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Title page

Novel cytochrome P450 2C94 (CYP2C94) functionally metabolizes diclofenac and omeprazole in dogs

Yasuhiro Uno[a, *], Saho Morikuni[b], Mitsuya Shiraishi[a], Atsushi Asano[a], Norie Murayama[b], and Hiroshi Yamazaki[b,*]

[a] Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima-city, Kagoshima 890-0065, Japan

[b] Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan

Running title: Novel Dog P450 2C94 Enzyme

* All correspondence should be sent to**:**

1) Yasuhiro Uno, D.V.M., Ph.D.
Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima-city, Kagoshima 890-0065, Japan
Phone/Fax: +81-99-285-8715. E-mail address: unoxx001@vet.kagoshima-u.ac.jp

or

2) Hiroshi Yamazaki, Ph.D.
Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, 3-3165 Higashi-Tamagawa Gakuen, Machida, Tokyo 194-8543, Japan
Phone/Fax: +81-42-721-1406. E-mail address: hyamazak@ac.shoyaku.ac.jp

Abbreviations

CYP2C, cytochromes P450 2C; P450, cytochrome P450; PCR, polymerase chain reaction; RT, reverse transcription; SRS, substrate recognition site.

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Abstract

Cytochromes P450 (P450s or CYPs) are important drug-metabolizing enzymes. Because dogs are frequently used in drug metabolism studies, knowledge of dog CYP2C enzymes is essential because, in humans, these enzymes are abundant and play major roles in liver and intestine. The present study identified and characterized novel dog CYP2C94 along with previously identified dog CYP2C21 and CYP2C41. Dog CYP2C21, CYP2C41, and CYP2C94 cDNAs, respectively, contained open reading frames of 490, 489, and 496 amino acids and shared high sequence identities (70%, 75%, and 58%) with human CYP2Cs. Dog CYP2C94 mRNA was preferentially expressed in liver, just as dog CYP2C21 and CYP2C41 mRNAs were. In dog liver, CYP2C21 mRNA was the most abundant, followed by CYP2C94 and CYP2C41 mRNAs. Moreover, the hepatic expressions of all three dog CYP2C mRNAs varied in four individual dogs, two of which did not express CYP2C41 mRNA. The three dog *CYP2C* genes had similar gene structures, and *CYP2C94*, although located on the same chromosome, was in a genomic region far from the gene cluster containing *CYP2C21* and *CYP2C41*. Metabolic assays with recombinant proteins showed that dog CYP2C94, along with CYP2C21 and CYP2C41, efficiently catalyzed oxidations of diclofenac, warfarin, and/or omeprazole, indicating that dog CYP2C94 is a functional enzyme. Novel dog *CYP2C94* is expressed abundantly in liver and encodes a functional enzyme that metabolizes human CYP2C substrates; it is, therefore, likely responsible for drug clearances in dogs.

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Significance Statement

Novel dog cytochrome P450 2C94 (CYP2C94) was identified and characterized, along with dog CYP2C21 and CYP2C41. Dog CYP2C94, isolated from liver, had 58% sequence identity and a close phylogenetic relationship with its human homologs and was expressed in liver at the mRNA level. Dog CYP2C94 (and CYP2C21 and CYP2C41) catalyzed oxidations of diclofenac and omeprazole, human CYP2C9 and CYP2C19 substrates, respectively, but CYP2C41 also hydroxylated warfarin. CYP2C94 is, therefore, a functional drug-metabolizing enzyme likely responsible for drug clearances in dogs.

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Introduction

Cytochromes P450 (P450s or CYPs) are essential enzymes that metabolize various endogenous and exogenous substrates; in humans, P450s are encoded by 57 functional genes (Nelson et al., 2004). Human CYP2C enzymes (CYP2C8, CYP2C9, and CYP2C19) metabolize >20% of prescription drugs, e.g., diclofenac, warfarin, and omeprazole (Goldstein, 2001; Zanger and Schwab, 2013). Of the total P450 content in human liver and small intestine, respectively, ~19% and ~25% are CYP2C proteins (Shimada et al., 1994; Paine et al., 2006). Among individuals and populations, CYP2C-dependent metabolic activity is variable, partly as a result of genetic polymorphisms. Alleles *CYP2C9*2* and *CYP2C9*3* are responsible for poor-metabolizer phenotypes common (Ingelman-Sundberg et al., 2007; Zanger and Schwab, 2013). Therefore, the CYP2C enzymes are essential for drug metabolism in humans and are responsible for a wide range of drug clearances.

Dogs are frequently used as preclinical species during drug development, and dog P450s have been analyzed, including two CYP2Cs, CYP2C21 and CYP2C41 (Uchida et al., 1990; Blaisdell et al., 1998). Expressions of dog CYP2C21 and CYP2C41 have been detected in liver at both the mRNA and protein levels (Graham et al., 2003; Heikkinen et al., 2015; Martinez et al., 2019). Dog CYP2C21 and CYP2C41 have been reportedly functional enzymes that metabolize typical human CYP2C substrates such as diclofenac and mephenytoin (Shou et al., 2003; Locuson et al., 2009). In comparison studies of preclinical species, dogs showed similar hepatic metabolic activities toward human CYP2C substrates phenytoin and mephenytoin to those in humans (Shimada et al., 1997). Our previous systematic investigation reported that new dog CYP3A98 and CYP3A99 are functional drug-metabolizing enzymes (Uno et al., 2023).

In the present study, we identified novel dog CYP2C94 and analyzed it along with previously identified CYP2C21 and CYP2C41. These P450s were found to be similar to human CYP2Cs and were characterized in terms of sequence and phylogenetic analyses, gene and genomic structures, expression patterns, and drug oxidation activities of recombinant P450 proteins. We report herein the functions of all three dog CYP2Cs, including CYP2C94, in comparison with human CYP2Cs using

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typical substrates of human CYP2Cs.

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Materials and Methods

Materials

Synthesized oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Pooled liver microsomes from pooled male and female dogs were purchased from Corning Life Sciences (Woburn, MA). Liver microsomes were prepared as described previously (Yamazaki et al., 2014) from four individual beagle dogs (males, 2 years of age, weighing ~10 kg; designated as dogs 3, 5, 6, and 7). Omeprazole, *R,S*- and *S*-warfarin, diclofenac, and paclitaxel and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Preparation of RNA

Samples of livers were collected from four beagle dogs, one of which also provided tissue samples of adrenal gland, brain, heart, ileum, jejunum, kidney, lung, and testis. Total RNAs of these samples were extracted as described previously (Uno et al., 2022b) and were used for molecular cloning and real-time polymerase chain reaction (PCR). This study was reviewed and approved by the Institutional Animal Care and Use Committee at Kagoshima University.

Molecular cloning

To isolate cDNAs, reverse transcription (RT)-PCR was carried out using liver total RNAs, as described previously (Uno et al., 2006). Briefly, the RT reaction was performed using 1 μg of total RNA, oligo (dT), and ReverTra Ace (TOYOBO, Osaka, Japan) at 42°C for 1 h. The resulting RT products were used as the template for PCR with primers (**Table 1**) and Q5 Hot Start High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA) in a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA). PCR conditions were an initial denaturation at 98°C for 30 s; 35 cycles at 98°C for 10 s, 60–65°C for 20 s, and 72°C for 50 s; followed by a final extension at 72°C for 2 min. The PCR product was cloned into pMiniT2.0 vectors using a PCR Cloning Kit (New England BioLabs) according to the manufacturer's instructions. Sequencing the insert was conducted using an

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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

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

Quantitative PCR

Expression levels of dog CYP2C mRNAs in samples of adrenal gland, brain, heart, ileum, jejunum, kidney, liver, lung, and testis were measured by real-time RT-PCR performed as reported previously (Uno et al., 2006). Briefly, RT reactions were carried out using a ReverTra Ace qPCR RT kit (TOYOBO), followed by PCR using the RT product as template. A 20-µL PCR reaction mixture with a THUNDERBIRD SYBR qPCR Mix Kit (TOYOBO) was subjected to a CFX Connect real-time PCR detection system (BioRad Laboratories, Hercules, CA). The annealing step was carried out at 60°C. The primers (**Table 1**) were used at final concentrations of 300 nM. 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).

Preparation of CYP2C proteins

Dog CYP2C protein expression was carried out with prepared expression plasmids in *Escherichia coli* as described previously (Iwata et al., 1998; Uno et al., 2006). Briefly, the *N*-terminus was modified by PCR-amplification of the coding region using the primers shown in **Table 1** and Q5 Hot Start

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High-Fidelity DNA Polymerase. The forward and reverse primers, respectively, contained *Nde*I and *Xba*I sites (for subcloning into the pCW vector, which contained human NADPH-P450 reductase cDNA) except that the dog CYP2C21 forward primer contained an *Ase*I site. Bacterial membranes were prepared, and the P450 protein and reductase contents in each membrane preparation were measured as described previously (Iwata et al., 1998; Uno et al., 2006).

The relative intensity on coupled SDS-PAGE/immunochemical development of CYP2C enzymes in dog liver microsomal samples was estimated in comparison with recombinant dog CYP2C21 using anti-human CYP2C9 antibodies (Shimada et al., 1986) and anti-rat CYP2C13 antibodies (Corning Life Sciences).

Enzymatic characterization of dog CYP2Cs

Paclitaxel 6α-hydroxylation, diclofenac 4′-hydroxylation, *S*- and *R,S*-warfarin 7-hydroxylation, and omeprazole 5-hydroxylation activities of recombinant dog CYP2C proteins were determined as described previously (Uno et al., 2011; Uehara et al., 2015; Uehara et al., 2016; Nakanishi et al., 2018; Uno et al., 2022a). Briefly, typical reaction mixtures (0.20 mL) contained dog liver microsomes (0.50 mg protein/mL) or recombinant protein (5.0 pmol), an NADPH-generating system, and substrate (10 or 100 μM) in 50 mM potassium phosphate buffer (pH 7.4). The lower substrate concentrations were adopted according to the known values of the Michaelis-Menten constant (K_m) for human CYP2C8/9/19 enzymes (Uehara et al., 2015; Uehara et al., 2016; Nakanishi et al., 2018). Reaction mixtures were incubated at 37°C for 15 min (paclitaxel 6α-hydroxylation, diclofenac 4′-hydroxylations, and omeprazole 5-hydroxylations) or 30 min (*S*- and *R,S*-warfarin 7-hydroxylations) and then terminated with 20 μL of 60%(v/v) $HClO_4$ (except for the case of ethyl acetate extraction of paclitaxel). Supernatants centrifuged at 900 × *g* for 5 min were analyzed by high-performance liquid chromatography with a fluorescence detector or a UV detector and a reverse phase analytical column (C_{18}) and eluents (Uno et al., 2022a).

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Results

Isolation of dog CYP2C cDNAs

Novel dog CYP2C94 cDNA was identified in this study, along with previously identified dog CYP2C21 and CYP2C41 cDNAs. Dog CYP2C21, CYP2C41, and CYP2C94 cDNAs, respectively, contained open reading frames of 490, 489, and 496 amino acid residues and had primary sequence structures characteristic of P450 proteins, i.e., a heme-binding region and putative substrate recognition sites (Gotoh, 1992) (**Fig. 1**). In terms of amino acids, dog CYP2C21 and CYP2C41, respectively, had 70% and 75% sequence identities to human CYP2Cs, whereas dog CYP2C94 had a lower sequence identity of 58% (**Table 2**). Phylogenetic analysis of CYP2C amino acid sequences revealed that dog CYP2C94 was closely clustered with pig CYP2C33 and rat CYP2C23, dog CYP2C21 was clustered with cynomolgus CYP2C76 and rat CYP2C22, and dog CYP2C41 was clustered with several pig CYP2Cs (**Fig. 2**). The CYP2C cDNAs identified in this study were deposited in GenBank, namely, dog CYP2C21 (OP493566), CYP2C41 (OP493567), and CYP2C94 (OP493568).

Genome organization and exon–intron structure of *CYP2C* genes

Analysis of the genome data revealed that dog *CYP2C21* and *CYP2C41* formed a gene cluster, similar to that of human *CYP2C* genes, located in the corresponding genomic region in humans and dogs; however, the number, direction, and location of the *CYP2C* genes within the gene cluster did not correspond well (**Fig. 3**). In contrast, dog *CYP2C94* was a monocistronic gene located adjacent to *CYP2E1* in the same chromosome as the other dog *CYP2C* genes; however, no *CYP2C* gene was found adjacent to *CYP2E1* in the human genome (**Fig. 3**). Dog *CYP2C* genes had similar gene structures to human *CYP2C* genes with nine coding exons (**Fig. 4**). The sizes of the coding exons were well conserved in dog and human *CYP2C* genes, containing the same number of bases in exons 2–8, i.e., 163, 150, 161, 177, 142, 188, and 142 bp respectively; in contrast, the size of the coding part of exons 1 and 9 varied in the ranges 165–180 and 182–188 bp, respectively. Introns of all these

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CYP2C genes begin with the dinucleotide GU and end with AG, consistent with the consensus sequences for splice junctions in eukaryotic genes.

Tissue expression patterns of CYP2C mRNAs

The levels of dog CYP2C mRNA expression were quantified using real-time RT-PCR in tissue samples of adrenal gland, brain, heart, ileum, jejunum, kidney, liver, lung, and testis. Among these tissues, dog CYP2C21 mRNA was predominantly expressed in liver (**Fig. 5**). Dog CYP2C41 and CYP2C94 mRNAs were also preferentially expressed in liver, followed by jejunum and testis, respectively. In liver, of the dog CYP2C mRNAs analyzed, CYP2C21 mRNA was the most abundant, followed by CYP2C94 and CYP2C41 mRNA (**Fig. 6**). In small intestine and kidney, respectively, CYP2C41 and CYP2C94 mRNAs were the most abundant, whereas, in lung and heart, CYP2C21 mRNA was the most abundant, followed by CYP2C94 mRNA (**Fig. 6**). In four individual dogs, the hepatic expressions of CYP2C mRNAs varied: CYP2C41 mRNA was undetectable in two dogs, and CYP2C21 mRNA was the most abundant among the CYP2C mRNAs analyzed (**Fig. 6**).

Drug-metabolizing activities

Hydroxylation activities toward typical human CYP2C8/9/19 substrates paclitaxel, diclofenac, *S*- and *R,S*-warfarin, and omeprazole were determined for recombinant dog CYP2C21, CYP2C41, and CYP2C94 proteins and dog liver microsomes. Dog CYP2C21, CYP2C41, and CYP2C94 exhibited high diclofenac 4′-hydroxylation and omeprazole 5-hydroxyaltion activities, and CYP2C41 additionally had high paclitaxel 6α-hydroxylation and warfarin 7-hydroxyaltion activities (**Fig. 7A**).

Pooled and individual liver microsomes showed activities toward *S*-warfarin and omeprazole, but activity levels varied between the substrates. Liver microsomes from dogs 3 and 6, in which CYP2C41 mRNA was not detectable (**Fig. 6B**), showed *S*-warfarin 7-hydroxylation activities comparable with those of the other individual or pooled dog liver microsomes (**Fig. 7**). *S*-Warfarin 7-hydroxylation activities at substrate concentrations of 10 μM and 100 μM were significantly correlated ($p < 0.05$, n = 4) with the sum of CYP2C21, CYP2C41, and CYP2C94 mRNA levels (**Fig.**

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8A). In contrast, omeprazole 5-hydroxylation activities at substrate concentrations of 10 μM and 100 μM were significantly correlated (p <0.05, n = 4) with CYP2C21 mRNA levels (**Fig. 8B**). The intensities of the immunochemical bands using anti-rat CYP2C13 antibodies in individual dog liver microsomes were well correlated (p <0.05, n = 4) with *S*-warfarin 7-hydroxylation activities (**Fig. 8C**) and omeprazole 5-hydroxylation activities (**Fig. 8D**). However, the relative intensities of CYP2C enzymes in individual dog liver microsomes using anti-human CYP2C9 antibodies was inversely correlated with the drug oxidation activities tested (not shown). These results indicated that dog CYP2C21, CYP2C41, and CYP2C94 were functional drug-metabolizing enzymes.

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Discussion

By analysing the dog genome, we found the novel *CYP2C94* gene sequence in addition to the previously identified genes *CYP2C21* and *CYP2C41*. We isolated CYP2C94 cDNA, along with CYP2C21 and CYP2C41 cDNAs, and characterized them using sequence analysis, tissue expression, and drug-metabolizing assays. All three dog CYP2Cs analyzed here were homologous to human CYP2Cs; however, CYP2C94 showed less sequence identity to human CYP2Cs than dog CYP2C21 and CYP2C41 did (**Table 2)**. Phylogenetic analysis showed that dog CYP2C94 was closely related to rat CYP2C23 and pig CYP2C33, whereas dog CYP2C21 and CYP2C41 were more closely related to human CYP2Cs (**Fig. 2**). Moreover, unlike the arrangement of human *CYP2C* genes, dog *CYP2C94* was located in the genomic region adjacent to *CYP2E1*, which is far from the gene cluster formed by dog *CYP2C21* and *CYP2C41* on the same chromosome (**Fig. 3**). This divergence in *CYP2C* gene arrangement is probably accounted for by different gene duplication events having taken place in the two species during evolution (Nelson et al., 2004). Similarly, pig *CYP2C33* is located distant from the main *CYP2C* gene cluster, and CYP2C33 has amino acid sequences less homologous to human CYP2Cs than those of other pig CYP2Cs (Uno et al., 2022a). Consequently, a one-to-one orthologous relationship to human CYP2Cs was not clearly evident for dog CYP2Cs, including for novel CYP2C94.

Dog CYP2C21, CYP2C41, and CYP2C94 mRNAs were preferentially expressed in liver among the tissues analyzed (**Fig. 5**). Among the dog CYP2C mRNAs, CYP2C21 mRNA was the most abundant in liver, followed by CYP2C94 and CYP2C41 mRNAs (**Fig. 6**). Expressions of dog CYP2C21 and CYP2C41 mRNAs and proteins have been detected in liver, with expression of CYP2C21 protein being more abundant than that of CYP2C41 (Graham et al., 2003; Heikkinen et al., 2015; Martinez et al., 2019). Dog CYP2C mRNA expressions were different in four individual dogs: indeed, CYP2C41 mRNA was not detected in two dogs (**Fig. 6**). The absence of CYP2C41 mRNA in liver has been noted in previous dog studies (Graham et al., 2003), likely resulting from the gene sequence being absent in some individuals and breeds (Blaisdell et al., 1998; Karakus et al., 2021).

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The different expression levels of dog CYP2Cs in liver from different individuals partly accounts for the inter-individual differences in CYP2C-dependent drug metabolism, as described below. Dog CYP2C94 mRNA was the most abundant among the three dog CYP2C mRNAs in kidney, adrenal gland, and testis (**Fig. 6**). Considering that CYP2C enzymes metabolize steroid hormones such as dehydroepiandrosterone and testosterone, which are produced in adrenal cortex and testis, respectively (Monostory and Dvorak, 2011; Niwa et al., 2015), dog CYP2C94 might have roles in these tissues.

Under the present conditions, all three dog CYP2Cs, including novel CYP2C94, were functional enzymes able to metabolize human CYP2C substrates diclofenac, warfarin, and omeprazole; nonetheless, their activities were different toward these substrates (**Fig. 7**). Dog CYP2C21, CYP2C41, and CYP2C94 all catalysed diclofenac 4′-hydroxylation, a marker reaction of human CYP2C9 (**Fig. 7**), and the catalytic activities of CYP2C21 and CYP2C94 were similar to those seen in previous studies of the known CYP2C form (Shou et al., 2003; Locuson et al., 2009). Diclofenac 4′-hydroxylation is also catalyzed by dog CYP2B6 (Shou et al., 2003; Locuson et al., 2009), indicating that diclofenac might not be a selective substrate of dog CYP2Cs. *R,S*-Warfarin was metabolized by all dog CYP2Cs, but the activity of CYP2C41 was greater than those of CYP2C21 and CYP2C94 (**Fig. 7**), possibly indicating that this substrate is relatively selective to CYP2C41. Omeprazole, a typical human CYP2C19 substrate, was also metabolized by all dog CYP2Cs, of which the activity of CYP2C21 was somewhat higher than that of CYP2C41 and CYP2C94 (**Fig. 7**). Paclitaxel, a typical human CYP2C8 substrate, was hydroxylated by dog CYP2C41, but the rate was one order of magnitude less than that of recombinant human CYP2C8 (Uehara et al., 2015; Uehara et al., 2016; Nakanishi et al., 2018). *S*-Warfarin and omeprazole hydroxylation activities in dog liver microsomes varied somewhat among the animals analyzed in the current study (**Fig. 7**). However, these hydroxylation activities were correlated with total CYP2C mRNA levels, CYP2C21 mRNA levels, and/or the relative immunochemical CYP2C intensity levels (**Fig. 8**). These results collectively suggest the major roles of dog CYP2Cs, especially CYP2C21, for the oxidation of typical human CYP2C9 and CYP2C19 substrates (*S*-warfarin and omeprazole) in liver; however, the common

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selectivities of dog CYP2Cs were slightly different from the apparent substrate specificities of human CYP2Cs. It would be of interest to find selective or specific substrates of dog CYP2C94, as this would help to further understand the importance of this enzyme in drug metabolism in dogs.

In dog liver microsomes, omeprazole was more efficiently metabolized than *S*-warfarin (**Fig. 7**). This might have resulted from differences of amino acid sequences, just as in the case of human CYP2Cs. For example, in human CYP2C19, His99, Pro220, and Thr221 are reportedly key residues for omeprazole 5-hydroxylation (Ibeanu et al., 1996). His99 is also present in dog CYP2C21, whereas Pro220 is present in all the dog CYP2Cs; however, Thr221 was not found in any dog CYP2C (**Fig. 1**). The compound mutation Glu241Lys;Asn286Ser;Ile289Asn in human CYP2C19 appears to increase the catalytic efficiency of both *S*- and *R*-warfarin metabolism (Jung et al., 1998). The substitution Glu241Lys was found in dog CYP2C21 and CYP2C94. It would be of interest to investigate the importance of these amino acid residues for dog CYP2C enzyme function. In humans, the enzyme activities of CYP2Cs vary among individuals and/or populations partly because of genetic polymorphisms. Human *CYP2C19*2* and *CYP2C19*3* alleles are responsible for poor metabolizer phenotypes as a result of aberrant splicing and the generation of a premature stop codon, respectively (Ingelman-Sundberg et al., 2007; Zanger and Schwab, 2013). In dogs, CYP2C41 is highly polymorphic, and the gene is deleted in some individuals of certain breeds (Blaisdell et al., 1998; Graham et al., 2003; Martinez et al., 2019). Extensive analysis of 1089 individual dogs from 36 different breeds established that the *CYP2C41* gene was absent in all the individuals examined from the following breeds: Bearded Collie, Bernese Mountain, Boxer, Briard, French Bulldog, and Irish Wolfhound. In contrast, the *CYP2C41* gene was present in 34–67% of animals among Chinese Char-Pei, Siberian Husky, Schapendoes, and Kangal breeds (Karakus et al., 2021). Future studies are needed to investigate the genetic polymorphisms of *CYP2C* genes in various breeds and individuals and to determine whether other dog CYP2C enzymes might show compensatory function when some genes are missing, as seen in **Figure 7**.

In conclusion, dog CYP2C21, CYP2C41, and CYP2C94 exhibited high sequence identities to

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human CYP2Cs and were shown to be functional enzymes that metabolize typical human CYP2C substrates warfarin, and omeprazole. These dog CYP2C mRNAs were abundantly expressed in liver, and expression of CYP2C41 mRNA was also detected in jejunum. The hepatic expressions of dog CYP2C mRNAs varied in four dogs, and hepatic expression of CYP2C41 mRNA was not detected in two dogs. Therefore, CYP2C21, CYP2C41, and CYP2C94 are expected to play roles as drug-metabolizing enzymes in dog liver.

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Authorship Contributions

Participated in research design: Uno, Yamazaki.

Conducted experiments: Morikuni, Murayama, Uno.

Contributed new reagents or analytic tools: Shiraishi, Asano, Uno.

Performed data analysis: Uno, Yamazaki.

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

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Footnotes

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Declaration of Interest

The authors have no competing interests to declare.

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Figure captions

Fig. 1. Dog and human CYP2C amino acid sequences. Human (h) and dog (d) sequences were aligned using ClustalW. Solid and broken lines above the sequences show the putative substrate recognition sites (SRSs) and the heme-binding region, respectively. Dots and asterisks under the sequences show conservatively changed and identical amino acids, respectively.

Fig. 2. Phylogenetic tree of dog CYP2Cs. The neighbor joining method was used to create the phylogenetic tree with CYP2C amino acid sequences from humans (h), cynomolgus macaques (mf), dogs (d), pigs (p), and rats (r). Chicken (ck) CYP2C45 was used as the outgroup. The scale bar represents 0.1 amino acid substitutions per site.

Fig. 3. Genomic organization of dog *CYP2C* genes. The human and dog genomes were analyzed by BLAT and Sequence Viewer. The sizes of the genes and the distances between them are not to scale. HELLS, lymphoid-specific helicase; PDLIM1, PDZ and LIM domain 1; ACSM6, acyl-CoA synthetase medium chain family member 6; SYCE1, synaptonemal complex central element protein 1; and ZNF717, zinc finger protein 717.

Fig. 4. Gene structures of dog *CYP2Cs*. The coding region of each CYP2C cDNA sequence was aligned with the dog (d) or human (h) genome using BLAT. Boxes and lines represent exons and introns, respectively.

Fig. 5. Expression of dog CYP2C mRNAs in various tissue types. Expression of dog CYP2C mRNAs was quantified by real-time RT-PCR in adrenal gland, brain, heart, ileum, jejunum, kidney, liver, lung, and testis. Each mRNA expression level was normalized to the 18S rRNA level. 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. Comparison of dog CYP2C mRNA levels in each tissue. Expressions of dog CYP2C mRNAs were compared in liver, jejunum, ileum, kidney, lung, heart, brain, adrenal gland, and testis

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(A), and in the liver of four dogs (B). Relative expression levels were determined by normalizing the raw data to the 18S rRNA level; the average ± SD values from three independent amplifications are shown. The most abundant expression level was arbitrarily set to 1, and all other expression levels were adjusted accordingly. N.D., not detectable.

Fig. 7. Oxidation activities of recombinant dog CYP2C proteins and liver microsomes. Paclitaxel 6α-hydroxylation, diclofenac 4′-hydroxylation, *S*- and *R*,*S*-warfarin 7-hydroxylation, and omeprazole 5-hydroxylation activities were determined for recombinant proteins (A, B) and for liver microsomes (C, D) using paclitaxel (open bars), diclofenac (light grey bars), *S*- warfarin (grey bars), *R*,*S*-warfarin (dark grey bars), and omeprazole (open pink bars) at substrate concentrations of 10 μM (A, C) and 100 μM (B, D) in triplicate reactions.

Fig. 8. Relationship of metabolic activities to CYP2C mRNA or protein levels in livers of four individual dogs. *S*-Warfarin and omeprazole hydroxylation activities at substrate concentrations of 10 μM (open circles) and 100 μM (grey circles) were significantly correlated ($p < 0.05$, n = 4) with the sum of CYP2C21, 2C41, and 2C94 mRNA levels (A), CYP2C21 mRNA levels alone (B), and the relative immunochemical intensity using anti-rat CYP2C13 antibodies (C and D). The amounts of dog CYP2C proteins were determined by immunochemical quantification by comparison with recombinant dog CYP2C21 using anti-human CYP2C9 antibodies (E) and anti-rat CYP2C13 antibodies (F). Lane 1, pooled male liver microsomes (20 μg), marked with an arrow; lanes 2–5, liver microsomes from individual dogs 3, 5, 6, and 7 (20 μg); and lane 6, dog CYP2C21 in bacterial membranes (0.40 pmol equivalent).

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Table 1

Primer sequences.

			Sequence (5′ → 3′)
RT-PCR			
CYP2C21	F	dCYP2C21 (5rt1)	GCAATGGATCTCTTCATAGTTCTGGTGATA
	R	dCYP2C21 (3rt1)	TTCAGACTGGAACAAAACAGAGCTTATAGGA
CYP2C41	F	dCYP2C41 (5rt1)	CATGGATCCAGTTGTGGTTCTGGTGCTT
	R	dCYP2C41 (3rt1)	CTCACACGGGAATGAAGCAGAGCTGGTA
CYP2C94	F	dCYP2C23L (5rt1)	GTGACATGGCTCTGCTCGGTCT
	R	dCYP2C23L (3rt1)	CTCTGGTTTGTCTTTTATTTCAATCTGGGTAA
Expression plasmid			
CYP2C21	F	dCYP2C21 (5exp1)	CGCATTAATGGCTCTGTTATTAGCAGTTTTTATATGTCTTTCTTG TTTGATTTCTTTCTT
	R	dCYP2C21 (3exp1)	GCTCTAGATTCAGACTGGAACAAAACAGAGCTT
CYP2C41	F	dCYP2C41 (5exp1)	GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTCTTTGTCTCT CCTGTTGCCTTCT
	R	dCYP2C41 (3exp1)	GCTCTAGACTCACACGGGAATGAAGCA
CYP2C94	F	dCYP2C23L (5exp1)	GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTTTCCTGGTGG CTTGTGTGG
	R	dCYP2C23L (3exp1)	GCTCTAGACTTTTATTTCAATCTGGGTAAGAAGCA
Quantitative PCR			
CYP2C21	F	dCYP2C21 (5qrt1)	TCCCTAAGCAAGCTAGCAGAGAA
	R	dCYP2C21 (3qrt1)	CGCTTCATACCCGTACAACAC
CYP2C41	F	dCYP2C41 (5qrt1)	CTTTTATTGGAAATATCCTACAGTTAGATAAGG
	R	dCYP2C41 (3qrt1)	GTCATACCCATGCAGTACAACAG
CYP2C94	F	dCYP2C23L (5qrt1)	TTGCAGCAGCTGATTATGTTG
	R	dCYP2C23L (3qrt1)	CCAGGAAGATAGTGAATGAATGATG

F, forward primer; R, reverse primer.

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Table 2

Amino acid sequence identities of CYP2Cs in dogs compared with humans.

		Human			
		CYP2C8	CYP2C9	CYP2C18	CYP2C19
		%			
Dog	CYP2C21	66	69	70	70
	CYP2C41	71	75	75	75
	CYP2C94	54	58	56	58

Dog CYP2C amino acid sequences were compared with human CYP2C sequences using BLAST.

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Fig. 1

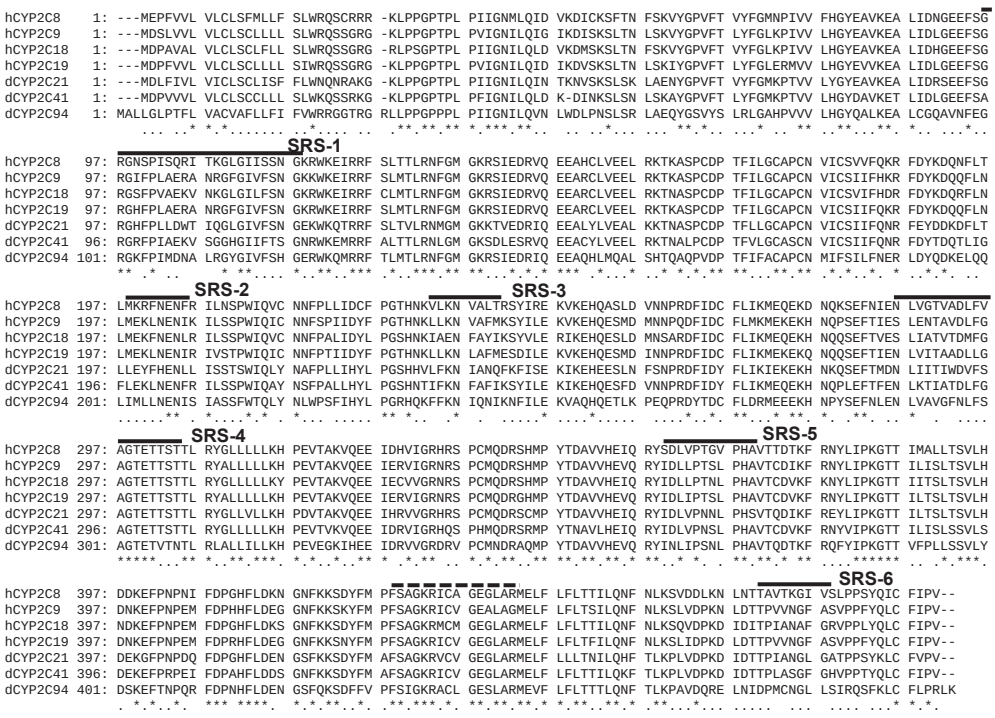

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Fig. 2

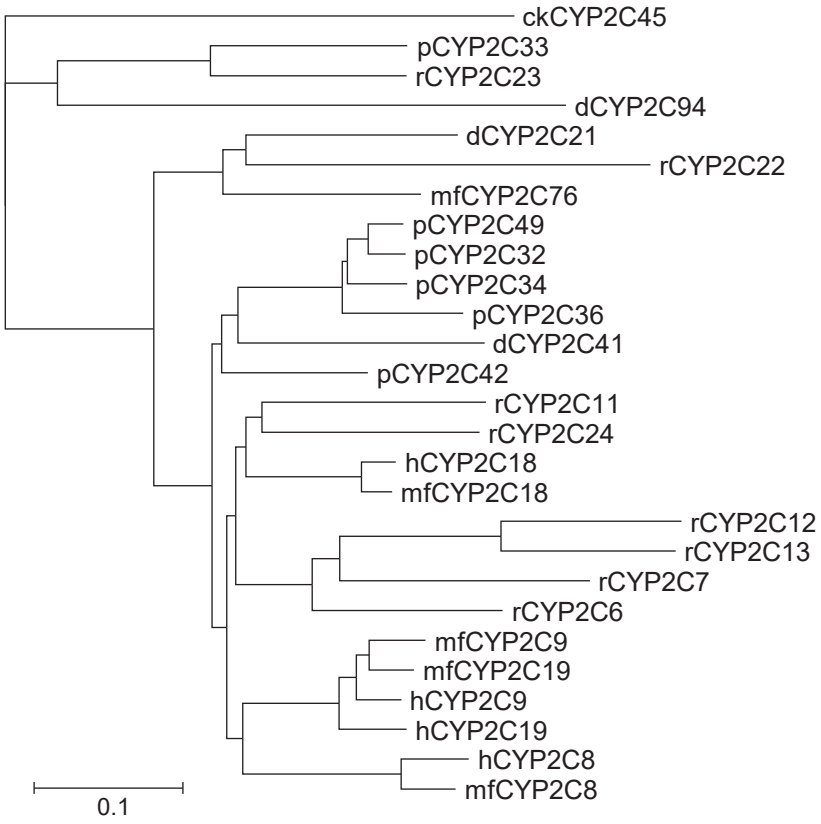

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Fig. 3

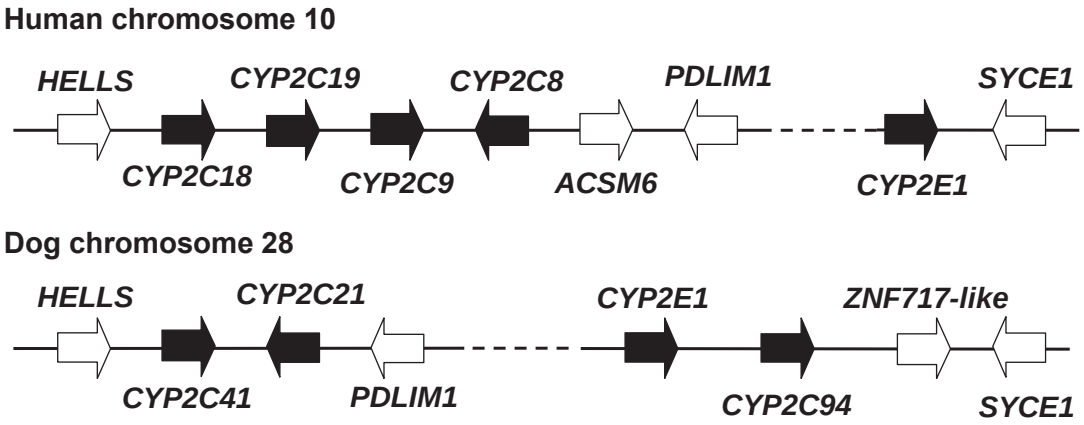

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Fig. 4

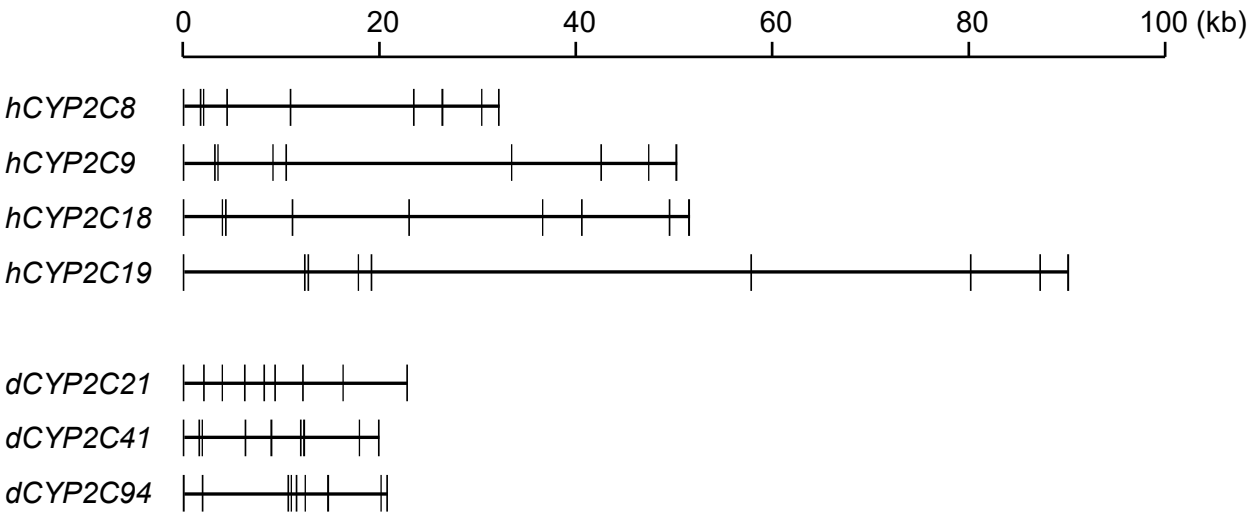

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Fig. 5

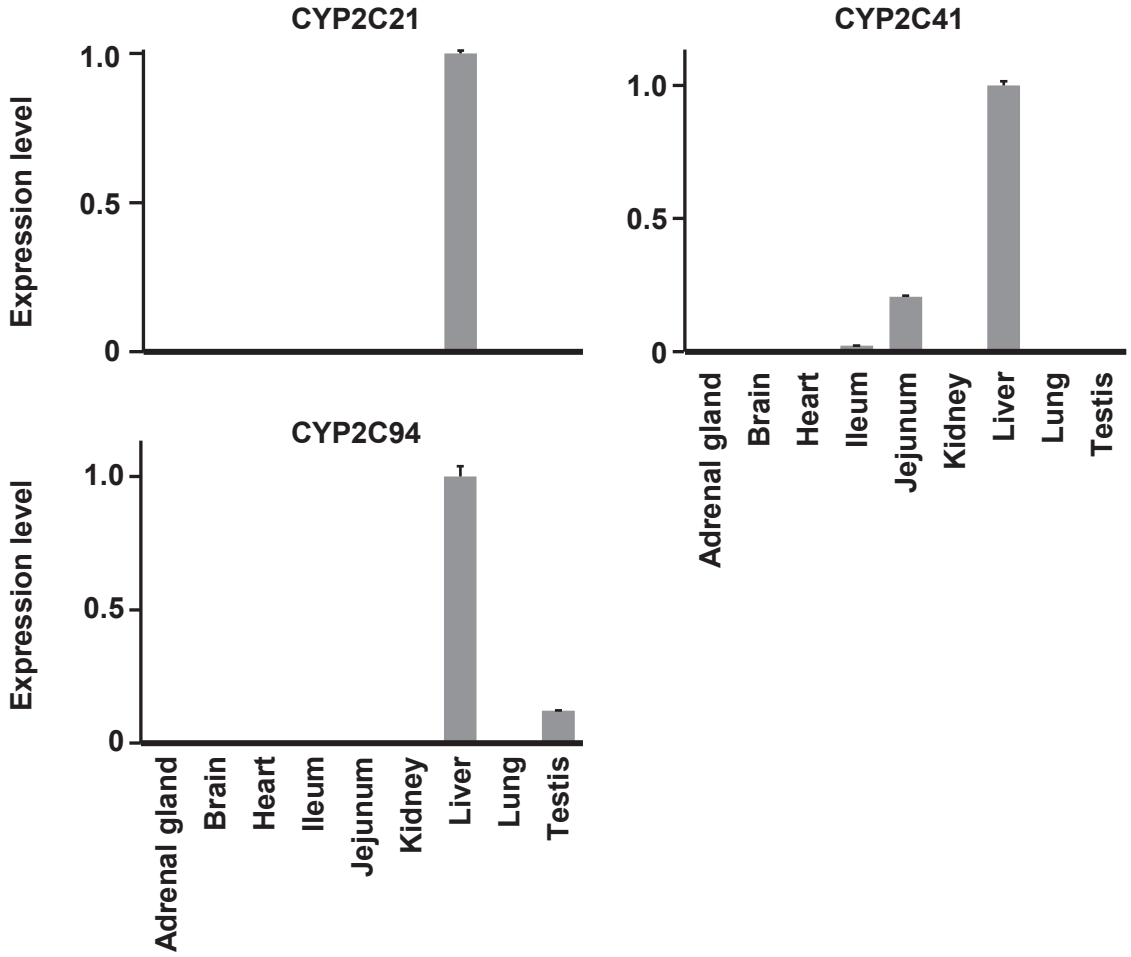

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Fig. 6

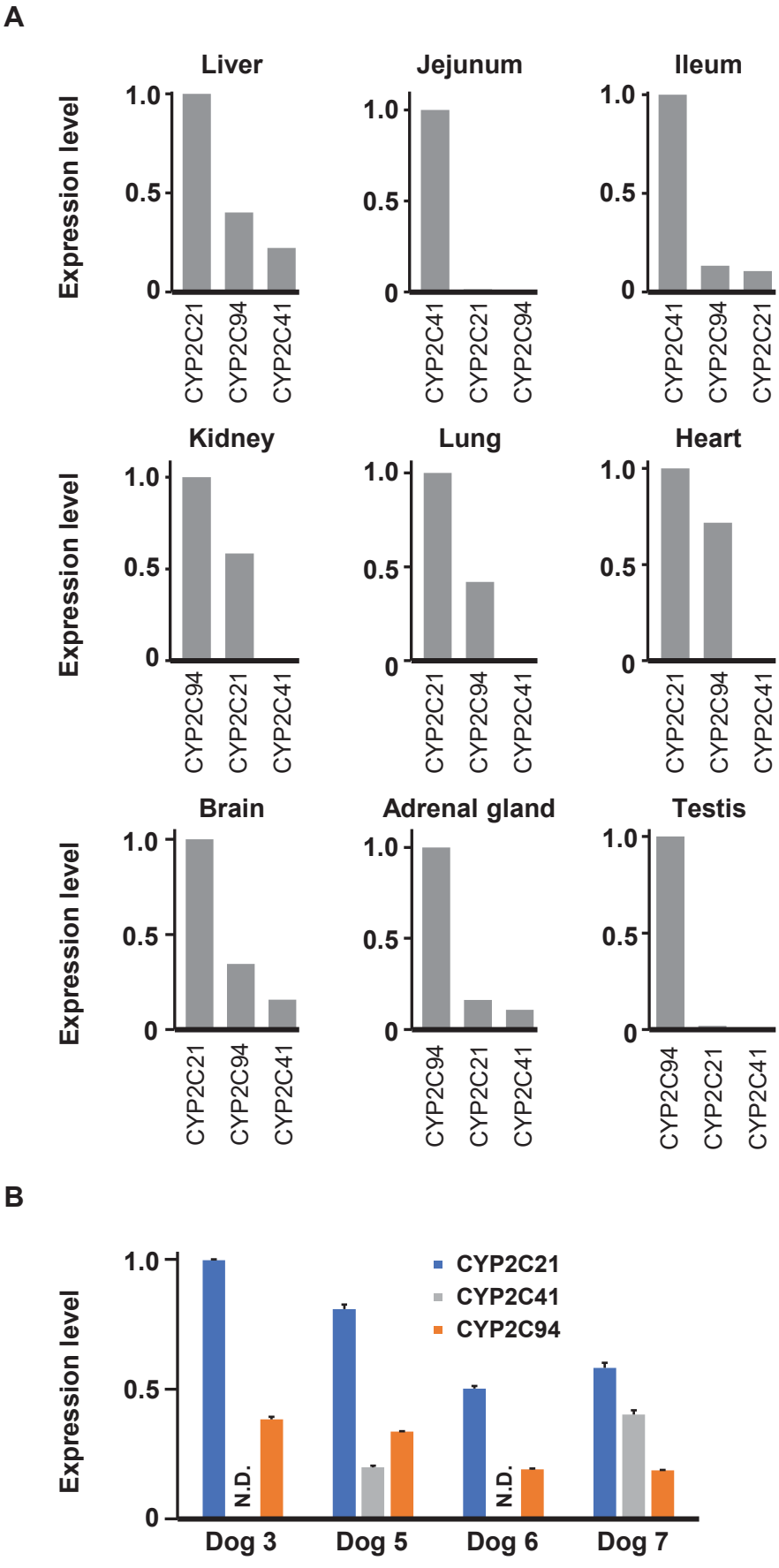

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Fig. 7

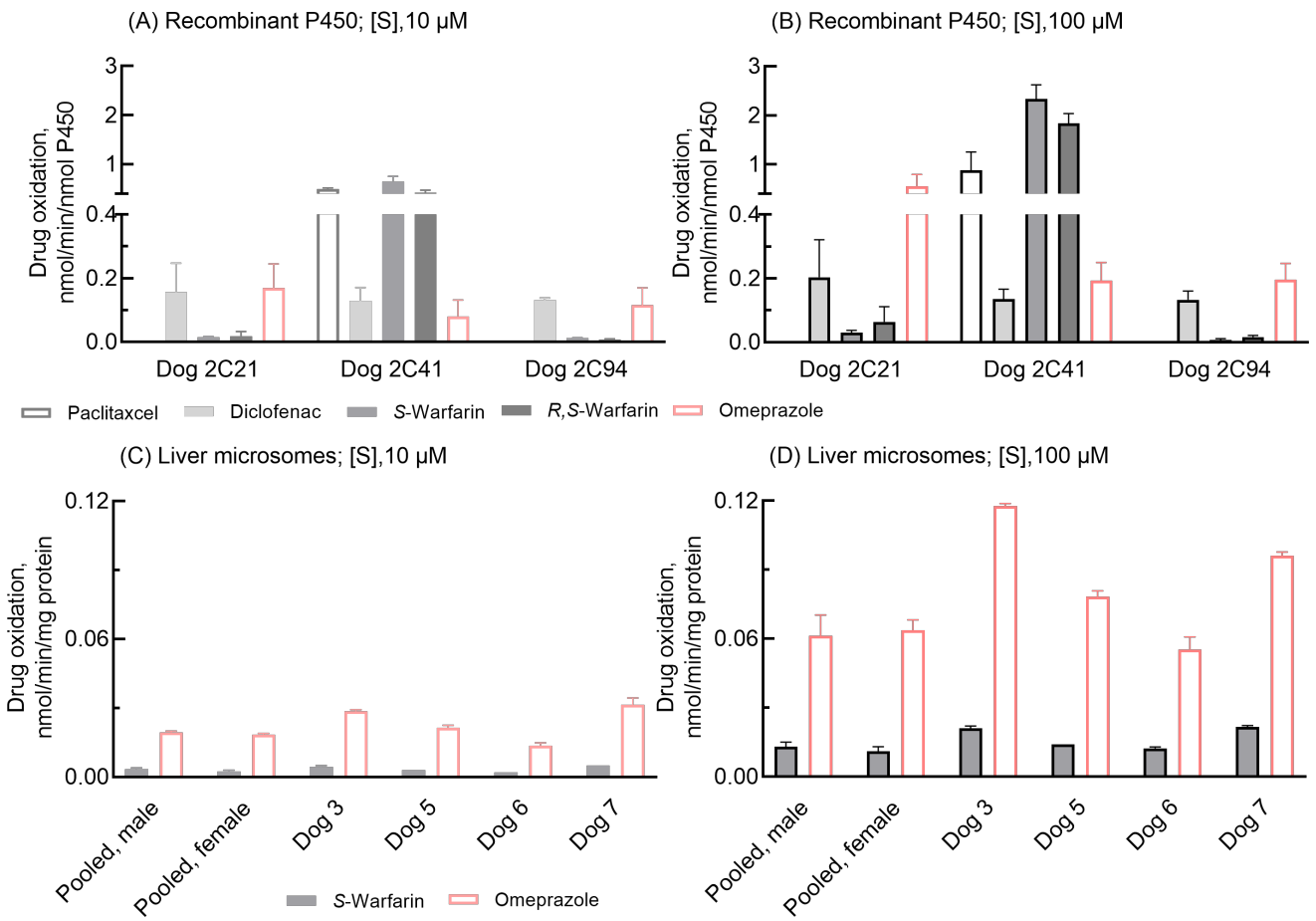

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Fig. 8