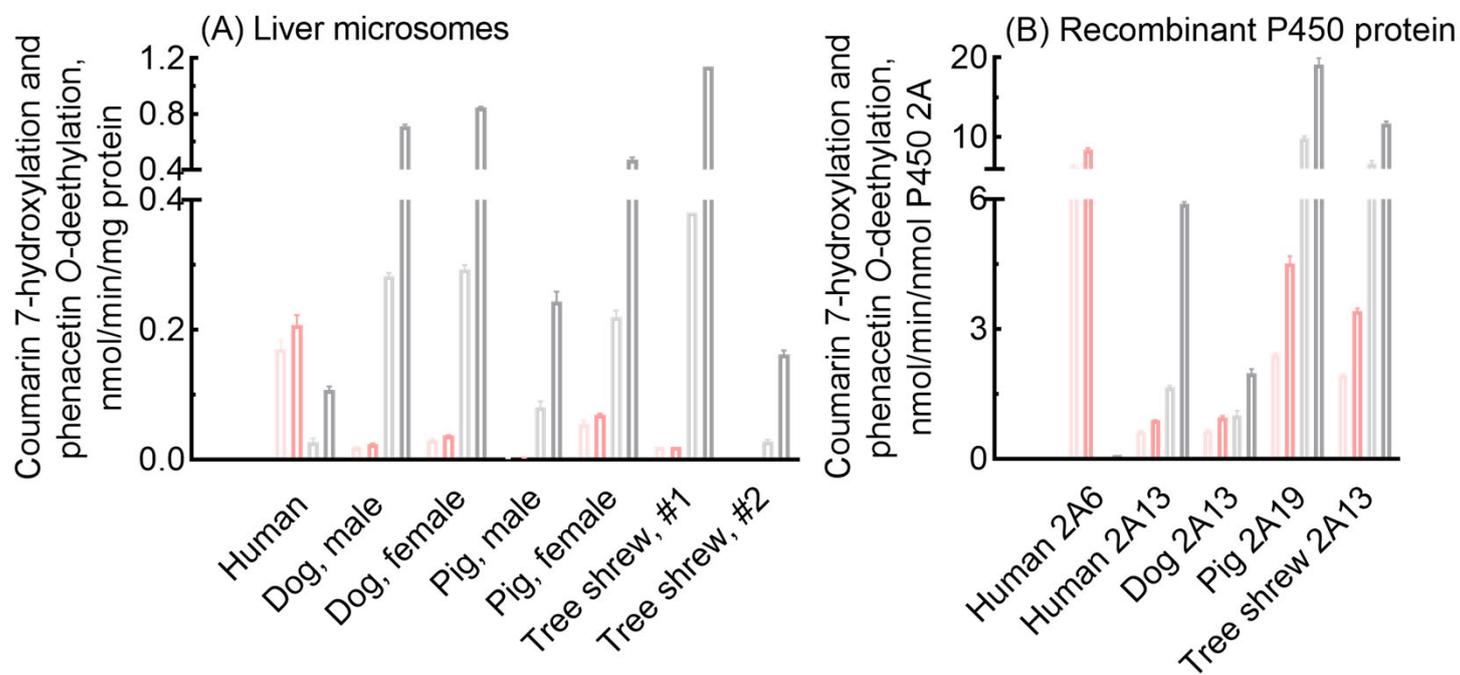


# Fig. 5



# Fig. 6

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# Fig. 7

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