

Title Page

**Novel marmoset cytochrome P450 2C19 in livers efficiently metabolizes human P450
2C9 and 2C19 substrates, S-warfarin, tolbutamide, flurbiprofen, and omeprazole**

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Running title page

Running title: Characterization of new marmoset P450 2C enzymes

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Number of Text Pages : 29

Number of Tables : 4

Number of Figures : 5

Number of References : 41

Number of Words in Abstract : 253

Number of Words in Introduction : 449

Number of Words in Discussion : 1,296

Abbreviations: CYP, individual forms of cytochrome P450 (EC 1.14.14.1); HPLC, high performance liquid chromatography; P450, general term for cytochrome P450; PCR, polymerase chain reaction; RT, reverse transcription; UV, ultraviolet.

Abstract

The common marmoset (*Callithrix jacchus*), a small New World monkey, due to their evolutionary closeness, has the potential for use in human drug development. Four novel cDNAs, encoding cytochrome P450 (P450) 2C18, 2C19, 2C58, and 2C76 were cloned from marmoset livers to characterize P450 2C molecular properties including P450 2C8 previously reported. The deduced amino acid sequence showed high sequence identities (>86%) to those of human P450 2Cs, except for marmoset P450 2C76 that has low sequence identity (~70%) to any human P450 2Cs. Phylogenetic analysis showed that marmoset P450 2Cs were more closely clustered with those of humans and macaques than other species investigated. Quantitative polymerase chain reaction analysis showed that all the marmoset P450 2C mRNAs were predominantly expressed in liver than among the other tissues tested. Marmoset P450 2C proteins were detected in liver by immunoblotting using antibodies against human P450 2Cs. Among marmoset P450 2Cs heterologously expressed in *Escherichia coli*, marmoset P450 2C19 efficiently catalyzed human P450 2C substrates, *S*-warfarin, diclofenac, tolbutamide, flurbiprofen, and omeprazole. Marmoset P450 2C19 had high V_{\max} and low K_m values for *S*-warfarin 7-hydroxylation comparable to those in human liver microsomes, indicating similar warfarin stereoselectivity to findings in humans. Faster *in vivo* *S*-warfarin clearance than *R*-warfarin after intravenous administration of racemic warfarin (0.2 mg/kg) to marmosets was consistent with the *in vitro* kinetic parameters. These results indicated that marmoset P450 2C enzymes had functional characteristics similar to those of humans and also that P450 2C-dependent metabolic properties are likewise similar between marmosets and humans.

Introduction

The cytochrome P450 (P450 or CYP) superfamily comprises 57 functional genes and 58 pseudogenes in humans (Nelson et al., 2004) and encodes enzymes mainly involved in the oxidative metabolism of drugs, toxic chemicals, and endogenous compounds. The human P450 2C enzymes, including P450 2C8, 2C9, and 2C19, metabolize 24% of all pharmaceutical drugs, such as diazepam, ibuprofen, phenytoin, tolbutamide, warfarin, and omeprazole (Rendic and Guengerich, 2015).

Non-human primates are used for preclinical studies to predict the toxicity and pharmacokinetics of new drugs due to their genetic similarity to humans. The Old World monkeys such as cynomolgus monkeys (*Macaca fascicularis*) and rhesus monkeys (*Macaca mulatta*) are the most commonly used among non-human primates. The common marmoset (*Callithrix jacchus*), a New World monkey, is beneficial in various research fields such as neuroscience, stem cell research, drug metabolism, toxicology, and immune and autoimmune diseases (Kishi et al., 2014; Tokuno et al., 2015) because of their small body size, availability, high reproductive efficiency, and early sexual maturity (Sasaki, 2015).

In the marmoset P450 2C subfamily, P450 2C8 cDNA has been identified and characterized. Marmoset P450 2C8 shares high sequence identity (87%) with human P450 2C8 and catalyzes tolbutamide methyl hydroxylation (Narimatsu et al., 2006). In cynomolgus monkeys, five P450 2C cDNAs, namely P450 2C8, 2C9, 2C18, 2C19, and 2C76 have been identified (Uno et al., 2011a). Monkey P450 2C9 and 2C19 share similar metabolic properties with human P450 2C9 and 2C19 in the metabolism of tolbutamide, *S*-mephenytoin, flurbiprofen, and diclofenac (Uno et al., 2011b). P450 2C19 stereoselectively catalyzes *R*-warfarin 7-hydroxylation (Hosoi et al., 2012). Cynomolgus

monkey P450 2C76, not orthologous to human P450s, catalyzes tolbutamide methyl hydroxylation and is partly responsible for the differences in drug metabolism between macaques and humans (Uno et al., 2011a). P450 2C93 is a functional enzyme in rhesus monkeys, but not in cynomolgus monkeys (Uno et al., 2011b).

We previously reported genes expressions of marmoset *P450 1A, 2A, 2B, 2C, 2D, 2E, 2J, and 3A* in livers (Shimizu et al., 2014) and enzymatic characteristics of marmoset P450 3A, 2D, and 2A isoforms (Uehara et al., 2015a; Uehara et al., 2015b; Uehara et al., 2015c). The characterization of marmoset P450 enzymes is useful for understanding the metabolic properties of marmosets. In the present study, we report the identification of four novel P450 2C cDNAs isolated from marmoset livers. Four novel marmoset P450 2Cs and previously reported P450 2C8 (Narimatsu et al., 2006) were analyzed for their amino acid sequence identity, tissue distribution of mRNA expression, drug-metabolizing activity compared with human and/or cynomolgus monkey P450 2Cs.

Materials and Methods

Chemicals and enzymes

Ethoxyresorufin, resorufin, 7-hydroxycoumarin, 7-pentoxoresorufin, diclofenac sodium, 4'-hydroxydiclofenac, tolbutamide, flurbiprofen, *S/R*-warfarin, 7-hydroxywarfarin, chlorzoxazone, 6-hydroxychlorzoxazone, and 1'-hydroxymidazolam were purchased from Sigma-Aldrich (Tokyo, Japan). Coumarin, 7-hydroxycoumarin, paclitaxel, omeprazole, and midazolam were purchased from Wako Pure Chemicals (Osaka, Japan). The 6 α -Hydroxypaclitaxel, methylhydroxy tolbutamide, and bufuralol were purchased from Corning Life Sciences (Woburn, MA). The 4'-Hydroxyflurbiprofen, 5-hydroxyomeprazole, and 1'-hydroxybufuralol were purchased from Toronto Research Chemicals (Toronto, Canada). Oligonucleotides were synthesized by Greiner Japan (Tokyo, Japan). Pooled liver microsomes from marmosets, cynomolgus monkeys, and humans were purchased from Corning Life Sciences. Marmoset liver microsomes were also prepared from individual marmoset tissue samples as described previously (Uehara et al., 2014). The other chemicals were obtained in the highest grade commercially available.

Animals and tissue or plasma preparations

Four marmosets (males, >2 years old) were purchased from CLEA Japan (Tokyo, Japan). The animals were kept in cages (40×610×1578 mm) at 24–27°C, 40–60% relative air humidity with a 12/12-h light/dark cycle, and had free access to a balanced diet (CMS-1M; CLEA Japan) with added vitamins and water. This study was approved by the animal ethics committee and the gene recombination experiment safety management committee of the Central Institute for Experimental Animals and was carried out according to the Guidelines

for Proper Conduct of Animal Experiments by the Science Council of Japan (2006). Animal care was conducted in accordance with the recommendation of the Guide for the Care and Use of Laboratory Animals (National Research Council of the National Academies, 2011). Tissue samples including brains, lungs, livers, kidneys, and small intestines were collected from 16 marmosets (9 males and 7 females) after euthanasia by exsanguination under ketamine (60 mg/kg) and isoflurane deep anesthesia as described previously (Shimizu et al., 2014).

Stocked marmoset plasma samples (Uehara et al., 2015a) after intravenous administration of five-drug composites of caffeine, warfarin, omeprazole, metoprolol, and midazolam (cassette dosing) at doses of 0.20 mg kg⁻¹ each, were re-analyzed for *R/S*-warfarin using LC-MS/MS (Nexera UHPLC System, Shimadzu, Kyoto, Japan; TSQ Vantage, Thermo Fisher Scientific Inc., Waltham, MA, USA) as described previously (Utoh et al., 2015). Briefly, *R/S*-warfarin was determined separately using a Chiralcel OD-RH column (Daicel, Tokyo, Japan) with 6% (v/v) methanol and 54% (v/v) acetonitrile in 0.040% (v/v) formic acid for 7 min. The mass transitions (*m/z*) monitored were 307 → 250 for *R/S*-warfarin.

Sequence analysis

Methods for cDNA cloning are shown in Supplemental methods. The amino acid sequences of marmoset P450 2Cs were compared with known P450 2C amino acid sequences of various species from GenBank using BLAST (National Center for Biotechnology Information). Multiple alignment of amino acid sequences was performed using Genetyx (Software Development, Tokyo, Japan). The analysis of the marmoset, rhesus monkey, and human genome data was performed using BLAT. The phylogenetic tree was constructed by the neighbor-joining method using DNASIS Pro (Hitachi Software, Tokyo, Japan). The P450 2C amino acid sequences from GenBank were used, including marmoset P450 2C8

(NP_001191366); human P450 2A6 (NP_000753), 2C8 (NP_000755), 2C9 (NP_000762), 2C18 (NP_000763), and 2C19 (NP_000760); cynomolgus monkey P450 2C8 (NP_001270692), 2C9 (NP_001274554), 2C18 (ABB87194), 2C19 (NP_001270219), and 2C76 (NP_001271718); rhesus monkey P450 2C8 (NP_001035300), 2C9 (NP_001035329), 2C18 (NP_001180995), 2C19 (NP_001035301), 2C76 (NP_001171259), and 2C93 (NP_001232885); dog P450 2C21 (NP_001183973) and 2C41 (NP_001003334); rabbit P450 2C1 (P00180), 2C2 (NP_001164584), 2C3 (NP_001164736), 2C4 (NP_001177360), 2C5 (NP_001164397), 2C14 (NP_001164591), 2C16 (NP_001164593), 2C30 (NP_001164737); pig P450 2C33 (NP_999579), 2C42 (NP_001161307), and 2C49 (NP_999585); rat P450 2C6 (NP_001013926), 2C7 (NP_058854), 2C11 (NP_062057), 2C12 (NP_113760), 2C13 (NP_612523), 2C22 (NP_612521), 2C23 (NP_114027), and 2C24 (NP_001258283); mouse P450 2C29 (NP_031841), 2C37 (NP_034131), 2C38 (NP_034132), 2C39 (NP_034133), 2C40 (NP_034134), 2C44 (NP_001001446), 2C50 (NP_598905), 2C54 (NP_996260), 2C55 (NP_082365), 2C65 (NP_082467), 2C66 (NP_001011707), and 2C70 (NP_663474). The marmoset P450 2C18, 2C19, 2C58, and 2C76 amino acid sequences were deduced from the cDNAs identified in this study.

Quantitative real-time RT-PCR

The mRNA expressions of marmoset *P450 2C* genes in brain, lung, liver, kidney, and small intestine were measured by quantitative real-time RT-PCR. RNA isolation and cDNA synthesis were carried out as described earlier. Briefly, total RNA samples were pooled in equal quantities from six marmosets for each tissue. The RT samples were prepared in a total volume of 20 μ L containing 1 μ g of pooled total RNA, SuperScript III RT reverse transcriptase, and random primers (Invitrogen) according to the manufacturer's instructions. PCR amplification was performed in triplicate with the total volume of 20 μ L using Fast

SYBR Green Master Mix (Applied Biosystems) on an ABI PRISM 7500 sequence detection system (Applied Biosystems). The PCR primers were used at the final concentrations of 200 nM, including cjCYP2C8 (5qrt1) 5'-CAGTAAAGGAAGCCCTGATTGATA-3' and cjCYP2C8 (3qrt1) 5'-ATTGCTGGAAATGATTCCAAGAT-3' for P450 2C8, cjCYP2C19L (5qrt1) 5'-CAGAGATACATTGACCTCATTCTACTAAC-3' and cjCYP2C19L (3qrt1) 5'-AGGCCCTCTCCTACACACATC-3' for P450 2C18, cjCYP2C26L (5qrt1) 5'-ACCAAGAATCGGTAGACATTAACAAC-3' and cjCYP2C26L (3qrt1) 5'-TCCAAACAAGTCTAATGCAGTGTTTAC-3' for P450 2C19, cjCYP2C20L (5qrt1) 5'-GGCAACTTTAAGAAAAGTGACCAT-3' and cjCYP2C20L (3qrt1) 5'-GGGTGGCAAGGAAAAAATCC-3' for P450 2C58, and cjCYP2C21L (5qrt1) 5'-AAAGGGCACAACAATATTAGCAGAT-3' and cjCYP2C21L (3qrt1) 5'-GAACTGGGGTGGTGTCAATATC-3' for P450 2C76. PCR cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. A calibration curve was generated using each target marmoset P450 2C cDNA and the amplification efficiency was confirmed. Marmoset P450 2C mRNA expression levels were normalized with 18S rRNA level.

Expression and preparation of recombinant P450 2Cs in *Escherichia coli*

Marmoset P450 2Cs were expressed by a heterologous expression system in *Escherichia coli* using the methods described previously (Uehara et al., 2010). Briefly, to enhance protein expression, marmoset P450 2C cDNAs were modified at the *N*-terminus by PCR using forward and reverse primers containing the restriction sites of the NdeI and XbaI sites (underlined), respectively. PCR primers were cjCYP2C8/20L (5exp1bov) 5'-GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTCTCTGTCTCTCTTTTTTGCTTCTCTTTTCAC-3' and cjCYP2C8 (3exp1) 5'-

GCTCTAGATCAGACAGGAATGAAGCAGATCTGGTA-3' for P450 2C8/2C58,
cjCYP2C19L (5exp1bov) 5'-

GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTTTCTGTCTCTCCTGTTTGCTTCTC
CTT-3' and cjCYP2C19L (3exp1) 5'-

GCTCTAGATCAGACTGGAATGAAGCAGAGCTGGTA-3' for P450 2C18, cjCYP2C26L
(5exp1bov) 5'-

GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTTCTCTCTCTCCTGTTTGCTTCTC
CTT-3' and cjCYP2C26L (3exp1) 5'-

GCTCTAGATCTTCAGATAGGAATGAAGCACAACTGAT-3' for P450 2C19, and
cjCYP2C21L (5exp1bov) 5'-

GGAATTCCATATGGCTCTGTTATTAGCAGTTTTTACTTGTCTTTCTTGTCTGACTCTT
CTTTTTCTG-3' and cjCYP2C21L (3exp1) 5'-

GCTCTAGATCATCAGACTGGAATAAAACAAAGCTCATAG-3' for P450 2C76. PCR products were purified, double-digested, and ligated with the pCW vector, which contained the human nicotinamide adenine dinucleotide phosphate-P450 (NADPH-P450) reductase cDNA, and sequences of the inserts were confirmed by sequencing. Plasmids for expression of human P450 2C8, 2C9, and 2C19, and cynomolgus monkey P450 2C19 were constructed as described previously (Yamazaki et al., 2002; Uno et al., 2006). The membrane fractions were prepared from *E. coli* DH5 α expressing P450 2C proteins: the concentrations of P450 protein and NADPH-P450 reductase were measured as described previously (Yamazaki et al., 2002). The detection of P450 2C proteins in recombinant and marmoset liver microsomes was performed by immunoblotting as shown in Supplemental methods.

Activity characterization and kinetic analysis

Ethoxyresorufin *O*-deethylation, 7-ethoxycoumarin *O*-deethylation, coumarin

7-hydroxylation, 7-pentoxoresorufin *O*-deethylation, paclitaxel 6 α -hydroxylation, diclofenac 4'-hydroxylation, tolbutamide methylhydroxylation, flurbiprofen 4-hydroxylation, *S/R*-warfarin 7-hydroxylation, omeprazole 5-hydroxylation, chlorzoxazone 6-hydroxylation, bufuralol 1'-hydroxylation, and midazolam 1'-hydroxylation by recombinant P450 2C proteins and liver microsomes from marmosets, cynomolgus monkeys, and humans were measured as described previously (Yamazaki et al., 2002; Uno et al., 2011b) with some minor changes. Briefly, each mixture containing recombinant protein (20 pmol/mL) or liver microsomes (0.40 mg/ml), an NADPH-generating system (0.25 mM NADP⁺, 2.5 mM glucose 6-phosphate, and 0.25 units/ml glucose 6-phosphate dehydrogenase), and substrate (10 μ M ethoxyresorufin, 100 μ M 7-ethoxycoumarin, 100 μ M coumarin, 10 μ M 7-pentoxoresorufin, 100 μ M paclitaxel, 100 μ M diclofenac, 2500 μ M tolbutamide, 100 μ M flurbiprofen, 100 μ M *S* and *R*-warfarin, 100 μ M omeprazole, 500 μ M chlorzoxazone, 100 μ M bufuralol, or 100 μ M midazolam) in 100 mM potassium phosphate buffer (pH 7.4) was incubated at 37°C for the optimal time (10 min for ethoxyresorufin 7-ethoxycoumarin, coumarin, 7-pentoxoresorufin and midazolam; 15 min for paclitaxel, tolbutamide, diclofenac, flurbiprofen, *S* and *R*-warfarin, omeprazole, chlorzoxazone, and bufuralol). For metabolites from 7-ethoxycoumarin, coumarin, flurbiprofen, *S*- and *R*-warfarin, bufuralol, and midazolam, reactions were stopped by addition of 25 μ l of 60% HClO₄ (w/v) or 0.4 ml of methanol. Reaction mixtures were centrifuged at 10,000g for 5 minutes, and metabolites of supernatants were analyzed directly by HPLC with fluorescence or a UV detector. For metabolites of other substrates, reactions were stopped with ethyl acetate and centrifuged at 2,500g for 5 minutes. The organic phases were evaporated and the residues were dissolved in the HPLC mobile phase, and then subjects were analyzed by HPLC with a UV detector. For kinetic analysis, reactions were performed over a range of concentrations for each substrate (5–400 μ M paclitaxel, 5–2500 μ M tolbutamide, 1–800 μ M *S/R*-warfarin, 0.5–300 μ M flurbiprofen,

and 1–1000 μM omeprazole). Data were fitted to an integrated form of the Michaelis-Menten equation using Kaleidagraph (Synergy Software, Reading, PA).

Results

Identification of novel marmoset *P450 2C* genes

To identify marmoset *P450 2C* cDNAs except for *P450 2C8* that was reported previously (Narimatsu et al., 2006), a search for the sequences highly homologous to human *P450 2C* cDNAs were performed on the marmoset genome data using BLAT. To clone the four novel marmoset *P450 2C* cDNAs, RT-PCR was performed with total marmoset liver RNA to amplify the region containing the open reading frame of marmoset *P450 2C* cDNA. Marmoset *P450 2C18*, *2C19*, *2C58*, and *2C76* cDNAs, encoding the proteins of 490 amino acids for *P450 2C18*, *2C19*, and *2C58*, or 489 amino acids for *P450 2C76*, were obtained (Fig. 1). The deduced amino acids of all marmoset *P450 2C* cDNAs contained the six potential substrate recognition sites (SRSs) and heme-binding region. Marmoset *P450 2C18*, *2C19*, and *2C58* showed high sequence identities to human *P450 2C18* (93%), *2C9/2C19* (86%), and *2C8* (87%) at the amino acid level (Table 1). Marmoset *P450 2C76* showed high sequence identity (87%) with cynomolgus monkey *P450 2C76*, but low sequence identity with the other human *P450 2Cs* (68-70%).

Phylogenetic analysis of *P450 2C* amino acid sequences in marmosets and other species indicated that marmoset *P450 2C18*, *2C19*, and *2C58* were closely clustered with human *P450 2Cs*, together with cynomolgus monkey and rhesus monkey *P450 2Cs*, unlike dog, pig, rabbit, rat, and mouse *P450 2Cs* (Fig. 2), while marmoset *P450 2C76* was closely clustered with cynomolgus monkey and rhesus monkey *P450 2C76*.

The genomic location of the *P450 2C* genes in the marmoset genome was determined by analyzing the marmoset genome data using BLAT, together with the macaque (rhesus monkey) and human genomes. The analysis showed that *P450 2C8*, *2C18*, *2C19*, *2C58*, and

2C76 formed the *P450 2C* cluster in marmoset chromosome 12, indicating that the *P450 2C* cluster was conserved among marmosets, macaques, and humans with some differences (Fig. 3). In the macaque genome, seven genes, *P450 2C8*, *2C9*, *2C18*, *2C19*, *2C58P*, *2C76*, and *2C93*, were found, whereas five genes, *P450 2C8*, *2C9*, *2C18*, *2C19*, and *2C58P* were located in the human genome. *P450 2C58* was the pseudogene in human and macaque, but not in marmoset. In the marmoset and macaque genomes, *P450 2C76* was found in the location corresponding to the intergenic region in the human genome.

Tissues expression pattern of marmoset P450 2Cs

To measure the expression level of P450 2C mRNAs in marmoset tissues, real-time RT-PCR was performed with gene-specific primers using total RNA of brains, kidneys, livers, lungs, and small intestines. Among the five tissues examined, all four marmoset P450 2C mRNAs were expressed predominantly in the liver where P450 2C8 mRNA was most abundant, followed by P450 2C19, 2C58, 2C18, and 2C76 mRNA (Fig. 4).

Expression of marmoset P450 2C proteins in livers was confirmed by immunoblotting using anti-human or anti-cynomolgus monkey P450 2C antibodies. The cross-reactivity of each P450 2C antibodies was investigated using recombinant proteins, including marmoset P450 2C8, 2C18, 2C19, 2C58, and 2C76 (Supplemental Figure 1A). Recombinant marmoset P450 2C8 and 2C58, 2C8, 2C18, and 2C19, and 2C76 were detected by anti-human P450 2C8, anti-human P450 2C9, and anti-cynomolgus monkey P450 2C76 antibodies, respectively. Immunoblot analysis using five individual marmoset liver microsomes detected bands of approximately 56 kDa (Fig. Supplemental Figure 1B), indicating that marmoset P450 2C proteins were expressed in marmoset liver with a large individual variation.

Kinetic analysis for drug oxidations mediated by marmoset P450 2Cs

To investigate catalytic function of marmoset P450 2C enzymes, ethoxyresorufin *O*-deethylation, 7-ethoxycoumarin *O*-deethylation, coumarin 7-hydroxylation, 7-pentoxoresorufin *O*-deethylation, paclitaxel 6 α -hydroxylation, diclofenac 4'-hydroxylation, tolbutamide methyl hydroxylation, flurbiprofen 4-hydroxylation, *S/R*-warfarin 7-hydroxylation, omeprazole 5-hydroxylation, chlorzoxazone 6-hydroxylation, bufuralol 1'-hydroxylation, and midazolam 1'-hydroxylation were measured using recombinant marmoset P450 2C enzymes (Table 2). Marmoset P450 2C enzymes substantially metabolized human P450 2C probe substrates, paclitaxel (P450 2C8), diclofenac (P450 2C18, 2C19, and 2C76), tolbutamide (P450 2C8, 2C18, 2C19, 2C58, and 2C76), flurbiprofen (P450 2C8, 2C19, and 2C58), *S*- and *R*-warfarin (P450 2C18 and 2C19), and omeprazole (P450 2C19). Marmoset P450 2C enzymes metabolized other non-P450 2C substrates of human, ethoxyresorufin, 7-ethoxycoumarin, and 7-pentoxoresorufin (marmoset P450 2C8, 2C18, 2C19, 2C58 and 2C76), coumarin and chlorzoxazone (P450 2C8 and 2C58), and bufuralol (P450 2C8, 2C18, 2C19, and 2C58).

Kinetic analysis for paclitaxel 6 α -hydroxylation, tolbutamide methyl hydroxylation, flurbiprofen 4-hydroxylation, and omeprazole 5-hydroxylation were performed with recombinant P450 2C enzymes and liver microsomes from marmosets and humans (Table 3). Marmoset P450 2C8 indicated low affinity (K_m , 42 μ M) and capacity (V_{max} , 1.6 min^{-1}) for paclitaxel 6 α -hydroxylation, showing a lower V_{max}/K_m value (0.038 $\mu\text{M}^{-1} \text{min}^{-1}$) than that of human P450 2C8 (7.2 $\mu\text{M}^{-1} \text{min}^{-1}$). Marmoset liver microsomes showed an apparent K_m value of 44 μ M toward paclitaxel 6 α -hydroxylation, similar to marmoset P450 2C8. For tolbutamide methyl hydroxylation, marmoset P450 2C19 showed an apparent K_m (217 μ M) lower than that of marmoset P450 2C8 (2210 μ M) and 2C58 (1320 μ M), and human P450

2C9 (284 μM), resulting in a $V_{\text{max}}/K_{\text{m}}$ value ($0.092 \mu\text{M}^{-1} \text{min}^{-1}$) lower than that of human P450 2C9 ($0.19 \mu\text{M}^{-1} \text{min}^{-1}$). Marmoset liver microsomes showed an apparent K_{m} value of 373 μM toward tolbutamide methyl hydroxylation, similar to marmoset P450 2C19. For flurbiprofen 4-hydroxylation, marmoset P450 2C8, 2C19 and 2C58 showed apparent K_{m} values of 155, 161, and 85 μM , and $V_{\text{max}}/K_{\text{m}}$ values of 0.0090, 0.075, and $0.0094 \mu\text{M}^{-1} \text{min}^{-1}$, respectively, indicating low affinity and $V_{\text{max}}/K_{\text{m}}$ values of marmoset P450 2C enzymes for flurbiprofen 4-hydroxylation, compared with human P450 2C9 (K_{m} , 15 μM ; $V_{\text{max}}/K_{\text{m}}$, $1.8 \mu\text{M}^{-1} \text{min}^{-1}$). Marmoset liver microsomes also showed an apparent K_{m} value of 34 μM and a $V_{\text{max}}/K_{\text{m}}$ value of $0.0021 \mu\text{M}^{-1} \text{min}^{-1}$, compared with those of human liver microsomes. For omeprazole 5-hydroxylation, marmoset P450 2C19 showed an apparent K_{m} value of 23 μM and $V_{\text{max}}/K_{\text{m}}$ value of $2.3 \mu\text{M}^{-1} \text{min}^{-1}$, similar to human P450 2C9. Marmoset liver microsomes showed a lower K_{m} value of 30 μM toward omeprazole 5-hydroxylation, similar to marmoset P450 2C19.

S/R-warfarin oxidation was further analyzed both *in vitro* (Table 4) and *in vivo* (Fig. 5) in marmosets. Recombinant marmoset P450 2C19 had higher affinity (K_{m} , 14 μM) and $V_{\text{max}}/K_{\text{m}}$ of $14 \text{mM}^{-1} \text{min}^{-1}$ for *S*-warfarin 7-hydroxylation than those for *R*-warfarin 7-hydroxylation (K_{m} , 755 μM ; $V_{\text{max}}/K_{\text{m}}$, $0.26 \text{mM}^{-1} \text{min}^{-1}$), but comparable to those for marmoset liver microsomes (Table 4). Similarly, human P450 2C9 had low K_{m} value of 3.1 μM and high $V_{\text{max}}/K_{\text{m}}$ value of $55 \text{mM}^{-1} \text{min}^{-1}$ for *S*-warfarin 7-hydroxylation compared with those for *R*-warfarin 7-hydroxylation (K_{m} , 123 μM ; $V_{\text{max}}/K_{\text{m}}$, $0.057 \text{mM}^{-1} \text{min}^{-1}$). By contrast, cynomolgus monkey P450 2C19 had a high K_{m} value of 124 μM and a low $V_{\text{max}}/K_{\text{m}}$ value of $10 \text{mM}^{-1} \text{min}^{-1}$ for *S*-warfarin 7-hydroxylation compared with those for *R*-warfarin 7-hydroxylation (K_{m} , 51 μM ; $V_{\text{max}}/K_{\text{m}}$, $137 \text{mM}^{-1} \text{min}^{-1}$). To investigate the pharmacokinetics of *S*- and *R*- warfarin in marmosets, plasma concentrations of *S*- and *R*-warfarin after a single intravenous administration at doses of 0.20 mg racemic warfarin kg^{-1}

each to four male marmosets were measured using LC-MS/MS. Plasma concentrations of *S*-warfarin decreased significantly faster than *R*-warfarin in a monophasic manner in marmosets (Fig. 5). Calculated mean clearance values (and ranges) in four marmosets for *S*- and *R*- warfarin were 22.2 (13.0-29.8) mL/h/kg and 15.3 (12.8-17.0) mL/h/kg, respectively.

Discussion

Human P450 2C enzymes in liver account for the metabolism of approximately 20% of the drugs on the market, and thus P450 2C enzymes are important in drug metabolism. Despite the potential importance of marmosets in drug development, marmoset P450 2C enzymes have not been fully identified and characterized. We previously reported that a number of *P450 2C* genes were expressed in marmoset livers (Shimizu et al., 2014). In the present study, four novel P450 2C cDNAs were cloned from marmoset livers (Figs. 1 and 3) based on the marmoset genome data. Marmoset P450 2C18, 2C19, and 2C58 had high sequence identities (>86%) at amino acid level with human P450 2Cs (Table 1) while marmoset P450 2C76 had a high sequence identity (87%) with cynomolgus monkey P450 2C76. In phylogenetic analysis, marmoset P450 2C18, 2C19, and 2C58 were closely clustered with human, cynomolgus, and rhesus monkey P450 2Cs, while marmoset P450 2C76 was clustered with cynomolgus and rhesus monkey P450 2C76 (Fig. 2). All marmoset P450 2C mRNAs were abundant in livers (Fig. 4) where P450 2C proteins were also expressed (Supplemental Figure 1). Marmoset P450 2C enzymes metabolized human P450 2C9 and 2C19 probe substrates, but also non-P450 2C substrates (Table 2). Marmoset P450 2C19 catalyzed many drug oxidations, including *S*-warfarin 7-hydroxylation, diclofenac 4'-hydroxylation, tolbutamide methyl hydroxylation, flurbiprofen 4-hydroxylation, and omeprazole 5-hydroxylation (Tables 3 and 4) and substantially catalyzed bufuralol 1'-hydroxylation, which is also catalyzed by marmoset P450 2D6 and 2D7 (Uehara et al., 2015b). Similarly, human P450 2C19 catalyzes bufuralol 1'-hydroxylation with low efficiency compared with human P450 2D6 (Mankowski, 1999). Marmoset P450 2C19 might be partly responsible for bufuralol 1'-hydroxylation in livers (Locuson et al., 2009). Marmoset P450 2C8, 2C18, 2C19, 2C58, and 2C76 also catalyzes 7-pentoxeresorufin *O*-deethylation. Similarly, cynomolgus monkey P450 2C9 and 2C19 metabolize many human

P450 2C substrates, but also show some limited differences in drug oxidations of human P450 2C substrates (Hosaka et al., 2015b). Interestingly, marmoset P450 2C58 had the highest catalytic activities among five marmoset P450s tested toward non-P450 2C substrates, such as ethoxyresorufin, 7-ethoxycoumarin, coumarin, 7-pentoxoresorufin, and chlorzoxazone, while cynomolgus monkey P450 2C58 and human P450 2C58 were both pseudogenes. These findings might account for some limited species differences of drug metabolizing enzymes between marmosets and cynomolgus monkeys or humans. Although monkey specific P450 2C76 had a high impact on drug oxidations (Hosaka et al., 2015a), marmoset P450 2C76 showed few unique characteristics of human P450 2C substrates under the present conditions. These findings suggested that the substrate selectivity of marmoset P450 2C19 may reflect the functional similarity of P450 2C enzyme between marmosets and humans.

Marmoset liver microsomes had efficiently catalyzed 7-hydroxylation for *S*-warfarin, but not for *R*-warfarin (Table 4), similar to those of humans but not those of cynomolgus monkeys. We reported previously that *S*-warfarin 7-hydroxylation in human liver microsomes was mainly catalyzed by P450 2C9, although cynomolgus monkey P450 2C19 preferentially catalyzed 7-hydroxylation of *R*-warfarin (Hosoi et al., 2012). Among all marmoset P450 2C enzymes, marmoset P450 2C19 efficiently catalyzed 7-hydroxylation for *S*-warfarin, but not *R*-warfarin, similar to human P450 2C9 (Table 4). Additionally, plasma concentrations of *S*-warfarin after intravenous administration of warfarin decreased faster than *R*-warfarin (Fig. 5), suggesting that *S*-warfarin was stereoselectively metabolized in marmoset livers, comparable to human livers. However, the metabolic clearances of *S*-warfarin showed large interindividual variations in comparison with those of *R*-warfarin, suggesting the possible genetic polymorphism of marmoset P450 2C19 involved in drug oxidations in marmosets. The similarity of stereoselectivity for warfarin 7-hydroxylation

between marmosets and humans indicated that the drug-metabolizing properties of marmoset P450 2C19 were similar to humans under the present conditions.

Marmoset P450 2C19 showed 86% amino acid sequence identity with both human P450 2C9 and 2C19 (Table 1). Marmoset P450 2C19 efficiently metabolized the human P450 2C9 and 2C19 probe substrates, tolbutamide, flurbiprofen, *S*-warfarin, and omeprazole (Tables 3-5). Human P450 2C9 and 2C19 amino acid sequences showed 91% sequence identities, however, metabolic activities and/or substrate selectivities are generally different. Some critical key residues for substrate specificity have been identified by studies with chimeric constructs and amino acid substitutions between human P450 2C19 and 2C9. Human P450 2C19 His99, Pro220, and Thr221 have been reportedly key residues for omeprazole 5-hydroxylation (Ibeanu et al., 1996). Moreover, human P450 2C19 triple mutant (Glu241Lys/Asn286Ser/Ile289Asn) has reportedly shown high catalytic efficiency for warfarin metabolism both *S*- and *R*-warfarin with a broadened regioselectivity (Jung et al., 1998). In the case of marmoset P450 2C19, Pro220, Lys241, and Asn289 might partly account for the impaired catalytic potential of P450 2C19 with both omeprazole 5-hydroxylation (human P450 2C19 selective activity) and *S*-warfarin 7-hydroxylation (human P450 2C9 selective activity). In this context, further study would be interesting.

Human P450 2C8, 2C9, and 2C19 proteins are predominantly expressed in human livers (Klose et al., 1999; Glaeser et al., 2005). In contrast, human P450 2C18 is expressed at the mRNA level, but its expression has not been detected at the protein level in any tissues (Klose et al., 1999). Regarding gene expression modulations, hepatic expression of human *P450 2C* genes is constitutively regulated by a number of liver transcription factors (Jover et al., 1998; Bort et al., 2004; Ferguson et al., 2005; Kamiyama et al., 2007; Wortham et al., 2007; Surapureddi et al., 2008). Human *P450 2C8* gene has been post-transcriptionally

regulated by microRNAs (Zhang et al., 2012). It is of great interest to investigate the transcriptional regulation of marmoset P450 2C19, which is important for drug metabolism as a functional counterpart of both human P450 2C9 and 2C19.

In terms of marmoset P450 2C expression level, all marmoset P450 2C mRNAs were predominantly expressed in livers (Fig. 3). Two marmoset P450 2C-like proteins have been detected in livers (Igarashi et al., 1997; Schulz et al., 2001) and induced by phenobarbital and rifampicin treatments (Schulz et al., 2001): tolbutamide methyl hydroxylation activities in liver microsomes have been greatly increased by phenobarbital (4.3-fold) and rifampicin (6.1-fold) (Schulz et al., 2001). Similarly, all three human *P450 2C* genes have been induced in the liver by phenobarbital, rifampicin, and dexamethasone (Raucy et al., 2002; Madan et al., 2003), which have been mediated through the xenobiotic-induced transcriptional activation by the nuclear receptors (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Ferguson et al., 2005). In the present study, marmoset P450 2C8 and 2C19 were major tolbutamide methyl hydroxylase in marmoset livers. Detailed analysis is needed to elucidate that marmoset liver P450 2C8 and/or 2C19 may be induced by phenobarbital and rifampicin via these nuclear receptors in the future. The similarity of tissue expression patterns possibly by common transcriptional regulation and induction properties in *P450 2C* genes might suggest that marmoset are potentially a suitable model for preclinical safety testing for humans.

Human *P450* genes are highly polymorphic (<http://www.cypalleles.ki.se/>) and these genetic variants would cause inter-individual differences in pharmacokinetics of drugs. Whole-genome sequencing has shown the genetic divergence of marmosets among multiple colonies (The Marmoset Genome Sequencing and Analysis Consortium, 2014). Indeed, in our preliminary study, genetic variants of marmoset *P450 2C19* were found with 20

marmoset genomes by direct sequencing (data not shown). Genetic polymorphisms of marmoset *P450 2C* genes might account for the inter-animal variability in *P450 2C*-dependent drug metabolism.

In conclusion, four novel marmoset *P450 2C18*, *2C19*, *2C58*, and *2C76* were predominantly expressed in marmoset livers. Among five marmoset *P450 2C* enzymes, marmoset *P450 2C19* efficiently metabolized human *P450 2C9* and *2C19* probe substrates. Additionally, marmoset *P450 2C19* preferentially metabolized *S*-warfarin both *in vitro* and *in vivo*, similar to the stereoselectivity of human live microsomal *P450 2C9*. These results indicate that marmoset *P450 2C* enzymes had functional characteristics similar to those of humans, indicating that *P450 2C*-dependent metabolic properties are relatively similar between marmosets and humans.

Acknowledgments

The authors thank Drs. Norie Murayama and Masayuki Mogi for their technical help.

Authorship contribution

Participated in research design: Uehara, Uno, and Yamazaki,

Conducted experiments: Uehara, Uno, Kawano, and Toda.

Contributed new reagents or analytic tools: Inoue and Sasaki,

Performed data analysis: Uehara, Shimizu, Uno, and Utoh.

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

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Footnotes

Shotaro Uehara and Yasuhiro Uno equally contributed.

This work was resulted from "Construction of System for Spread of Primate Model Animals" under the Strategic Research Program for Brain Sciences of Japan Agency for Medical Research and Development. Shotaro Uehara was also supported partly by JSPS Grant-in-Aid for Young Scientists (B) 15K18934.

Legends for figures

Fig. 1. Multiple sequence alignment of the deduced amino acid sequences of marmoset P450 2Cs.

Amino acid sequences of marmoset (cj) P450 2Cs (2C8, 2C18, 2C19, 2C58, and 2C76) and human (h) P450 2Cs (2C8, 2C9, 2C18, and 2C19) were aligned using Genetyx. Six substrate recognition sites (SRS) are marked by a solid line. The heme-binding domain is marked by a broken line. An asterisk indicates perfectly conserved. A dot indicates conservatively changed amino acids.

Fig. 2. Phylogenetic tree of marmoset P450 2C amino acid sequences.

The neighbor-joining method was used to create a phylogenetic tree with P450 2C amino acid sequences of marmoset (cj), human (h), cynomolgus monkey (mf), rhesus monkey (mm), dog (d), pig (p), rabbit (rab), rat (r), and mouse (m), together with human P450 2A6 as an outgroup. Branch length is proportional to the number of amino acid substitutions. The scale bar indicates 10% amino acid substitution in the sequence.

Fig. 3. Genomic structures of P450 2C cluster in marmosets, macaques, and humans.

The genomic structures were analyzed using the marmoset, macaque (rhesus monkey), and human genome data on BLAT. *P450 2C* genes were located between *HELLES* and *PDLIM1* in the marmoset genome, similar to rhesus monkey and human. The length of the genes and the distance between the genes are not proportionate to actual measurement.

Fig. 4. Expression of P450 2C mRNAs in marmoset tissues.

Expression of marmoset P450 2C mRNAs in brains, lungs, livers, kidneys, and small

intestines were measured by real-time RT-PCR. The raw data was normalized to 18S rRNA expression level. Values are presented as the means plus standard deviations ($n = 3$). Expression level of P450 2C8 mRNA was adjusted to 1, and expression levels of other P450 2C mRNAs are shown as the expression values relative to P450 2C8 mRNA.

Fig. 5. Plasma concentrations of *S*- and *R*- warfarin after intravenous administration of racemic warfarin (0.2 mg/kg) in marmosets.

Plasma concentrations of *S*- (A) and *R*- (B) warfarin after intravenous administration of racemic warfarin (0.2 mg/kg) were measured in four marmosets. The average and individual values are marked by solid and broken lines, respectively. Results are expressed with circles (means) and bars (\pm SDs) obtained with four marmosets ($*p < 0.05$, two-way ANOVA with Bonferroni post test).

Table 1

Similarities of the deduced amino acid sequences of marmoset P450 2Cs with cynomolgus monkey and human P450 2C isoforms

	P450	Marmoset P450				
		2C8	2C18	2C19	2C58	2C76
				%		
Human	2C8	86	78	76	87	68
	2C9	78	82	86	79	68
	2C18	77	93	77	78	70
	2C19	77	81	86	78	69
Cynomolgus monkey	2C8	87	77	75	87	68
	2C9	77	81	84	78	68
	2C18	77	93	77	78	70
	2C19	76	81	85	76	68
Marmoset	2C76	71	73	70	72	87
	2C8		77	74	96	67
	2C18			78	78	70
	2C19				76	67
	2C58					68

The BLAST program was used to compare sequences with marmoset, cynomolgus monkey, and human P450 2C isoforms as described in Materials and Methods.

Table 2

Catalytic activities by recombinant marmoset P450 2C enzymes.

Drug oxidation	Substrate concentration (μM)	Catalytic activities (min ⁻¹)				
		2C8	2C18	2C19	2C58	2C76
Ethoxyresorufin <i>O</i> -deethylation	10	0.06	0.19	0.45	0.52	0.05
7-Ethoxycoumarin <i>O</i> -deethylation	100	0.20	0.06	0.31	0.65	0.01
Coumarin 7-hydroxylation	100	0.002	<0.001	<0.001	0.013	<0.001
7-Pentoxoresorufin <i>O</i> -deethylation	10	0.06	0.11	0.17	0.18	0.02
Paclitaxel 6α-hydroxylation	100	1.0	<0.5	<0.5	<0.5	<0.5
Diclofenac 4'-hydroxylation	100	<0.0001	0.001	0.002	<0.0001	0.001
Tolbutamide methyl hydroxylation	2500	62	0.25	19	0.34	0.17
Flurbiprofen 4-hydroxylation	100	0.54	<0.01	4.7	0.42	<0.01
<i>Racemic</i> warfarin 7-hydroxylation	100	<0.0001	0.0023	0.037	<0.0001	<0.0001
<i>S</i> -Warfarin 7-hydroxylation	100	<0.0001	0.0056	0.17	<0.0001	<0.0001
<i>R</i> -Warfarin 7-hydroxylation	100	<0.0001	0.0028	0.0053	<0.0001	<0.0001
Omeprazole 5-hydroxylation	100	<0.01	<0.01	50	<0.01	<0.01
Chlorzoxazone 6-hydroxylation	500	0.96	<0.05	<0.05	1.6	<0.05
Bufuralol 1'-hydroxylation	100	1.5	0.73	10	6.2	<0.01
Midazolam 1'-hydroxylation	100	<0.3	<0.3	<0.3	<0.3	<0.3

Table 3

Kinetic parameters for oxidation activities of paclitaxel, tolbutamide, flurbiprofen, and omeprazole by recombinant P450 2C enzymes and liver microsomes from marmosets and humans.

Drug oxidation	Enzyme source	K_m μM	V_{max} min^{-1}	V_{max}/K_m $\mu\text{M}^{-1} \text{min}^{-1}$
Paclitaxel 6 α -hydroxylation	Marmoset 2C8	42 \pm 10	1.6 \pm 0.2	0.038 \pm 0.010
	Human 2C8	11 \pm 3	79 \pm 11	7.2 \pm 2.2
	MLM	44 \pm 8	0.020 \pm 0.001	0.00045 \pm 0.00009
	HLM	27 \pm 5	0.22 \pm 0.01	0.0081 \pm 0.0016
Tolbutamide methyl hydroxylation	Marmoset 2C8	2210 \pm 313	98 \pm 5	0.044 \pm 0.007
	Marmoset 2C19	217 \pm 27	20 \pm 1	0.092 \pm 0.012
	Human 2C8	1040 \pm 403	41 \pm 6	0.039 \pm 0.016
	Human 2C9	284 \pm 45	55 \pm 2	0.19 \pm 0.03
	MLM	373 \pm 103	1.0 \pm 0.1	0.0027 \pm 0.0008
	HLM	165 \pm 14	0.79 \pm 0.01	0.0048 \pm 0.0004
Flurbiprofen 4-hydroxylation	Marmoset 2C8	155 \pm 30	1.4 \pm 0.1	0.0090 \pm 0.0019
	Marmoset 2C19	161 \pm 57	12 \pm 2	0.075 \pm 0.029
	Marmoset 2C58	85 \pm 11	0.80 \pm 0.10	0.0094 \pm 0.0017
	Human 2C9	15 \pm 4	27 \pm 2	1.8 \pm 0.5
	MLM	34 \pm 5	0.070 \pm 0.003	0.0021 \pm 0.0003
	HLM	9.1 \pm 0.3	0.33 \pm 0.01	0.036 \pm 0.002
Omeprazole 5-hydroxylation	Marmoset 2C19	23 \pm 13	52 \pm 8	2.3 \pm 1.3
	Human 2C19	16 \pm 3	34 \pm 2	2.1 \pm 0.4
	MLM	30 \pm 2 (K_{m1})	0.58 \pm 0.03 ($V_{\text{max}1}$)	0.019 \pm 0.002 ($V_{\text{max}1}/K_{m1}$)
		325 \pm 18 (K_{m2})	1.7 \pm 0.1 ($V_{\text{max}2}$)	0.0052 \pm 0.0004 ($V_{\text{max}2}/K_{m2}$)
	HLM	36 \pm 3 (K_{m1})	0.33 \pm 0.02 ($V_{\text{max}1}$)	0.0092 \pm 0.0009 ($V_{\text{max}1}/K_{m1}$)
727 \pm 40 (K_{m2})		1.8 \pm 0.1 ($V_{\text{max}2}$)	0.0025 \pm 0.0002 ($V_{\text{max}2}/K_{m2}$)	

MLM, marmoset liver microsomes; HLM, human liver microsomes.

Table 4

Kinetic parameters for 7-hydroxylation of racemic, *S*- and *R*-warfarin by recombinant P450 2C enzymes and liver microsomes from marmosets, cynomolgus monkeys, and humans.

Enzymes	Warfarin 7-hydroxylation			
	<i>R</i> -/ <i>S</i> -	K_m μM	V_{max} min ⁻¹	V_{max}/K_m mM min ⁻¹
Marmoset P450 2C19	<i>racemic</i>	12 ± 1	0.04 ± 0.01	3.3 ± 0.9
	<i>S</i>	14 ± 1	0.20 ± 0.01	14 ± 1
	<i>R</i>	755 ± 84	0.20 ± 0.02	0.26 ± 0.04
Cynomolgus monkey P450 2C19	<i>racemic</i>	37 ± 5	2.5 ± 0.1	68 ± 10
	<i>S</i>	124 ± 27	1.3 ± 0.1	10 ± 2
	<i>R</i>	51 ± 6	7.0 ± 0.2	137 ± 17
Human P450 2C9	<i>racemic</i>	22 ± 8	0.018 ± 0.001	0.82 ± 0.30
	<i>S</i>	3.1 ± 0.1	0.17 ± 0.02	55 ± 2
	<i>R</i>	123 ± 35	0.007 ± 0.001	0.057 ± 0.018
MLM	<i>racemic</i>	150 ± 39	0.0014 ± 0.0002	0.0093 ± 0.0028
	<i>S</i>	9.5 ± 1.6	0.0058 ± 0.0003	0.61 ± 0.11
	<i>R</i>	240 ± 55	0.0037 ± 0.0005	0.015 ± 0.004
CLM	<i>racemic</i>	44 ± 2	0.0033 ± 0.0001	0.075 ± 0.004
	<i>S</i>	73 ± 6	0.0051 ± 0.0002	0.070 ± 0.006
	<i>R</i>	16 ± 3	0.0059 ± 0.0003	0.37 ± 0.07
HLM	<i>racemic</i>	7.7 ± 0.3	0.0016 ± 0.0001	0.21 ± 0.02
	<i>S</i>	5.0 ± 0.3	0.0028 ± 0.0001	0.56 ± 0.04
	<i>R</i>	237 ± 22	0.0016 ± 0.0001	0.0068 ± 0.008

MLM, marmoset liver microsomes; CLM, Cynomolgus monkey liver microsomes, and HLM, human liver microsomes.

Fig. 2

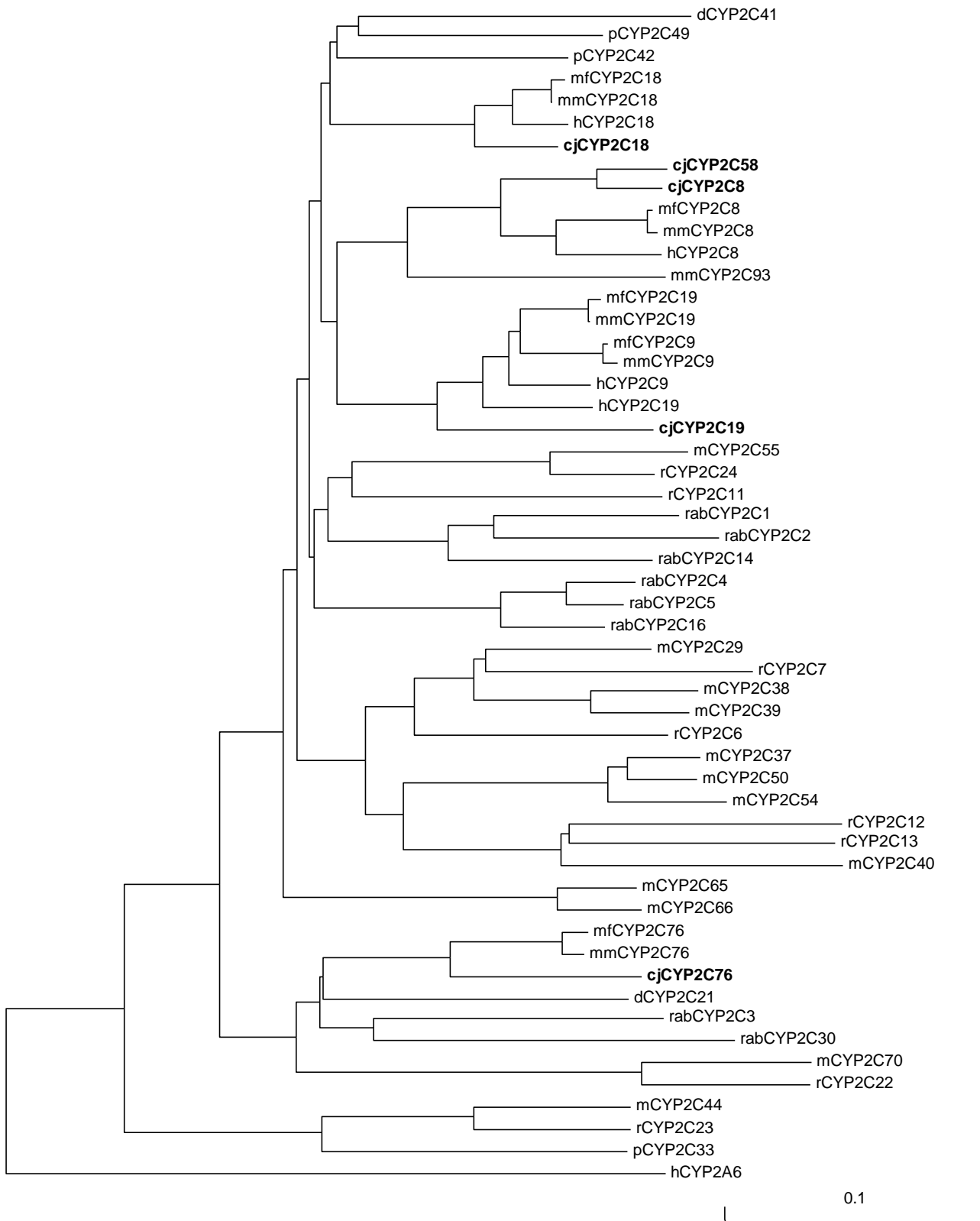
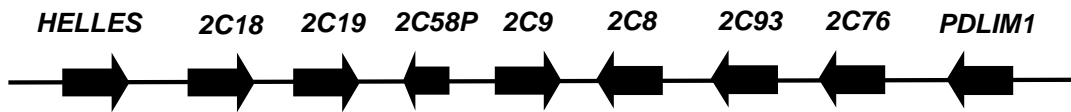


Fig. 3

Marmoset chromosome 12



Macaque chromosome 9



Human chromosome 10

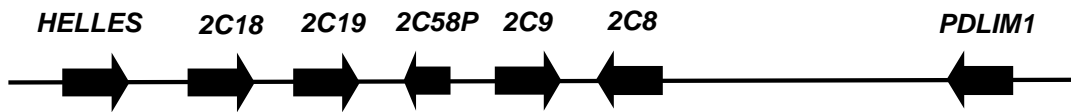


Fig. 4

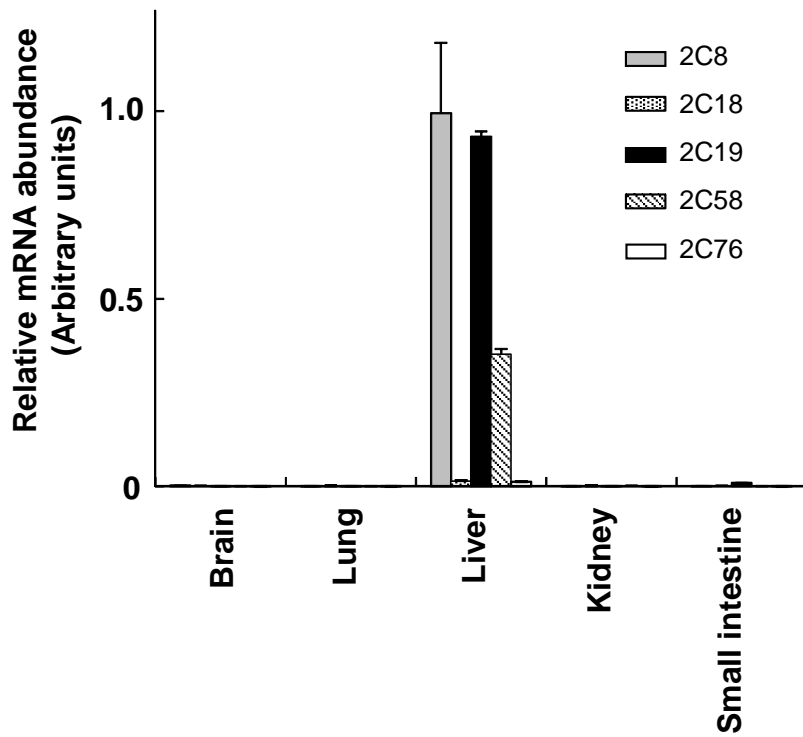
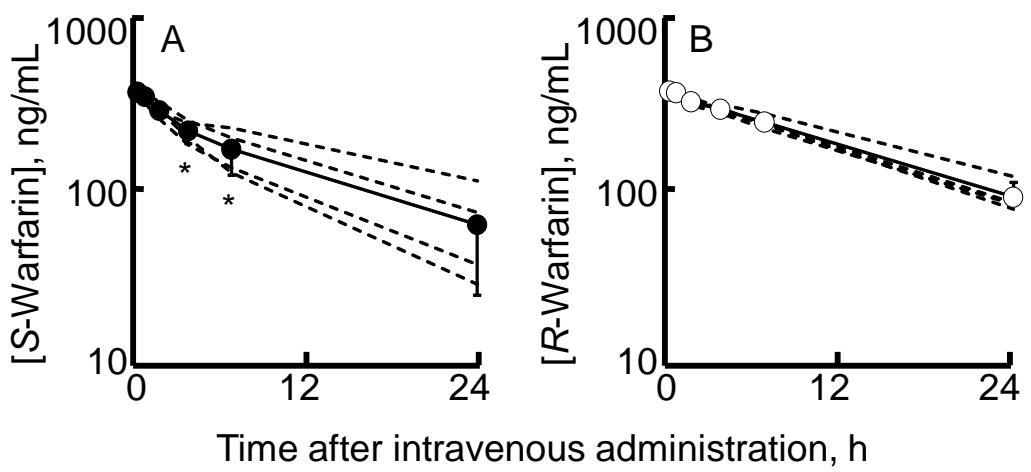


Fig. 5



Supplemental Data

Novel marmoset cytochrome P450 2C19 in livers efficiently metabolizes human P450 2C9 and 2C19 substrates, S-warfarin, tolbutamide, flurbiprofen, and omeprazole

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Supplemental Methods

cDNA cloning

Five full-length cDNAs encoding marmoset P450 2Cs were isolated by reverse transcription (RT)- polymerase chain reaction (PCR) using marmoset total liver RNA prepared using RNeasy Mini Kit (Qiagen, Valencia, CA). The first-strand cDNA was synthesized in a mixture of 20 μ L containing 1 μ g of total RNA, oligo(dT), and SuperScript III RT reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The full-length cDNA amplification was carried out by PCR using KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan) on an ABI GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. PCR cycles included: a 2-min denaturation at 94 °C followed by 35 cycles of 98 °C for 10 seconds, annealing at 65 °C for 30 seconds and 68 °C for 2 minutes, and a final cycle of 68 °C for 5 min. PCR primers, designed based on the marmoset genome data of BLAT (UCSC Genome Bioinformatics), were cjCYP2C8 (5rt1) 5'-GAGCCAAAGTCCACTGGGAAAAAAAGC-3' and cjCYP2C8 (3rt1) 5'-TCAGACAGGAATGAAGCAGATCTGGTACGG-3' for P450 2C8, cjCYP2C19L (5rt1) 5'-AAAGCCTGCAGTTGTCTTACTAAGAGATGG-3' and cjCYP2C19L (3rt1) 5'-GGTGACAGCAGCAGCCAGAAAGTC-3' for P450 2C18, cjCYP2C26L (5rt1) 5'-GGCTTGGAGTGCAAGCTCACAATTATC-3' and cjCYP2C26L (3rt1) 5'-CACAGCAGCAGCCAGACCATTTG-3' for P450 2C19, cjCYP2C20L (5rt1) 5'-GAACCAAAGTCCACTGGGAAAAAAAAG-3' and cjCYP2C20L (3rt1) 5'-TCAGACAGGAATAAAGCAGATCTGGTATGAG-3' for P450 2C58, and cjCYP2C21L (5rt1) 5'-AGCTGCATTTCCACAAAGCACATCA-3' and cjCYP2C21L (3rt1) 5'-GGCCTTGCTCTAGCAGCCTGACAT-3' for P450 2C76. Amplified cDNAs were cloned

into pCR4 vectors using a Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen). The inserts were sequenced with an ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems).

Immunoblotting

The detection of P450 2C proteins in recombinant and marmoset liver microsomes was performed by immunoblotting using anti-human P450 2C8 antibodies (GeneTex, Irvine, CA), anti-human P450 2C9 antibodies (Nosan, Yokohama, Japan), and anti-cynomolgus monkey P450 2C76 antibodies. Recombinant marmoset P450 2C proteins or marmoset liver microsomes were separated on 10% sodium dodecyl sulphate-polyacrylamide gel and transferred electrophoretically to a polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA). The membranes were treated with 0.5% skim milk in Tris-buffered saline with 0.05% Tween 20 (TBST) at room temperature for 30 minutes to block nonspecific binding and incubated with polyclonal primary antibodies (1: 1000-5000) in 0.5% skim milk in TBST at room temperature for 1 hour, then incubated with goat anti-rabbit IgG secondary antibodies (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA) in 0.5% skim milk in TBST at room temperature for 20 minutes. Immunoreactive bands were visualized with an ECL Prime Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

References

Uno Y, Fujino H, Kito G, Kamataki T, and Nagata R (2006) CYP2C76, a novel cytochrome P450 in cynomolgus monkey, is a major CYP2C in liver, metabolizing tolbutamide and testosterone. *Mol Pharmacol* **70**:477-486.

Supplemental Figure 1**Immunoblot analysis of recombinant P450 2C proteins and marmoset liver microsomes.**

Immunoblotting was performed using anti-human (h) P450 2C8 and 2C9 antibodies and anti-cynomolgus monkey (mf) P450 2C76 antibodies to detect expression of P450 2C proteins in marmoset liver microsomes. A, the specificity of the primary antibodies were assessed using recombinant marmoset P450 2C proteins (1 pmol of P450/lane). B, liver microsomes (40 µg/lane) from males (lanes 1 and 3) and females (lanes 2, 4, and 5) were analyzed.

