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Title

A Comprehensive Investigation of Dog Cytochromes P450 3A (CYP3A) Reveals A Functional Role of Newly Identified CYP3A98 in Small Intestine

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Running title: New Dog P450 3A98 in Gut

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Abbreviations

CYP3A, cytochromes P450 3A; P450, cytochrome P450; PCR, polymerase chain reaction; RT, reverse transcription; SRS, substrate recognition site.

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Abstract

Dogs are frequently used in drug metabolism studies, and their important drug-metabolizing enzymes, including cytochromes P450 (P450 or CYP), have been analyzed. In humans, CYP3A4 is an especially important P450 due to its abundance and major roles in liver and intestine. In the present study, dog CYP3A98 and CYP3A99 were identified and characterized, along with previously identified CYP3A12 and CYP3A26. The dog CYP3A cDNAs contained open reading frames of 503 amino acids and shared high sequence identity (78–80%) with human CYP3As. Among the dog CYP3A mRNAs, CYP3A98 mRNA was expressed most abundantly in small intestine. In contrast, dog CYP3A12 and CYP3A26 mRNAs were expressed in liver, where CYP3A12 mRNA was the most abundant. The four *CYP3A* genes had similar gene structures and formed a gene cluster in the dog and human genomes. Metabolic assays of dog CYP3A proteins heterologously expressed in *Escherichia coli* indicated that the dog CYP3As tested were functional enzymes with respect to typical human CYP3A4 substrates. Dog CYP3A98 efficiently catalyzed oxidations of nifedipine, alprazolam, and midazolam, indicating major roles of CYP3A98 in small intestine. Dog CYP3A12 and CYP3A26 metabolizing nifedipine and/or midazolam would play roles in these reactions in liver. In contrast, dog CYP3A99 showed minimal mRNA expression and minimal metabolic activity, and its contribution to overall drug metabolism is therefore negligible. These results indicated that newly identified dog CYP3A98, a testosterone 6β- and estradiol 16α-hydroxylase, was abundantly expressed in small intestine and is likely the major CYP3A in small intestine in combination with liver-specific CYP3A12.

Significance Statement

Novel dog cytochromes P450 3A98 (CYP3A98) and CYP3A99 were identified and characterized to be functional and highly identical to human CYP3A4. Known CYP3A12 and new CYP3A98 efficiently catalyzed estradiol 16α-hydroxylation and midazolam 1′-hydroxylation. CYP3A98 mRNA was expressed in small intestine, whereas CYP3A12 mRNA was predominant in liver. Dog CYP3A12 and CYP3A98, in liver and small intestine, respectively, are the enzymes likely responsible for the metabolic clearances of orally administered drugs, unlike human CYP3A4/5, which are in both liver and intestine.

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Introduction

Cytochromes P450 (P450s or CYPs) are essential drug-metabolizing enzymes in humans; of these, CYP3As are abundantly expressed in liver (Shimada et al., 1994) and metabolizes nearly half of all prescribed drugs, including nifedipine and midazolam (Wilkinson, 2005). The drug-metabolizing capability of human CYP3As vary from person to person, and this variety is partially explained by genetic polymorphisms (Zanger and Schwab, 2013). For example, protein expression of human CYP3A5 in liver varies greatly and is highly correlated with the presence of defective allele *CYP3A5*3*; this defective allele causes aberrant splicing that generates a nonfunctional protein (Kuehl et al., 2001). The difference of abundance in CYP3As is due to ethnic differences in isoform expression, while CYP3A7 is mainly expressed in neonates (Shimada et al., 1994; Shimada et al., 1996; Zanger and Schwab, 2013).

P450s have been analyzed in dogs, a species that is frequently used as part of preclinical drug development. Two CYP3As have been identified in dogs, namely CYP3A12 (Ciaccio et al., 1991) and CYP3A26 (Fraser et al., 1997). Expression of dog CYP3A12 and CYP3A26 has been detected in liver at both the mRNA (Mealey et al., 2008; Haller et al., 2012) and protein levels, and CYP3A12 is the most abundant P450 protein in dog liver (Heikkinen et al., 2015; Martinez et al., 2019). Dog CYP3A12 and CYP3A26 are functional enzymes that metabolize human CYP3A substrates such as dextromethorphan, diazepam, and diclofenac (Shou et al., 2003; Locuson et al., 2009). Comparisons of human enzymes with those of species used in preclinical studies have shown that dogs exhibit hepatic metabolic activities toward human CYP3A substrates erythromycin and nifedipine at levels similar to those of humans, whereas cynomolgus macaques, guinea pigs, and rat enzymes show substantially greater activities (Shimada et al., 1997).

It is important to understand the roles of human CYP3A4/5 in both liver and small intestine in the metabolic clearances of orally administered drugs. In preclinical studies, to obtain some insight into the likely metabolic clearances in humans, the effects *in vitro* or *in vivo* of the responsible CYP3A enzymes in dog liver and intestine on orally administered drug candidates are investigated. In

this study, dog genome data were analyzed, and two novel CYP3A genes (*CYP3A98* and *CYP3A99*) were identified. Their cDNAs were isolated and characterized in terms of sequence and phylogenetic analyses, gene and genomic structures, tissue expression patterns, and metabolic assays. For comparison, the two previously identified dog P450s, CYP3A12 and CYP3A26, were similarly analyzed.

Materials and Methods

Materials

Alprazolam, estradiol, midazolam, and testosterone were purchased from Fujifilm Wako Pure Chemicals (Osaka, Japan). Nifedipine, oxidized nifedipine, 1′-hydroxymidazolam, 4-hydroxymidazolam, 4-hydroxyalprazolam, 2-/16α-hydoxyestradiol, and 6β-hydroxytestosterone were purchased from Sigma-Aldrich (St. Louis, MO). Pooled liver microsomes from dogs and humans were obtained from Corning Life Sciences (Woburn, MA) and intestinal microsomes were purchase from Sekisui Xenotech (Kansas City, KS). Recombinant human CYP3A4 in the bicistronic system with human NADPH-P450 reductase in bacterial membranes was prepared as described previously (Yamazaki et al., 2002). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). All other reagents were purchased from Sigma-Aldrich or Fujifilm Wako Pure Chemicals, unless otherwise specified.

Tissues and Nucleic Acid Preparation

Dog tissue samples (adrenal gland, brain, heart, ileum, jejunum, kidney, liver, lung, and testis) were collected from a beagle dog (male, 2 years of age, weighing approximately 10 kg) at Shin Nippon Biomedical Laboratories (Kainan, Japan). From these samples, total RNA was extracted using a mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's protocols and used for cDNA cloning and the analysis of tissue expression patterns. This study was reviewed and approved by the Institutional Animal Care and Use Committee at Kagoshima University.

Isolation of CYP3A cDNAs

Reverse transcription (RT)-polymerase chain reaction (PCR) was performed using total RNA extracted from dog liver or jejunum as previously described (Uno et al., 2006). Briefly, first-strand cDNA was synthesized in a reaction containing 1 μg of total RNA, oligo (dT), and ReverTra Ace (TOYOBO, Osaka, Japan) at 42°C for 1 h according to the manufacturer's protocols. PCR reactions

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were performed using the RT product as the template with Q5 Hot Start High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA) in a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocols. The thermal cycler conditions were an initial denaturation at 98°C for 30 s and 35 cycles at 98°C for 10 s, 60°C for 20 s, and 72°C for 50 s, followed by a final extension at 72°C for 2 min. The primers used were dCYP3A12&26 (5rt1) 5′-GTGAAAGTACAGAGAATTCACAGAGGACGA-3′ and dCYP3A12&98 (3rt1) 5′-TCTTATATTCCCTACATGAGTGAAACCACATAATC-3′ for CYP3A12, dCYP3A12&26 (5rt1) and dCYP3A26 (3rt1) 5′-GTCTCTGGTATTCTGGGATCCAGCTTCTTA-3′ for CYP3A26, dCYP3A98 (5rt1) 5′-CTGAACAAAAGTAAAGAGAATACGCAGAAGAAAAG-3′ and dCYP3A12&98 (3rt1) for CYP3A98, and dCYP3A99 (5rt1) 5′-CTGAACAAAAGTAGAGGAGACTCACAGAGAGAAAG-3′ and dCYP3A99 (3rt1) 5′-GTCTGTGGTGTTCTGGGATATAGCTTCTCG-3′ for CYP3A99. PCR products were cloned into pMiniT2.0 vectors using a PCR Cloning Kit (New England BioLabs) according to the manufacturer's protocol. The inserts were sequenced using an ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with an ABI PRISM 3500xl Genetic Analyzer (Applied Biosystems).

Sequence Analysis

Raw sequence data were analyzed using the Genetyx system (Software Development, Tokyo, Japan), including the ClustalW program, for multiple alignment of amino acid sequences. A phylogenetic tree was created by the neighbor-joining method. BLAST (National Center for Biotechnology Information) and BLAT (UCSC Genome Bioinformatics) were used for analysis of the homology and the genome data, respectively. Amino acid and cDNA sequences used for the analyses were from GenBank or the present study.

Measurement of mRNA Expression

Real-time RT-PCR was performed, as reported previously (Uno et al., 2006), in dog adrenal gland,

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brain, heart, ileum, jejunum, kidney, liver, lung, and testis. Briefly, RT reactions were carried out using ReverTra Ace qPCR RT kit (TOYOBO) according to the manufacturer's protocols, and one-twentieth of the reaction mixture was subsequently used for PCR. PCR amplification was carried out in a total volume of 20 µl using a THUNDERBIRD SYBR qPCR Mix Kit (TOYOBO) and a StepOnePlus Real-Time PCR System (Applied Biosystems), following the manufacturers' protocols. The following primers were used at final concentrations of 300 nM: dCYP3A12 (5qrt1) 5′-CCCAGAATTCCAAAGAAATGG-3′ and dCYP3A12 (3qrt1) 5′-GGCATCGTAAGTGGGCAAT-3′ for CYP3A12, dCYP3A26 (5qrt1) 5′-GTAAAGAAACACAGATCTCCCTGAGAA-3′ and dCYP3A26 (3qrt1) 5′-TTCAGGTTGAATAATCCCTCGAGTA-3′ for CYP3A26, dCYP3A98 (5qrt1) 5′-ATATCTGGCTCTTTCCAAAAAGTGT-3′ and dCYP3A98 (3qrt1) 5′-AAATCAGATAGAGCTTTATGAGTGTCTGT-3′ for CYP3A98, and dCYP3A99 (5qrt1) 5′-GAATTCTAAAGAAATGAATACCCACAA-3′ and dCYP3A99 (3qrt1) 5′-AACAACAAAGATAATAGATTGAGCAACA-3′ for CYP3A99. Relative expression levels were determined, based on three independent amplifications, by normalizing the raw data to the 18S ribosomal RNA level, which was measured using TaqMan Gene Expression Assays (Assay ID: Hs99999901_s1, Applied Biosystems) with THUNDERBIRD Probe qPCR Mix (TOYOBO).

Heterologous Protein Expression in *Escherichia coli*

Expression plasmids were generated with the CYP3A cDNAs isolated in this study, and the proteins were expressed in *Escherichia coli* DH5α as reported previously (Iwata et al., 1998; Uno et al., 2006). To enhance protein expression, the *N*-terminus was modified to that of bovine CYP17, MALLLAVF (Barnes et al., 1991), by PCR-amplification of the coding region using Q5 Hot Start High-Fidelity DNA Polymerase as described earlier with the CYP3A cDNA as the template, except that the annealing step was done at 55°C. The forward primers used were dCYP3A98 (5exp1) 5′-GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTATGGAAACCTGGCTTCTCCTGGCTA-3′ for CYP3A98, and dCYP3A12/26/99 (5exp1)

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5′-GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTACAGAAACCTGGCTTCTTCTGG-3′ for the remaining CYP3As. The reverse primer was dCYP3As (3exp1a) 5′-GCTCTAGAACAAAAGGGAAGTCCTTAGGGAAA for all the CYP3As. The NdeI and XbaI sites (underlined) of the forward and reverse primers, respectively, were utilized for subcloning into the pCW bicistronic vector, which contained human NADPH-P450 reductase cDNA in addition to the dog CYP3A cDNA. Protein expression using the generated expression plasmids and membrane preparations in *E.coli* were carried out as described previously (Iwata et al., 1998; Uno et al., 2006). The CYP3A protein and NAPDH-P450 reductase contents in membrane preparations were determined as previously described (Phillips and Langdon, 1962; Omura and Sato, 1964; Iwata et al., 1998). Molar ratios of dog NADPH-P450 reductase/CYP3A in bacterial membranes were in the range of 4.8 to 9.2.

Enzyme Assays

Drug oxidation activities of recombinant CYP3A enzymes and liver and small intestinal microsomes were determined for alprazolam, estradiol, midazolam, nifedipine, and testosterone as substrates using an HPLC-UV system as described previously (Uno et al., 2010; Uehara et al., 2017). Briefly, the incubation mixture consisted of 1.4–100 μM of midazolam, 10 or 100 μM of nifedipine or testosterone, or 20 or 200 μM of alprazolam or estradiol; recombinant CYP3A protein (20 pmol equivalent/mL) or tissue microsomes (0.25 mg/mL); 100 mM potassium phosphate buffer (pH 7.4); and an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 1 U/mL glucose-6-phosphate dehydrogenase) in a final volume of 0.25 mL. The reaction was carried out at 37°C for 10–30 min and was terminated as described previously (Uno et al., 2010; Uehara et al., 2017).. The samples of midazolam and alprazolam were centrifuged at 2000 *g* for 5 min, and then the resulting supernatant (20–50 μL) was injected into a reversed-phase C_{18} column (5 mm, 250 × 4.6 mm) using isocratic elution by a mobile phase of methanol/acetonitrile/10 mM potassium phosphate buffer (pH 7.4) (24:33:43, v/v/v) at 1.5 mL/min. For nifedipine, testosterone, and estradiol oxidation, the resulting mobile phase solutions of metabolites extracted with ethyl acetate were injected into a

reversed-phase C_{18} column (5 mm, 150 × 4.6 mm) using isocratic elution by 64% (v/v) methanol or 33% (v/v) acetonitrile in 1% (v/v) acetic acid (for estradiol) at a flow rate of 1.0 ml/min. Kinetic parameters were calculated from a curve fitted by nonlinear regression (mean ± standard error, n = 10 substrate concentrations, in duplicate) based on the Michaelis–Menten equation (with or without substrate inhibition) using Prism (GraphPad Software, La Jolla, CA): $v = V_{max} \times [S]/(K_m + [S] + [S]^2/K_s)$. One- or two-way analysis of variance was carried out using Prism (GraphPad Software, La Jolla, CA) to compare the activities among the groups.

Results

Identification of CYP3A cDNAs

Dog CYP3A cDNAs were successfully isolated from liver and jejunum by RT-PCR and were named by the P450 Nomenclature Committee (Nelson, 2009). These CYP3A cDNAs contained open reading frames of 503 amino acid residues with primary sequence structures characteristic of P450 proteins, such as six substrate recognition sites (SRSs) (Gotoh, 1992) and a heme-binding region **(Fig. 1**). The deduced amino acids of the CYP3As shared high sequence identity (78–80%) with human CYP3As (**Table 1**). Phylogenetic analysis of the CYP3A amino acid sequences showed that the dog enzymes were more closely related to those of humans than to those of rats (**Fig. 2**). The dog CYP3A cDNAs identified in the current study have been deposited in GenBank under the accession numbers ON164792, ON164793, ON164794, and ON164795 for CYP3A12, CYP3A26, CYP3A98, and CYP3A99, respectively.

Genome Organization and Gene Structure of *CYP3A* Genes

The human and dog genomes were analyzed using BLAT to determine the genomic location of the *CYP3A* genes. The analysis indicated that the four *CYP3A* genes formed a gene cluster at corresponding locations in the dog and human genomes, but three of the genes were oriented in opposite directions in the two species (**Fig. 3**). Analysis revealed that dog and human *CYP3A* genes contained similar gene structures with 13 coding exons (**Fig. 4**). Dog *CYP3A12*, *CYP3A26*, *CYP3A98*, and *CYP3A99* were approximately 35, 38, 32, and 41 kb, respectively. The sizes of the coding exons were well conserved in all the dog and human *CYP3A* genes, i.e., 71, 94, 53, 100, 114, 89, 149, 128, 67, 161, 227, 163, and 96 bp for exons 1–13, respectively, except for human *CYP3A5* exon 12, which was 160 bp. Virtually all the dog and human *CYP3A* genes begin with the dinucleotide GU and end with AG, consistent with the consensus sequences for splice junctions in eukaryotic genes. These results indicated highly conserved gene structures among dog and human *CYP3A* genes.

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CYP3A mRNA Expression in Tissues

To measure the mRNA expressions of dog CYP3As, real-time RT-PCR was performed with gene-specific primers in samples of adrenal gland, brain, heart, ileum, jejunum, kidney, liver, lung, and testis. Of the tissues analyzed, dog CYP3A12 and CYP3A26 mRNAs were expressed only in liver, whereas dog CYP3A98 mRNA was predominantly expressed in jejunum and ileum (**Fig. 5**). Dog CYP3A99 mRNA was preferentially expressed in liver and small intestine (**Fig. 5**), but the expression level was minimal compared with the other three dog CYP3A mRNAs (**Fig. 6**). Among the dog CYP3A mRNAs, CYP3A12 mRNA was the most abundant in liver, followed by CYP3A26 mRNA **(Fig. 6**). In jejunum, ileum, and lung, CYP3A98 mRNA was the most abundant. In kidney, CYP3A12 mRNA was the most abundant, followed by CYP3A98 and CYP3A26 mRNAs (**Fig. 6**).

Drug-metabolizing Capability of CYP3A Proteins

To determine the enzymatic properties of dog CYP3As, metabolic assays were carried out using typical human CYP3A substrates testosterone and estradiol with dog CYP3A proteins heterologously expressed in *E. coli*. Among the dog CYP3As, CYP3A98 most efficiently catalyzed testosterone 6β-hydroxylation (a marker reaction of human CYP3A4 in the liver microsomes) (**Fig. 7**). Dog CYP3A98 also catalyzed estradiol 16α-hydroxylation (another marker reaction of human CYP3A4), along with dog CYP3A12/26, just as human CYP3A4 does (**Fig. 7**). Thus, dog CYP3A98 is a functional enzyme that contributes to the marker reactions of human CYP3A4 in dog small intestinal microsomes (**Fig. 7**).

Dog CYP3A98 was further investigated for its activity toward human CYP3A substrates nifedipine, alprazolam, and midazolam. Among the dog CYP3As, CYP3A98 most efficiently metabolized nifedipine and alprazolam, likely reflecting drug metabolism in small intestine (**Fig. 8**). CYP3A12 and CYP3A26 also metabolized nifedipine or alprazolam, thereby contributing to drug metabolism in liver (**Fig. 8**). Kinetic analyses were carried out on midazolam hydroxylation activities using recombinant dog P450 enzymes (**Fig. 9**). Dog CYP3A98 and CYP3A12 and human CYP3A4

showed high catalytic activities at low substrate concentrations. Dog CYP3A98 and CYP3A12 catalyzed midazolam 1′-hydroxylation, a typical marker reaction of human CYP3A enzymes in liver microsomes, more efficiently than they did midazolam 4-hydroxylation, similar to the process in human liver (**Fig. 9**). The kinetic parameters calculated using the Michaelis–Menten equation with and without substrate inhibition constants are summarized in **Table 2**. Under the present conditions, dog CYP3A98 and CYP3A12 and human CYP3A4 enzymes showed low K_m values of 3.3–5.3 μM with substrate inhibition constants (K_s) of 72–425 μM. The other dog P450 enzymes tested in the kinetic studies showed midazolam hydroxylation activities without K_s.

Discussion

The current analysis of the dog genome found two new *CYP3A* gene sequences in addition to the previously identified genes *CYP3A12* and *CYP3A26*. The cDNAs of the novel CYP3As (CYP3A98 and CYP3A99) were identified and characterized by sequence analysis, and tissue expression and metabolic assays were carried out to determine if these novel CYP3As are expressed and play roles as drug-metabolizing enzymes in liver and/or small intestine, which are the typical drug-metabolizing organs.

Analysis of the genome data showed that the four *CYP3A* genes formed a gene cluster at the corresponding region of the genome in dogs and humans (**Fig. 4**). Three of the genes ran in opposite directions in humans and dogs, probably as a result of differences in gene duplication event(s) that occur in each species during evolution (Nelson et al., 2004). Consequently, a one-to-one orthologous relationship of CYP3As was not clearly evident between the two species, despite some similarities in gene structures, tissue expression patterns, and metabolic properties.

Dog CYP3A98 mRNA was predominantly expressed in small intestine, whereas dog CYP3A12 and CYP3A26 mRNAs were predominantly expressed in liver (**Fig. 5**). We found that CYP3A12 mRNA was more abundant in liver than CYP3A26 mRNA (**Fig. 6**). One previous study reported that CYP3A26 mRNA was more abundant than CYP3A12 mRNA in dog liver (Mealey et al., 2008); this inconsistency could be due to inter-individual differences, as have also been noted for human P450s (Zanger and Schwab, 2013). In other studies, CYP3A12 was found to been more abundant at the protein level than CYP3A26 in liver (Heikkinen et al., 2015; Martinez et al., 2019), suggesting that CYP3A12 is likely the major CYP3A in dog liver. Because this study analyzed one animal for analysis of tissue expression, it is of great interest to investigate additional Beagle dogs and other dog breeds.

For small intestine, previous studies have reported that CYP3A12 is more abundant than CYP3A26 both in terms of mRNA (Mealey et al., 2008) and protein levels (Heikkinen et al., 2015).

However, the nucleotide and peptide sequences used to measure CYP3A12 abundance in those studies were identical to those for CYP3A98 (Uno, unpublished data); therefore, the abundance assumed to represent CYP3A12 likely reflects that of CYP3A98, considering that CYP3A98 mRNA was greatly more abundant than CYP3A12 mRNA in jejunum and ileum (**Fig. 6**). Moreover, the reported level of CYP3A12 protein decreased from the proximal to distal portion of the small intestine (Heikkinen et al., 2015), which is consistent with our finding that CYP3A98 mRNA was expressed more abundantly in jejunum than in ileum (**Fig. 5**). Therefore, CYP3A98 is likely the major CYP3A in dog small intestine.

All four dog CYP3As were functional enzymes that metabolize the human CYP3A substrates testosterone, estradiol, nifedipine, alprazolam, and midazolam (**Figs. 7-9**). Testosterone 6β-hydroxylation, a marker reaction of human CYP3A4, was efficiently catalyzed by CYP3A98 and CYP3A12, the latter of which likely accounts for the activity in liver microsomes because CYP3A98 is barely expressed in liver (**Fig. 7**). Testosterone 6β-hydroxylation activity was lower in dog liver than in human liver (**Fig. 7**), which is consistent with the findings of another study (Bogaards et al., 2000). All four dog CYP3As catalyzed both estradiol 16α- and 2-hydroxylations, just as human CYP3A4 does, and this fact accounts for the activities in liver and small intestinal microsomes (**Fig. 7**). Therefore, dog CYP3As participate in the metabolism of steroids in a similar manner to that of human CYP3As.

Other human CYP3A substrates, nifedipine and alprazolam, were metabolized most efficiently by CYP3A98 among the dog CYP3As, indicating the involvement of CYP3A98 in small intestine in orally administered drug metabolism (**Fig. 8**). CYP3A12 and CYP3A26 also catalyzed this reaction, albeit with lower activities than CYP3A98, and therefore likely contribute to drug metabolism in liver (**Fig. 8**). In both dog and human liver microsomes, midazolam 1′-hydroxylation was more efficient than midazolam 4-hydroxylation (**Fig. 9**). Dog CYP3A12 and CYP3A98 efficiently catalyzed midazolam 1′-hydroxylation at comparable levels to that of human CYP3A4, indicating their involvement in this reaction in liver and small intestine, respectively (**Fig. 9, Table 2**). Other studies

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have also shown the major involvement of CYP3A12 in midazolam 1′-hydroxylation and to a greater extent than CYP2B11 (Zeng et al., 2021; Wu et al., 2022), which also catalyzes this reaction (Locuson et al., 2009; Baratta et al., 2010) and is expressed in liver (Heikkinen et al., 2015; Martinez et al., 2019). CYP3A26 appears to be a minor CYP3A enzyme in dog liver because of its lower hepatic protein abundance (Heikkinen et al., 2015; Martinez et al., 2019). Therefore, midazolam 1′-hydroxylation could be a marker reaction of dog CYP3A, just as it is for human CYP3A.

The activity of dog CYP3A99 was minimal toward all the CYP3A substrates analyzed (**Figs. 7-9**), and this fact might be accounted for by amino acid substitutions of 113 residues, including 304V and 363S, compared with human CYP3A4 (**Fig. 1**). In human CYP3A4, F304 in SRS-2 is predicted to be important for binding at the active site and the substitution F304A abolishes the CYP3A4-dependent metabolism of aflatoxin B_1 (Xue et al., 2001). A human CYP3A4 mutant protein (T363M) is expressed at substantially lower levels by heterologous expression in bacterial membranes and shows lower catalytic activity toward testosterone 6β-hydroxylation (Eiselt et al., 2001; Murayama et al., 2002).

Human CYP3A4 and CYP3A5 are highly variable enzymes, partly due to genetic polymorphisms. These include *CYP3A5*3*, a defective allele causing aberrant splicing that results in a nonfunctional protein (Kuehl et al., 2001). This allele is partly responsible for the variability of hepatic CYP3A5 expression seen in different populations, i.e., it is present in about 5–10% of Caucasians and in 60% or more of Africans or African Americans (Zanger and Schwab, 2013). Similar to humans, individual dogs and/or breeds of dogs exhibit variability in drug disposition (Hay Kraus et al., 2000; Neff et al., 2004). For dog CYP3A12, cDNA with five different amino acid residues was isolated, and the mutant protein did not affect enzyme activity for testosterone 6β-hydroxylation (Paulson et al., 1999). It will be important to investigate the genetic polymorphisms of dog CYP3As in the future.

In conclusion, all four dog CYP3As, including novel CYP3A98 and CYP3A99, were highly identical to human CYP3A4 and were functional enzymes that metabolize typical human CYP3A

substrates testosterone, estradiol, nifedipine, alprazolam, and midazolam. In particular, dog CYP3A12 and CYP3A98 efficiently catalyzed marker reactions of human CYP3A, namely, testosterone 6β-hydroxylation, estradiol 16α-hydroxylation, and midazolam 1′-hydroxylation. CYP3A98 mRNA was expressed in small intestine with the highest abundance among the four dog CYP3A mRNAs, whereas CYP3A12 mRNA was predominant in liver. Therefore, dog CYP3A12 and CYP3A98 are likely the main CYP3A enzymes in liver and small intestine, respectively, responsible for the metabolic clearances of orally administered drugs; this contrasts with the situation in humans in which CYP3A4/5 are abundantly expressed in both liver and small intestine.

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Authorship Contributions

Participated in research design: Uno and Yamazaki

Conducted experiments: Uno, Jikuya, Noda, and Murayama

Contributed new reagents or analytic tools: Uno

Performed data analysis: Uno and Yamazaki

Wrote or contributed to the writing of the manuscript: Uno and Yamazaki

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Footnotes

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Declaration of Interest

The authors have no interests to declare.

Legends for figures

Fig. 1. Amino acid sequences of dog and human CYP3As. Amino acid sequences of dog (d) and human (h) CYP3As were aligned as described in *Materials and Methods*. A broken line above the sequences indicates the putative heme-binding region characteristic of P450 protein. The solid lines above the sequences indicate the six putative substrate recognition sites (SRSs). Asterisks and dots under the sequences indicate identical amino acids and conservatively changed amino acids, respectively.

Fig. 2. Phylogenetic tree of CYP3A amino acid sequences. The phylogenetic tree was created as described in *Materials and Methods* using CYP3A amino acid sequences from humans (h), cynomolgus macaques (mf), dogs (d), pigs (p), and rats (r). The scale bar indicates 0.1 amino acid substitutions per site for distance measurement. Chicken (ck) CYP3A37 was used as an outgroup.

Fig. 3. Genomic structure of *CYP3A* genes. The dog and human genomes were analyzed using BLAT. Four *CYP3A* genes formed gene clusters at corresponding regions in the dog and human genomes, but three of the dog *CYP3A* genes ran in opposite directions, compared to the human *CYP3A* genes. The sizes of the genes and the distances between the genes are not proportional to actual measurements.

Fig. 4. Gene structures of *CYP3A* genes. The coding region of each CYP3A cDNA sequence was aligned with the genome using BLAT to determine the gene structure of dog (d) and human (h) *CYP3A* genes.

Fig. 5. Tissue expression patterns of CYP3A mRNAs. Expression levels of dog CYP3A mRNAs were measured by real-time RT-PCR using gene-specific primers and probes in samples of adrenal gland, brain, heart, ileum, jejunum, kidney, liver, lung, and testis. Expression levels of each CYP3A mRNA were normalized to the 18S rRNA level and represent the average ± S.D. from three independent amplifications. The most abundant expression in each graph was arbitrarily adjusted to 1, and all other expression levels were adjusted accordingly.

Fig. 6. Expression levels of dog CYP3A mRNAs in liver, kidney, jejunum, ileum, and lung. Expression levels were compared in each tissue type using averaged quantitative values. The most abundant expression was arbitrarily adjusted to 1, with which all other values were adjusted accordingly.

Fig. 7. Testosterone 6β-hydroxylation (A, C) and estradiol 16α- and 2-hydroxylation (B, D) activities by dog liver or small intestine microsomes (A, B) and recombinant dog CYP3A enzymes (C, D). Steroid oxidation activities were determined at substrate concentrations of 10 and 100 μM testosterone and 20 and 200 μM estradiol in triplicate determinations. Testosterone 6β-hydroxylation and estradiol 16α- and 2-hydroxylation activities in dog (or human) liver microsomes (A, B) were higher than those in dog (or human) intestinal liver microsomes (p <0.05, two-way analysis of variance). A new CYP3A98 had higher testosterone 6β-hydroxylation activities (C) than the known CYP3A12 among four dog CYP3A enzymes tested (p <0.05, two-way analysis of variance).

Fig. 8. Nifedipine (A, C) and alprazolam (B, D) oxidation activities by dog liver or small intestine microsome (A, B) and recombinant dog CYP3A enzymes (C, D). Drug oxidation activities were determined at substrate concentrations of 10 and 100 μM nifedipine and 20 and 200 μM alprazolam in triplicate determinations. Nifedipine and alprazolam oxidation activities in dog (or human) liver microsomes (A, B) were higher than those in dog (or human) intestinal liver microsomes (p <0.05, two-way analysis of variance). A new CYP3A98 had higher nifedipine and alprazolam oxidation activities (C, D) than the known CYP3A12 among four dog CYP3A enzymes tested (p <0.05, two-way analysis of variance).

Fig. 9. Midazolam hydroxylation catalyzed by liver microsomes and recombinant dog CYP3A proteins. Midazolam 1′- and 4-hydroxylation by liver or small intestine microsomes were determined at substrate concentrations of 10 and 100 μM in triplicate determinations (A). Midazolam hydroxylation activities in dog (or human) liver microsomes (A) were higher than those in dog (or human) intestinal liver microsomes (p <0.05, two-way analysis of variance). Kinetic analyses were performed for 1′- (closed symbols) and 4- (open symbols) midazolam hydroxylation activities by

recombinant CYP3A enzymes of dogs (B, CYP3A98, circles; CYP3A99, inverted triangles; CYP3A12, triangles; CYP3A26, squares; and human CYP3A4 as a reference, diamonds). The substrate-dependent velocity curves (n = 10 substrate concentrations, in duplicate) were prepared by nonlinear regression analysis. Dog CYP3A98 and CYP3A12 catalyzed midazolam 1′-hydroxylation with statistically similar K_m and V_{max} values among four dog CYP3A enzymes tested (B).

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Table 1

Sequence identities of dog CYP3As compared with human CYP3As.

	Human			
	CYP3A4	CYP3A5	CYP3A7	CYP3A43
	%			
Dog				
CYP3A12	**80**	78	75	73
CYP3A26	**78**	77	74	72
CYP3A98	**80**	79	76	74
CYP3A99	**78**	76	74	72

Dog CYP3A amino acid sequences were compared with human CYP3A sequences using BLAST.

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Table 2

Kinetic analyses for midazolam hydroxylation by recombinant CYP3A enzymes.

P450	Midazolam hydroxylation	K_m, μM	K_s, μM	V_{max}, nmol/min/nmol P450	V_{max}/K_m, ml/min/nmol
3A98	1′-hydroxylation	5.3 ± 3.3	425 ± 30	47 ± 15	8.9
	4-hydroxylation	27 ± 6	N.A.	18 ± 2	0.67
3A99	1′-hydroxylation	130 ± 120	N.A.	8.6 ± 5.1	0.05
	4-hydroxylation	160 ± 130	N.A.	8.3 ± 4.6	0.05
3A12	1′-hydroxylation	3.3 ± 1.4	72 ± 34	63 ± 11	19
	4-hydroxylation	35 ± 8	N.A.	20 ± 2	0.57
3A26	1′-hydroxylation	27 ± 6	N.A.	2.1 ± 0.2	0.08
	4-hydroxylation	25 ± 4	N.A.	4.1 ± 0.2	0.16
Human 3A4	1′-hydroxylation	4.0 ± 0.9	185 ± 67	60 ± 5	15
	4-hydroxylation	52 ± 9	N.A.	41 ± 4	0.79

N.A., not available.

Midazolam (1.4–100 μM) was incubated with recombinant CYP3A enzymes (40 pmol equivalent/mL) at 37°C for 15 min. Kinetic parameters were calculated from curves fitted by nonlinear regression (mean ± standard error, n = 10 substrate concentrations, in duplicate) using the substrate inhibition equation: $v = V_{max} \times [S]/(K_m + [S] + [S]^2/K_s)$. Dog CYP3A98 and CYP3A12 catalyzed midazolam 1′-hydroxylation with statistically similar K_m and V_{max} values among four dog CYP3A enzymes tested.

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Fig. 1

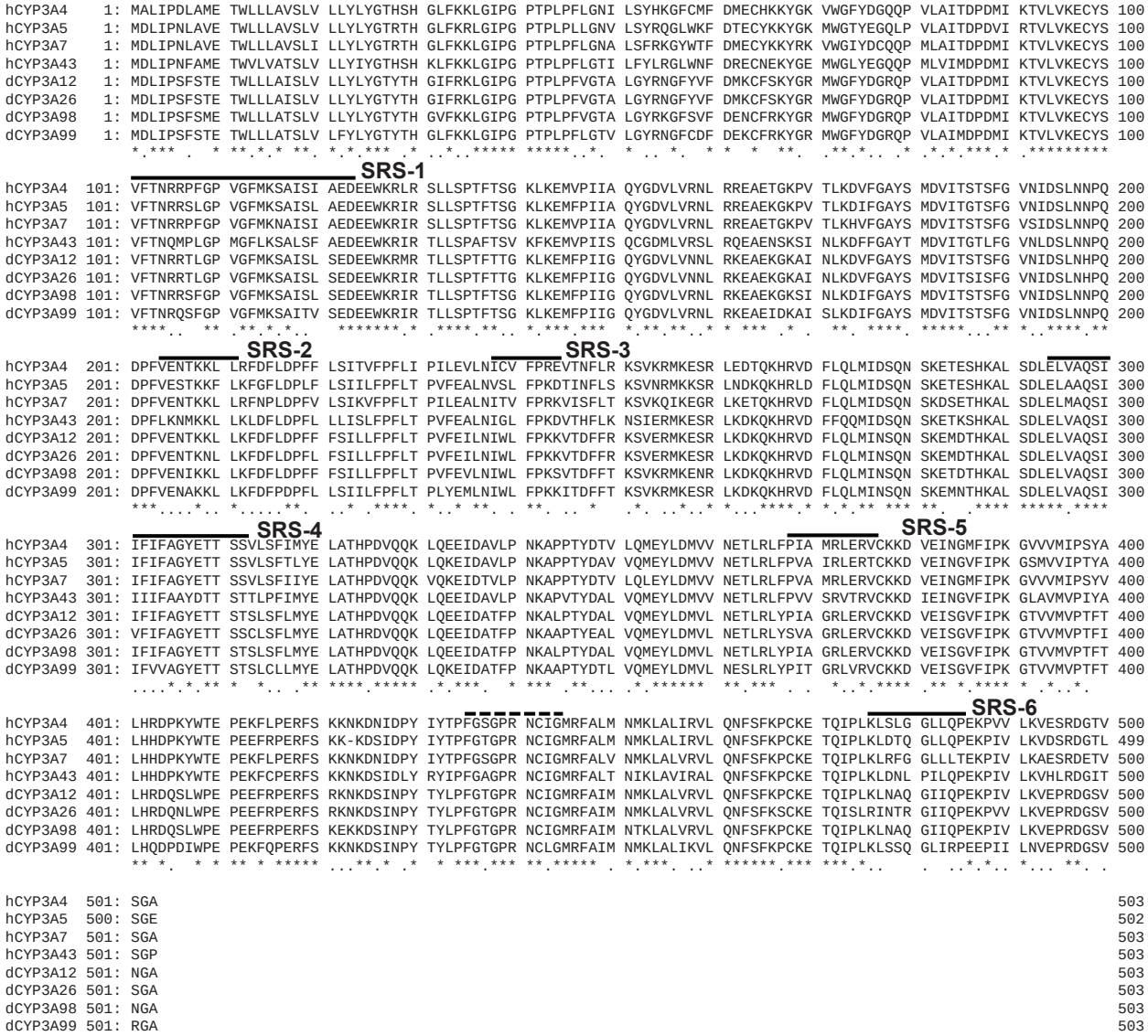

Fig. 2

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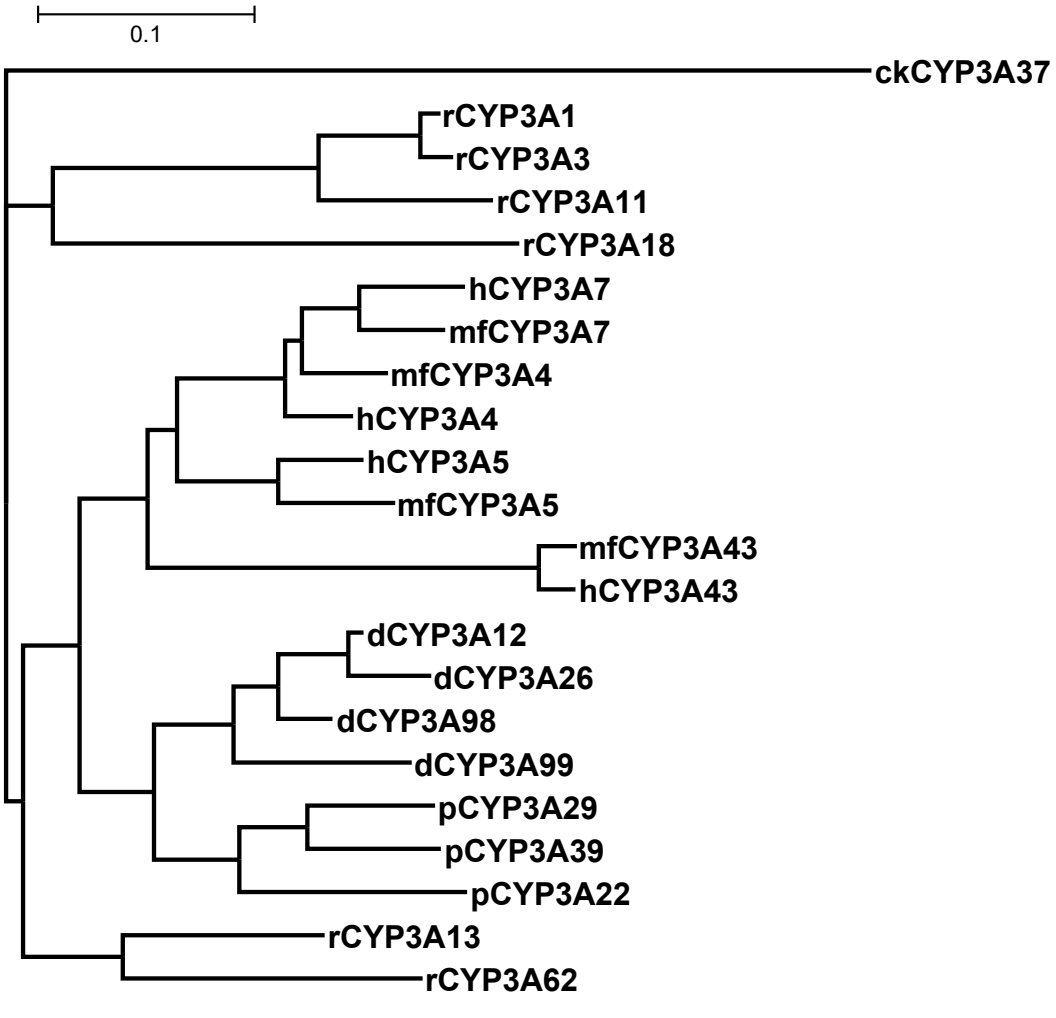

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Fig. 3

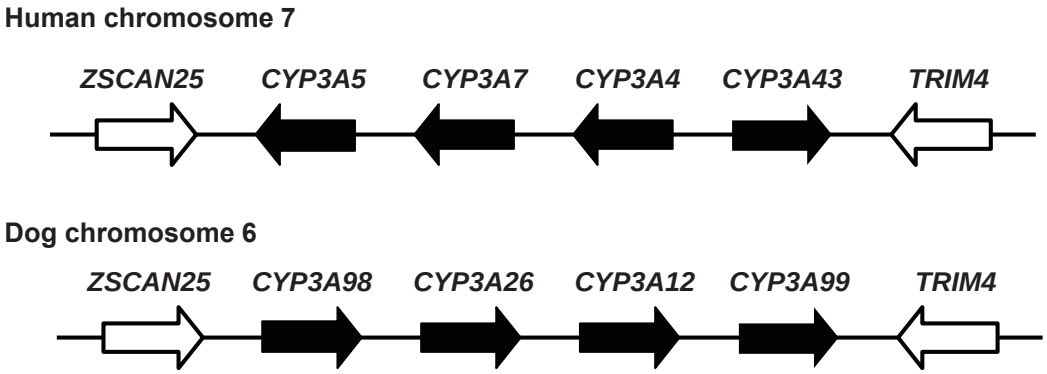

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Fig. 4

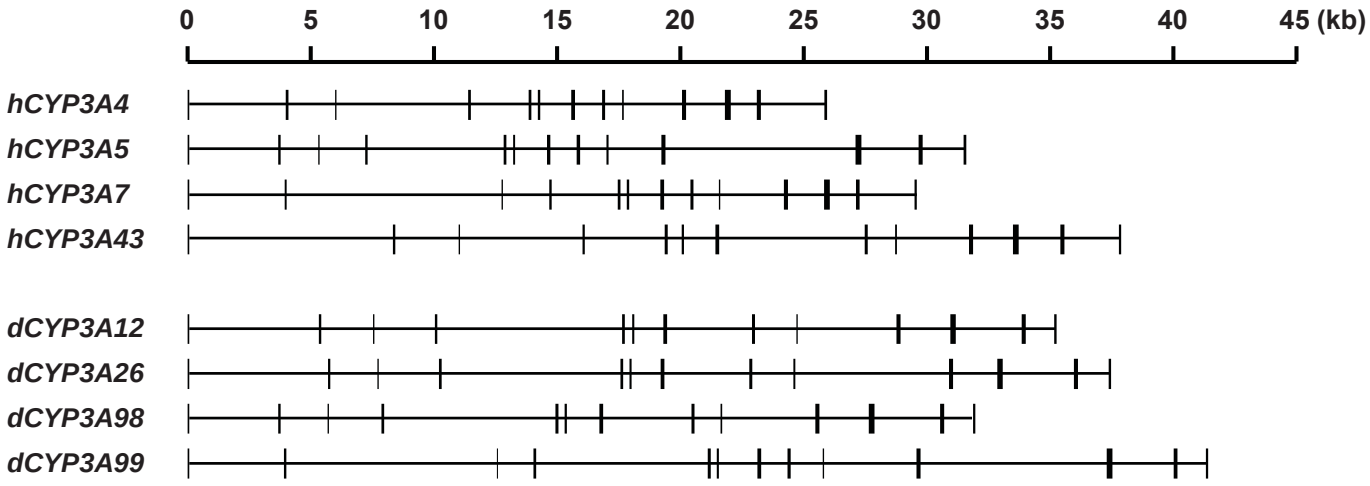

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Fig. 5

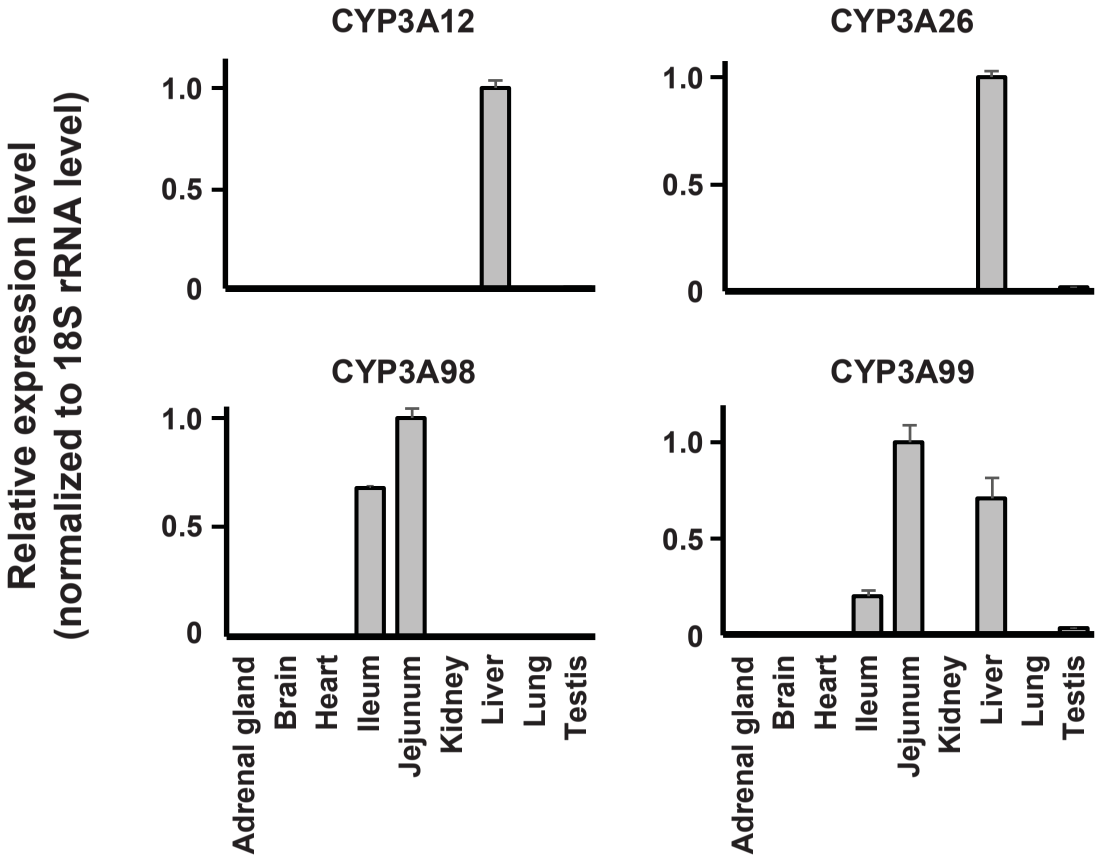

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Fig. 6

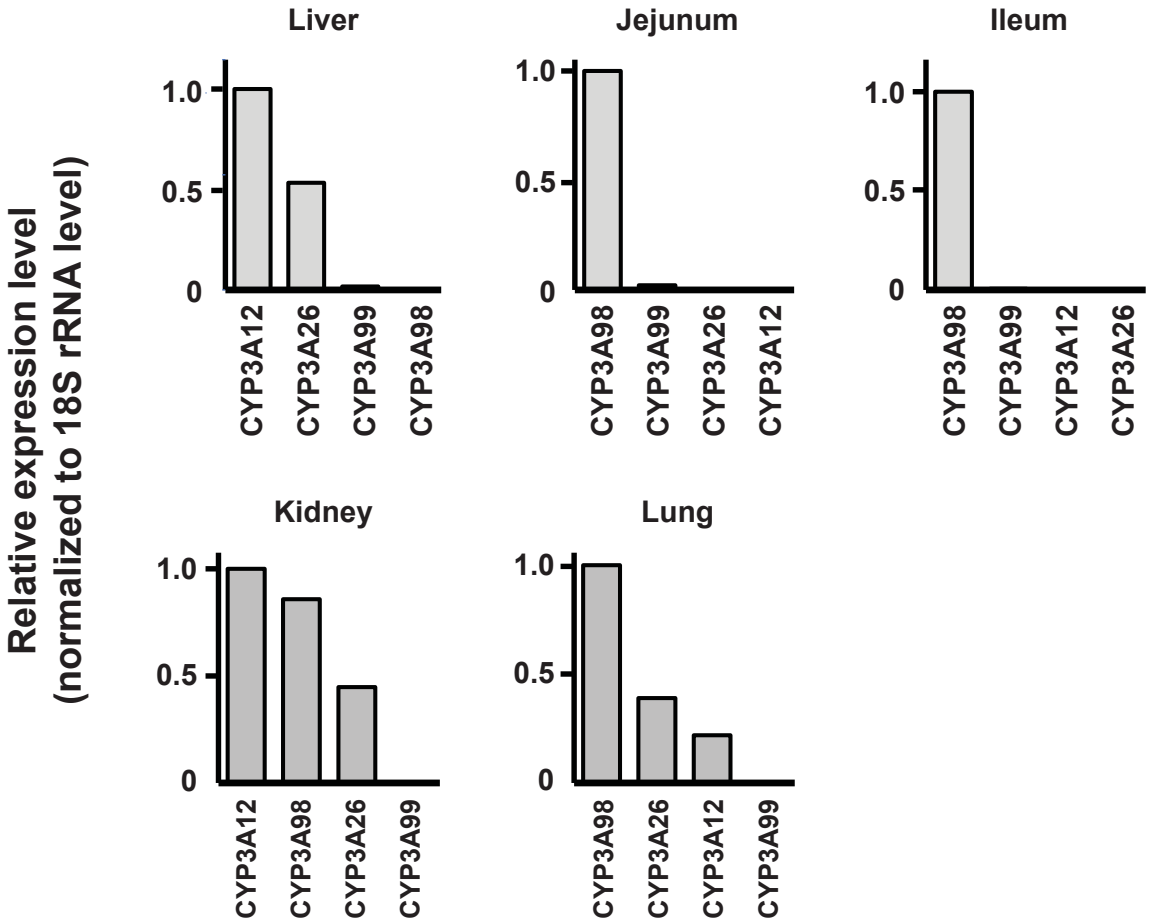

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Fig.7

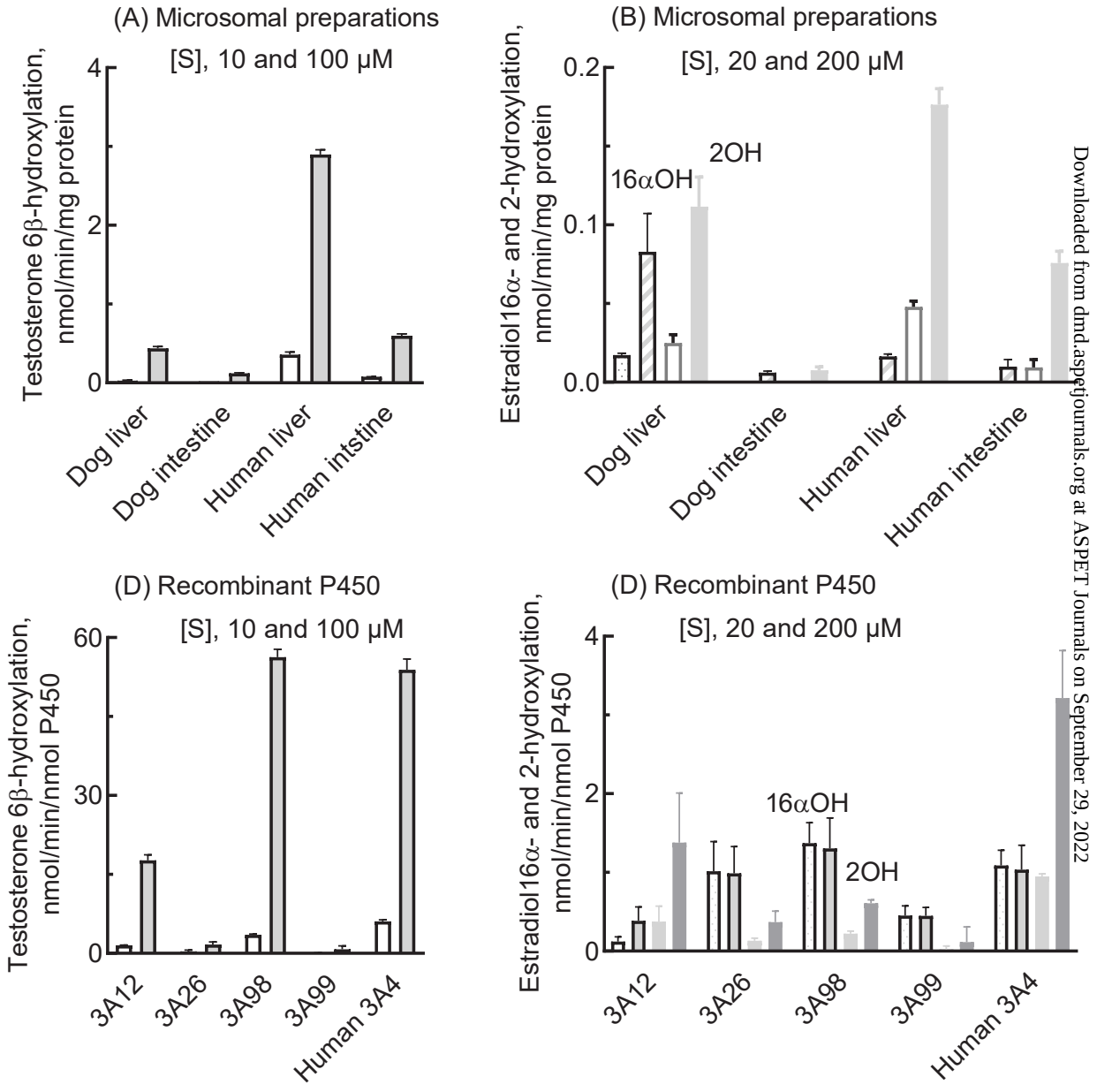

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Fig.8

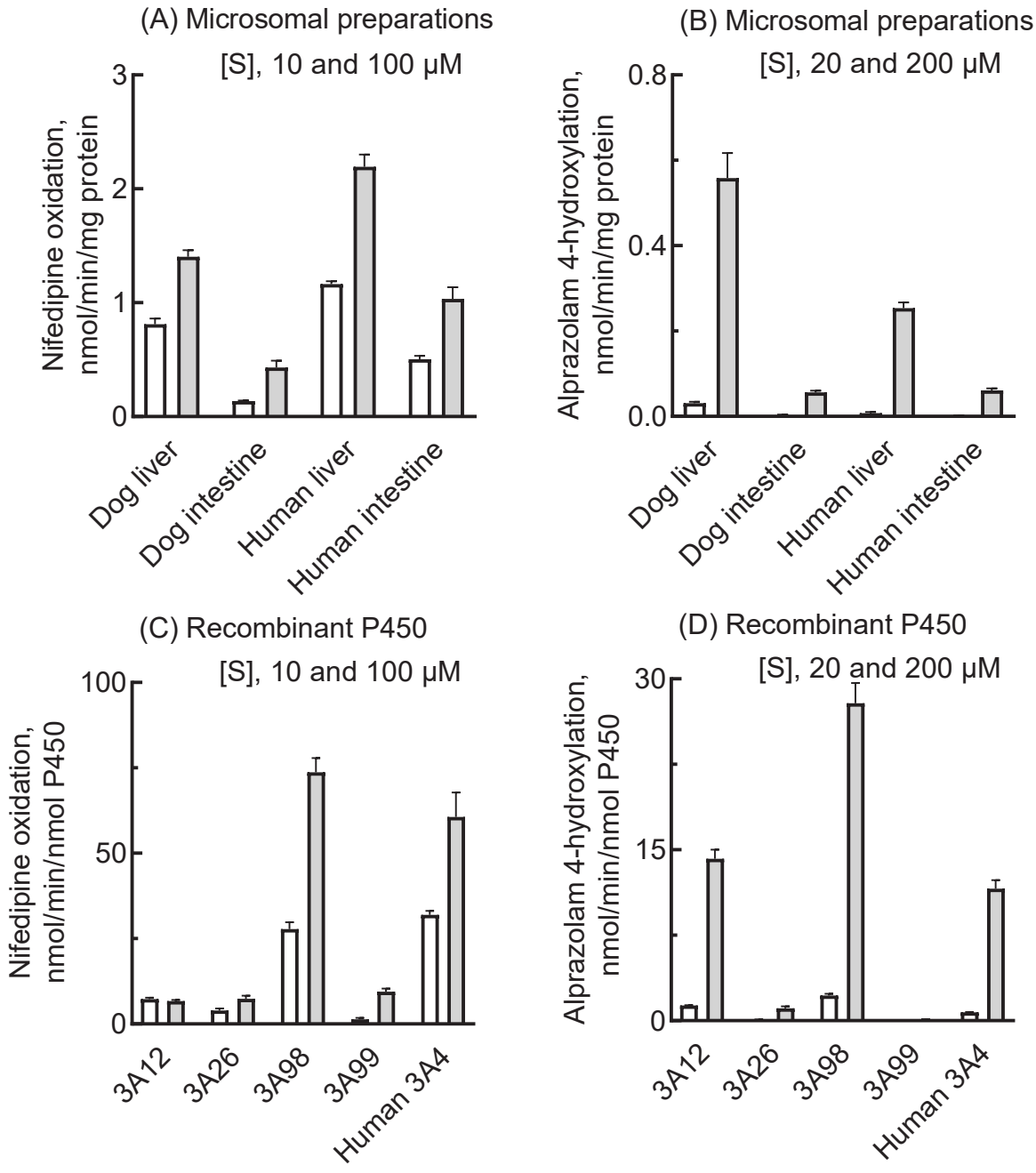

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Fig.9