DMD Fast Forward. Published on February 14, 2017 as DOI: 10.1124/dmd.116.074898
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Title Page

Marmoset cytochrome P450 3A4 orthologue expressed in liver and small intestine tissues efficiently metabolizes midazolam, alprazolam, nifedipine, and testosterone

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Running Title Page

Running title: Characterization of P450 3A enzymes in marmosets

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Number of Text Pages : 30

Number of Tables : 4

Number of Figures : 7

Number of References : 39

Number of Words in Abstract : 253

Number of Words in Introduction : 506

Number of Words in Discussion : 1,127

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; SRS, substrate recognition site.

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Abstract

Common marmosets (*Callithrix jacchus*), small New World primates, are increasingly attracting attention as potentially useful animal models for drug development. However, characterization of cytochrome P450 (P450) 3A enzymes involved in the metabolism of a wide variety of drugs has remained in marmosets. In this study, sequence homology, tissue distribution, and enzymatic property of marmoset P450 3A4 orthologue, 3A5 orthologue, and 3A90 were investigated. Marmoset P450 3A forms exhibited high amino acid sequence identities (88-90%) to the human and cynomolgus monkey P450 3A orthologues and evolutionary closeness to human and cynomolgus monkey P450 3A orthologues, compared with other P450 3A enzymes. Among the five marmoset tissues examined, P450 3A4 orthologue mRNA was abundant in livers and small intestines where P450 3A4 orthologue proteins were immunologically detected. Three marmoset P450 3A proteins heterologously expressed in *Escherichia coli* membranes catalyzed midazolam 1′- and 4-hydroxylation, alprazolam 4-hydroxylation, nifedipine oxidation, and testosterone 6β-hydroxylation, like cynomolgus monkey and human P450 3A enzymes. Among the marmoset P450 3A enzymes, P450 3A4 orthologue effectively catalyzed midazolam 1′-hydroxylation, comparable to microsomes from marmoset livers and small intestines. Correlation analyses with 23 individual marmoset liver microsomes suggested contributions of P450 3A enzymes to both 1′-hydroxylation of midazolam (human P450 3A probe) and bufuralol (human P450 2D6 probe), like cynomolgus monkey P450 3A enzymes. These results indicated that marmoset P450 3A forms had the functional characteristics roughly similar to cynomolgus monkeys and humans in term of tissue expression patterns and catalytic activities, suggesting marmosets as suitable animal models for P450 3A-dependent drug metabolism.

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Introduction

Common marmosets (*Callithrix jacchus*) have attracted increasing attention as a potentially useful non-human primate model in fields such as pharmacokinetics, toxicology, neuroscience, stem-cell research, immunology, and infectious disease because of their genetic closeness to humans, small body size (weighing 350–400 g on average), high reproductive efficiency (typically producing twins), early sexual maturity (reached within 18 months of age), and applicability of transgenic technologies(Orsi et al., 2011; Sasaki, 2015) . Cynomolgus monkeys (*Macaca fascicularis*) are the widely used non-human primate species for pharmacokinetics and drug safety testing in pharmaceutical companies.

Cytochrome P450s (P450s), the major drug-metabolizing enzymes comprising of multiple subfamilies, catalyze the oxidative biotransformation of potentially toxic compounds, including drugs and new chemical compounds (Wrighton and Stevens, 1992). In humans, it has reported that approximately 75% of the drugs on the market are cleared by P450s and P450 3A enzymes significantly are involved in metabolism of more than 50% of the drugs (Williams et al., 2004). In humans, the P450 3A subfamily consists of four members, namely P450 3A4, 3A5, 3A7, and 3A43 forms. Human P450 3A enzymes reportedly metabolize midazolam, alprazolam, nifedipine, and testosterone (Yamazaki et al., 2002; Ohtsuka et al., 2010). P450 3A4 and 3A5 mRNAs are highly expressed in livers, followed by small intestines (among 10 human tissues) (Nishimura et al., 2003), and their protein expression was also detected in livers (Yamazaki et al, 1995) and small intestines (Paine et al., 2006). P450 3A4 protein expression was approximately 10-fold higher than that of P450 3A5 in human livers (Yamaori et al, 2004, 2005; Wang et al., 2008).

Marmoset P450 3A forms identified to date are P450 3A4 orthologue (formerly 3A21), 3A5

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orthologue, and 3A90 (http://drnelson.uthsc.edu/CytochromeP450.html). An our previous study showed that marmoset P450 3A4 orthologue and 3A90 enzymes effectively catalyzed midazolam 1′-hydroxylation, similar to human and cynomolgus monkey P450 3As (Uehara et al., 2016a). Marmoset and cynomolgus monkey P450 3A4 orthologue also catalyzed omeprazole 5-hydroxylation and sulfoxidation reactions with high capacity (Uehara et al., 2016b). Quantitative analysis of gene expression for common marmoset transcriptomes indicated that P450 3A4 orthologue and 3A5 orthologue /3A90 mRNAs were expressed in livers and small intestines, similar to human P450 3A mRNA (Shimizu et al., 2014). P450 3A4 and 3A5 orthologue -like proteins were detected in marmoset livers (Schulz et al., 2001). In spite of the potential importance as non-human primate model in drug metabolism and toxicological research, the molecular characteristics of marmoset P450 3A forms has not been analyzed in detail.

More than 20 marmoset P450 cDNAs have been identified so far; these P450s have high sequence similarities (>85%) to their orthologous human P450s (Uno et al., 2016). Overall, substrate specificity and tissue expression of orthologous P450 enzymes are similar between marmosets and humans, except for some enzymes belonging to the P450 2 family. In this study, gene cluster organization, sequence similarity, tissue distribution, and enzymatic properties of marmoset P450 3A4 orthologue, 3A5 orthologue, and 3A90 were investigated. This work is of importance for understanding the metabolic characteristics of marmosets as animal models in drug development.

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Materials and Methods

Chemicals and enzymes

Alprazolam, midazolam, and testosterone were purchased from Wako Pure Chemicals (Osaka, Japan). 4-Hydroxyalprazolam were purchased from Enzo Life Sciences (Farmingdale, NY). Nifedipine, oxidized nifedipine, 1′-hydroxymidazolam, 4-hydroxymidazolam, and 6β-hydroxytestosterone were purchased from Sigma-Aldrich (Tokyo, Japan). Bufuralol and 1′-hydroxybufuralol were purchased from Toronto Research Chemicals (Toronto, Canada). Oligonucleotides were synthesized at Sigma Genosys (Ishikari, Japan). Pooled liver microsomes from marmosets (5 males, sexually mature) and humans (74 males and 76 females, 18-82 years old) were purchased from Corning Life Sciences (Woburn, MA). Pooled liver microsomes from cynomolgus monkeys (8 males, 3-8 years old) and pooled intestine microsomes from cynomolgus monkeys (15 males, 2-5 years old) and humans (4 males and 6 females, 14-65 years old) were purchased from Xenotech (Lenexa, KS). Pooled microsomes of brains, lungs, livers, kidneys, and small intestines were prepared from tissue samples of 20 marmosets (10 males and 10 females, >2 years old) at the Central Institution for Experimental Animals (Kawasaki, Japan) as described previously (Uehara et al., 2016c). Individual liver microsomes were prepared from 23 marmosets (14 males and 9 females, >2 years old). This study was reviewed and approved by the Institutional Animal Care and Use Committee (Central Institution for Experimental Animals). Anti-human P450 3A4 antibodies (WB-3A4) and anti-human P450 3A5 antibodies (WB-3A5) were purchased from Corning Life Sciences. Anti-human P450 2D6 antibodies were purchased from Nosan Corporation (Yokohama, Japan). Anti-human protein disulfide isomerase (PDI) antibodies (H-160) and goat anti-rabbit IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All

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other regents used were the highest quality commercially available.

Bioinformatics

The structure of marmoset P450 3A gene cluster was analyzed by BLAT (UCSC Genome Bioinformatics, University of California, Santa Cruz, CA) and by GGGenome (DNA Data Bank of Japan, National Institute of Genetics, Mishima, Japan). The amino acid sequence similarity of marmoset P450 3A forms compared with other species P450 3A members was determined by BLAST (National Center for Biotechnology Information, Bethesda, MD). The amino acid sequence alignment was performed using Genetyx system (Software Development, Tokyo, Japan). The phylogenetic tree was constructed with the aligned sequences by the neighbor-joining method using DNASIS Pro (Hitachi Software, Tokyo, Japan). P450 amino acid sequences used were from GenBank: human P450 3A4 (NP_059488), 3A5 (NP_000768), 3A7 (NP_000756), 3A43 (NP_073731), and 2D6 (NP_000097); chimpanzee P450 3A4 (NP_001116247), 3A5 (NP_001087246), and 3A7 (NP_001087243); orangutan P450 3A43 (ABU85093) and 3A67 (ABU85096); cynomolgus monkey P450 3A4 (NP_001271463), 3A5 (NP_001306440), 3A7 (NP_001306436), and 3A43 (NP_001306434); rhesus monkey P450 3A4 (NP_001035504), P450 3A5 (NP_001035309), and P450 3A7 (NP_001182687); marmoset P450 3A4 orthologue (NP_001191369), 3A5 orthologue (NP_001191371), and 3A90 (NP_001191372); dog P450 3A12 (NP_001003340) and 3A26 (NP_001003338); pig P450 3A22 (NP_001182438), 3A29 (NP_999588), 3A39 (NP_999587), and 3A46 (NP_001128296); rabbit P450 3A6 (NP_001164739); guinea pig P450 3A14 (NP_001166587), 3A15 (NP_001166588), and 3A17 (NP_001166540); rat P450 3A2 (NP_695224), 3A9 (NP_671739), 3A18 (NP_665725), 3A23 (NP_037237), and 3A62 (NP_001019403); mouse P450 3A11 (NP_031844), 3A13 (NP_031845), 3A16 (NP_031846), 3A25 (NP_062766), 3A41

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(NP_001098629), 3A44 (NP_796354), 3A57 (NP_001093650), and 3A59 (NP_001098630).

Quantitative reverse transcription PCR

The P450 3A mRNA distribution in marmoset tissues was analyzed by real-time reverse transcription (RT)-polymerase chain reaction (PCR) as described previously (Uehara et al., 2016d). Briefly, total RNAs were extracted from brains, lungs, livers, kidneys, and small intestines, each pooled from 12 adult marmosets (6 males and 6 females, >2 years old), using an RNeasy Mini Kit (Qiagen, Valencia, CA) and were used to synthesize cDNA using SuperScript III RT reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) with random hexamers in a 20-µl reaction. Quantitative RT-PCR was performed with SYBR Green-based detection system using gene-specific primers; 5′-GCTTTTGGAAGTTTGACATGGA-3′ and 5′-CAGGCTGTCGACCATCATAAATC-3′ for marmoset P450 3A4 orthologue, 5′-GTGAAGAAGTTCCTAAAATTTGATTTCC-3′ and 5′- GGGGTAAGGAACGGGAAGAA-3′ for marmoset P450 3A5 orthologue, and 5′- CCTAAAATTTGATGTATTAGCTCCACTG-3′ and 5′- GGATAAGGAACGGAAAGAGTACTACTGA-3′ for marmoset P450 3A90. The reaction mixture contained 400 nM of each primer, 12.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 2 µl of template DNA in in a total volume of 25 µl. The PCR conditions were as follows: an initial denaturation for 10 minutes at 95°C, followed by 40 cycles of 95 °C for 15 seconds and 60°C for 1 minute. Real-time PCR was performed with an ABI PRISM 7300 sequence detection system (Applied Biosystems). This study performed absolute quantification, displaying high PCR efficiency (>93%) comparable for three P450 genes with high correlation coefficient ($r > 0.99$). Standard curves were created by absolute amounts (10^2–10^6 copies) with 10-fold dilution series of purified PCR products of marmoset P450 3A cDNAs. The expression level of each P450 3A mRNA was normalized to

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the level of 18S rRNA measured using Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems).

Heterologous protein expression in *Escherichia coli*

Marmoset P450 3A4 orthologue, 3A5 orthologue, and 3A90 cDNAs were cloned from liver by RT-PCR as described previously (Uehara et al., 2015c). In order to enhance the efficiency of expression for recombinant marmoset P450 3A proteins, the N-terminus modification was performed for the expression plasmids constructed with the P450 3A cDNAs by PCR using the forward and reverse primers; cjCYP3A4 (5exp1) 5′-CATATGGCTCTGTTATTAGCAGTTTTCCTGGTGCTTCTGTATCTA-3′ and cjCYP3A4 (3exp1) 5′-TCTAGATTAGGCTCCACTTACAGTCC-3′ for marmoset P450 3A4 orthologue, cjCYP3A5 (5exp1) 5′-CATATGGCTAAGAAAACGAGCTCTAAAGGTAAGCTTATTCCAGGACCCGCACCT-3′ and cjCYP3A5 (3exp1) 5′-TCTAGATTATTCTCCACTTAGGGTTC-3′ for marmoset P450 3A5 orthologue, and cjCYP3A4 (5exp1) 5′-CATATGGCTCTGTTATTAGCAGTTTTCCTGGTGCTTCTGTATCTA-3′ and cjCYP3A5 (3exp1) 5′-TCTAGATTATTCTCCACTTAGGGTTC-3′ for marmoset P450 3A90. PCR amplification was carried out for 30 cycles (denaturation at 98°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 68°C for 2 minutes) using KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan) with an ABI GeneAmp PCR System 2720 thermocycler (Applied Biosystems). PCR products were cloned into pGEM-T easy vectors (Promega, Madison, WI), and subsequently subcloned into pCW vectors using the restriction sites of the NdeI and XbaI sites (underlined). Recombinant P450 3A proteins were prepared in *Escherichia coli* DH5α expression system, and the concentration of P450 and NADPH-P450 reductase in

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each membrane preparation was measured as described previously (Yamazaki et al., 2002). Recombinant proteins were produced on the yield of approximately 2 μmol/L culture medium. Recombinant marmoset P450 2D6 and 2D8, recombinant cynomolgus monkey 3A4 and 3A5, and recombinant human P450 3A4, 3A5, and 2D6 were prepared as described previously (Yamazaki et al., 2002; Uno et al., 2010; Uehara et al., 2015a).

Western blotting

Recombinant P450 proteins (1.0 pmol) or liver microsomes (10 μg) were subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and then electrophoretically transferred to polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA). After blocking with 0.5% non-fat milk in TBS (50 mM Tris, 138 mM NaCl, 2.7 mM KCl) containing 0.05% Tween 20 (v/v) (TBST) at room temperature for 30 minutes, the membrane was probed with anti-human P450 3A4 antibodies (1:2,000), anti-human P450 3A5 antibodies (1:5,000), anti-human P450 2D6 antibodies (1:10,000), or anti-human PDI antibodies (1:200) at room temperature for 1 hour, and then with goat-anti-rabbit IgG antibodies (1:5,000) at room temperature for 20 minutes. The antigen-antibody complex was visualized by an ECL Prime Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK).

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Enzyme assay

Midazolam 1′- and 4-hydroxylation, nifedipine oxidation, testosterone 6β-hydroxylation, and bufuralol 1′-hydroxylation activities by recombinant P450 proteins and tissue microsomes were measured as described previously (Yamazaki et al., 2002; Uehara et al., 2015b). For alprazolam hydroxylation, the incubation mixture consisted of 40 pmol/mL recombinant protein or 0.5 mg/mL microsomes (liver or small intestine), 200 μM alprazolam, an NADPH-

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generating system (0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, and 0.25 units/mL glucose 6-phosphate dehydrogenase), and 100 mM potassium phosphate buffer (pH7.4) in a total volume of 0.25 mL. Reactions were started by adding the NADPH-generating system, and performed with 100 rpm/ minute of shaking at 37°C for 10 minutes. Reactions were terminated by addition of methanol (0.40 mL), and then centrifuged at 20,000g for 10 minutes. The resulting supernatant was analyzed by reversed-phase HPLC using a Prominence-I LC-2030C HPLC system with a fluorescence detector (Shimadzu, Kyoto, Japan). HPLC analysis was performed on a C_{18} column (L-column2 ODS, 5 µm, 150 × 4.6 mm; Chemicals Evaluation and Research Institute, Tokyo, Japan) using isocratic elution by methanol/acetonitrile/10 mM potassium phosphate buffer (pH 7.4) (24:33:43, v/v/v) at a flow rate of 1.0 mL/min with monitoring the absorbance at 220 nm. Metabolite concentrations were quantified based on standard curves prepared with reference standards. Kinetic parameters for midazolam 1′- and 4-hydroxylation and nifedipine oxidation were estimated from the fitted curves employing Michaelis-Menten equations, substrate inhibition equations, or Hill equations (Emoto et al., 2001; Shimizu, et al., 2007; Okada, et al., 2009) using the KaleidaGraph program (Synergy Software, Reading, PA). Linear regression analysis was performed with Prism (Graphpad Software, La Jolla, CA).

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Results

Determination of *P450 3A* gene cluster structure and amino acid sequences identity in marmosets

The structure of marmoset *P450 3A* gene cluster was determined using marmoset genomic sequence by BLAT. Marmoset *P450 3A* cluster was localized in marmoset chromosome 2 (chr2: 12136811-12255660) (Fig. 1). Three marmoset *P450 3A* genes (*P450 3A4 orthologue*, *3A5 orthologue*, and *3A90*) form a cluster of total length 118850 bp (containing two gaps) between *ZSCAN25* and *TRIM4* genes on the short arm of chromosome 2. No marmoset *P450 3A* genes had one-to-one orthologous relationship to human or cynomolgus monkey *P450 3A* genes. A marmoset *P450 3A43* ortholog was not found in the genome as analyzed by BLAT, or GGGenome. The amino acid sequences of marmoset P450 3A4 orthologue, 3A5 orthologue, and 3A90 had six substrate recognition sites and a heme-binding site, similar to human and cynomolgus monkey P450 3A forms (Fig. 2). Amino acid sequences of marmoset P450 3A4 orthologue, 3A5 orthologue, and 3A90 showed high degrees of identity (>88%) to those of human and cynomolgus monkey P450 3A forms (Table 1). Phylogenetic analysis using amino acid sequences of the P450 3A forms from 12 species showed that marmoset P450 3A forms were evolutionarily closer to the P450 3A forms of primates, including humans, chimpanzees, orangutans, rhesus monkeys, and cynomolgus monkeys, than those of dogs, pigs, guinea pigs, rats, and mice (Fig. 3).

Tissue distribution of three P450 3A mRNAs and proteins in marmosets

Expression levels of marmoset P450 3A mRNAs in brains, lungs, livers, kidneys, and small intestines were analyzed by real-time RT-PCR. All three marmoset P450 3A mRNAs were abundantly expressed in livers and small intestines among the five tissues examined (Fig. 4).

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The expression level of P450 3A4 orthologue mRNA was >5-fold (livers) and >3-fold (small intestines) higher than those of P450 3A5 orthologue and 3A90 mRNAs, respectively, indicating that P450 3A4 orthologue was the major P450 3A form in livers and small intestines, the organs responsible for drug metabolism. P450 3A5 orthologue mRNA was >7-fold higher in small intestines than livers, different from P450 3A4 orthologue and 3A90 mRNAs expressed in these tissues at comparable levels. Tissue distribution of P450 3A proteins in marmosets was investigated by Western blotting using anti-human P450 3A4 and 3A5 antibodies capable of detecting selectively recombinant marmoset P450 3A proteins (Fig. 5A). P450 3A4 orthologue and 3A5 orthologue /3A90 proteins were detected in marmoset livers and small intestines (Fig. 5B); abundant P450 3A4 orthologue protein was detected in marmoset livers. P450 3A4 orthologue and 3A5 orthologue /3A90 proteins were constitutively expressed in livers from five individual marmosets (Fig. 5C).

Enzymatic activities of marmoset P450 3A proteins

To assess the enzymatic function of marmoset P450 3A enzymes, the drug oxidation activities by recombinant P450 3A proteins and tissue microsomes were measured using typical human P450 3A probe substrates (midazolam, alprazolam, nifedipine, and testosterone). Liver and small intestine microsomes from marmosets catalyzed midazolam 1′- and 4-hydroxylation, alprazolam 4-hydroxylation, nifedipine oxidation, and testosterone 6β-hydroxylation in the similar manner to those of humans and cynomolgus monkeys (Table 2); liver microsomes from marmoset and cynomolgus monkeys catalyzed these reactions more strongly compared with those from humans. All marmoset P450 3A4 orthologue, 3A5 orthologue, and 3A90 enzymes also metabolized above typical human P450 3A probe substrates. Catalytic activities of P450 3A4 orthologue enzyme were the highest among the marmoset P450 3A enzymes analyzed.

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Bufuralol 1′-hydroxylation activities, a typical human P450 2D probe activity, were higher for marmoset P450 3A4 orthologue, 3A5 orthologue, and 3A90 than human P450 3A4 and 3A5, similar to cynomolgus monkey P450 3A4 and 3A5 reported previously (Emoto et al., 2011). Kinetic analyses indicated that marmoset liver microsomes more effectively catalyzed midazolam 1′-hydroxylation (V_{max}/K_m, 0.74 mL/min/mg protein) with substrate inhibition kinetic, compared with those of human and cynomolgus monkey (V_{max}/K_m, 0.38 and 0.33 mL/min/mg protein, respectively) (Table 3 and Fig. 6). Marmoset P450 3A4 orthologue enzyme was the most effective catalytic enzyme (V_{max}/K_m, 14 mL/min/nmol) for midazolam 1′-hydroxylation with substrate inhibition kinetic among the marmoset, cynomolgus monkey, and human P450 3A enzymes, and showed low K_m value (2.5 μM), comparable to liver microsomes (8.1 μM) and small intestine microsomes (6.6 μM) from marmosets. Similarly, kinetic analyses indicated that marmoset liver microsomes effectively catalyzed nifedipine oxidation (V_{max}/K_m, 0.24 mL/min/mg protein) comparable to those from human and cynomolgus monkey (V_{max}/K_m, 0.25 and 0.35 mL/min/mg protein, respectively) (Table 4 and Fig. 6). Marmoset P450 3A4 orthologue enzyme also had high V_{max}/K_m (2.3 mL/min/nmol) for nifedipine oxidation and showed low K_m value (33 μM), roughly corresponding to those of liver microsomes (42 μM) and small intestine microsomes (84 μM) from marmosets. V_{max}/K_m values of marmoset P450 3A5 orthologue and 3A90 enzymes for midazolam 1′- and 4-hydroxylation and nifedipine oxidation were of different order of magnitude, compared with that of marmoset P450 3A4 orthologue.

Midazolam 1′-hydroxylation activities were significantly correlated with P450 3A4 orthologue contents (r = 0.76, $p < 0.05$, Fig. 7B), but not with P450 3A5 orthologue/3A90 contents (r = 0.23) in 23 individual marmoset liver microsomes (Fig. 7C). P450 3A4 orthologue, 3A5 orthologue/3A90, and sum of P450 3A contents in individual marmoset liver microsomes

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were 77-218 (average ± SD, 150 ± 45), 42-112 (average ± SD, 83 ± 18), and 137-321 (average ± SD, 233 ± 57) pmol/mg protein, respectively. The total contribution of P450 3A4 to P450 3A protein assumed to be 64 ± 7 % (range of 51 to 74 %). These results indicated that P450 3A4 orthologue was the major hepatic P450 3A enzyme in marmosets, similar to humans (Westlind-Johnsson et al., 2003). Hydroxylation activities of midazolam and bufuralol were also observed (Fig. 7D). Moreover, bufuralol 1´-hydroxylation activities in marmoset liver microsomes were correlated with midazolam 1´-hydroxylation activities ($r = 0.81$, $p < 0.01$, Fig. 7D), marmoset P450 2D contents ($r = 0.50$, $p < 0.05$, Fig. 7E), and sum of P450 3A contents ($r = 0.83$, $p < 0.01$, Fig. 7F), although bufuralol 1'-hydroxylation by cDNA-expressed marmoset P450 3As lower than cDNA-expressed marmoset P450 2D6 (a major hepatic P450 2D enzyme) (Table 2). When considered together with high levels of P450 3A proteins in livers, P450 3A enzymes might play another important role for bufuralol 1´-hydroxylation by marmoset livers.

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Discussion

In marmosets, three *P450 3A* genes were identified (Qiu et al., 2008); however, the molecular characteristics have not been clarified. In this study, we investigated the sequence identity, the tissue distribution, and the enzymatic function of marmoset P450 3A forms. Marmoset P450 3A forms were highly identical (>88%) to the human and cynomolgus monkey P450 3A orthologues (Table 1). A phylogenetic tree created using amino acid sequences showed that marmoset P450 3A forms were clustered with P450 3A forms from other primates, different from P450 3A forms found in preclinical animal species including dogs, pigs, and rodents (Fig. 3). The high similarity of P450 3A amino acid sequences between marmosets, cynomolgus monkeys, and humans suggested a possibility that the enzymatic function of P450 3A was conserved among these primate species.

Among the marmoset P450 3A enzymes, P450 3A4 orthologue most abundantly expressed in livers and small intestines effectively catalyzed the oxidation of midazolam and nifedipine (Table 3 and 4), and showed the significant correlation between P450 3A4 orthologue contents and midazolam 1′-hydroxylation activities in 23 individual marmoset liver microsomes (r = 0.76, $p < 0.05$; Fig. 7B), suggesting that P450 3A4 orthologue was the major catalyst for P450 3A-dependent drug metabolism in livers and small intestines. Moreover, K_m, V_{max}, and V_{max}/K_m values for the oxidation of midazolam and nifedipine were similar between marmoset, cynomolgus monkey, and human P450 3A4 enzymes. By analysis of site-directed mutagenesis based on a three-dimensional homology model of human P450 3A4, Phe-108, Ser-119, Ile-120, Leu-211, Asp-214, Ile-301, Phe-304, Ala-305, Thr-309, Ala-370, and Leu-373 were identified as key residues for substrate binding and regioselectivity (He et al., 1997; Fowler et al., 2000; Fowler et al., 2002; Khan et al., 2002). These amino acid residues on human P450 3A4 were

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completely shared with marmoset and cynomolgus monkey P450 3A4 orthologue, suggesting that the enzymatic function of P450 3A4 orthologue enzyme was highly conserved between marmosets, cynomolgus monkeys, and humans. Therefore, marmosets might be a suitable model for evaluating the P450 3A-dependent drug metabolism in preclinical studies.

Oxidation of bufuralol, a typical human P450 2D probe, were fast in marmoset livers than human livers (Table 2). Bufuralol 1′-hydroxylation activities were also higher for marmoset P450 3A enzymes than human P450 3A enzymes, although those activities by marmoset P450 2D6 enzyme (major P450 2D enzyme responsible for bufuralol 1′-hydroxylation in marmoset liver in term of expression level and enzyme kinetics) were comparable to human P450 2D6 (Uehara et al., 2015a). In correlation analyses with 23 individual marmoset liver microsomes (Fig. 7) suggested contributions of P450 3A enzymes to 1′-hydroxylation of midazolam (human P450 3A probe) and bufuralol (human P450 2D6 probe). Similarly, cynomolgus monkey P450 3A4 and/or 3A5 enzymes showed higher velocity than human P450 3A enzymes for bufuralol 1′-hydroxylation and dextromethorphan *O*-dealkylation (Emoto et al., 2011; Selvakumar et al., 2014). Hence, P450 3A enzymes might account for the higher velocity of bufuralol 1′-hydroxylation in marmoset and cynomolgus monkey livers.

In addition to drugs, P450 enzymes have various important physiological functions including the metabolism of steroids, bile acids, vitamins, and prostaglandins (Nebert and Dalton, 2006). In humans, P450 3A4 play major roles in testosterone 16β-, 6β-, and 2β-hydroxylation and progesterone 16α-, 6β-, and 2β-hydroxylation (Yamazaki and Shimada, 1997; Niwa et al., 2015). Progesterone metabolism in humans is most similar to those in cynomolgus monkeys and least similar to those in rats (Swinney, 1990). In this study, P450 3A4 orthologue, 3A5 orthologue, and 3A90 enzymes catalyzed testosterone 6β-hydroxylation, like human P450 3A enzymes (Table 2). For understanding the physiological similarity between marmosets and

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humans, further study is needed to investigate the role of P450 enzymes for the metabolism of various endogenous compounds.

In humans, P450 3A4 and 3A5 mRNAs are abundant in livers and small intestines among the ten human tissues (Nishimura et al., 2003), and these proteins are expressed in human livers and small intestines (Gibbs et al., 1999; Paine et al., 2006). Marmoset P450 3A4 orthologue, 3A5 orthologue, and 3A90 mRNAs and proteins were also expressed in livers and small intestines among the five tissues (Figs. 4 and 5), suggesting a possibility that the basal transcriptional regulation of *P450 3A* genes was shared between marmosets and humans. Indeed, the sequences of the proximal promoter and the xenobiotic-responsive enhancer module (XREM) of marmoset *P450 3A4* orthologue gene are highly identical (88%) to those of human *P450 3A4* gene (Koehler et al., 2006). Several putative transcriptional factor-binding sites conserved between marmoset P450 3A4 orthologue and human P450 3A4 promoter, including CCAAT/enhancer-binding protein binding site in the proximal promoter and hepatic nuclear factor-4 binding site in XREM, might play an important role for the basal transcriptional regulation. The similarity of tissue expression patterns possibly accounted for by common transcriptional regulation in *P450 3A* genes suggests that the marmoset, again, would be potentially a suitable model for preclinical safety testing in relation to P450 3A enzymes.

Marmoset *P450 3A* cluster contained two genes orthologous to human *P450 3A5* genes, *P450 3A5* orthologue and *3A90* (Fig. 1). By phylogenomics analysis of primate *P450 3A* locus structure, marmoset *P450 3A4* orthologue shared a common ancestry with catarrhine *P450 3A4*, while marmoset *P450 3A5* orthologue and *3A90* shared a common ancestry with catarrhine *P450 3A5*, suggesting that *P450 3A5* expanded in only New World Monkeys, in contrast to the

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repeated duplication of *P450 3A4-like* gene (*P450 3A4*, *3A7*, and *3A67*) in catarrhines (Qiu et al., 2008). In comparison with recombinant marmoset P450 3A4 orthologue, recombinant marmoset 3A5 orthologue and 3A90 moderately metabolized multiple typical human P450 3A probe substrates, such as midazolam, alprazolam, nifedipine, and testosterone (Table 2). It would be of great interest to compare the physiological significance of P450 3A5 orthologue and 3A90 with that of P450 3A4 orthologue in marmosets by exhaustive analysis of substrate specificity using xenobiotics and endogenous compounds.

In conclusion, amino acid sequences of three marmoset P450 3A forms showed high sequence identities (>87%) with P450 3A forms of cynomolgus monkeys, great apes, and humans, and phylogenetically had the close relationship with the human counterparts. P450 3A4 orthologue mRNA was abundant in marmoset livers and small intestines among three P450 3A mRNAs; P450 3A4 orthologue and 3A5 orthologue/3A90 proteins were also detected in these organs contributing to the drug metabolism. Recombinant P450 3A4 orthologue, 3A5 orthologue, and 3A90 enzymes prepared in *Escherichia coli* membranes catalyzed typical human P450 3A probe substrates, suggesting that P450 3A function was highly conserved between marmosets and humans. The significant correlation relationship between P450 3A4 orthologue contents and midazolam 1′-hydroxylation activities in marmoset livers showed that P450 3A4 orthologue enzyme greatly contributed to midazolam 1′-hydroxylation in marmoset liver microsomes. These results indicated that marmoset P450 3A forms have functional similarities with those of humans in terms of tissue expression and enzymatic properties.

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Acknowledgments

The authors thank Drs. Norie Murayama and Makiko Shimizu for their technical help and Mr. Lance Bell for his advice on English writing.

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Authorship contribution

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

Conducted experiments: Uehara, Uno, Nakanishi, and Ishii.

Contributed new reagents or analytic tools: Inoue and Sasaki.

Performed data analysis: Uehara, Uno, and Yamazaki.

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 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 the Japan Society for the Promotion of Science Grant-in-Aid for Young Scientists B [15K18934].

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Legends for figures

Fig. 1. *P450 3A* cluster in marmosets, cynomolgus monkeys, and humans.

The structure of *P450 3A* gene cluster in marmoset, cynomolgus monkey, and human genome was analyzed by BLAT. Three marmoset *P450 3A* genes were located adjacent to *ZSCAN25* and *TRIM4* in the genome region corresponding to human and cynomolgus *P450 3A* genes. This schematic diagram is not proportionate to actual size and distance on the chromosome.

Fig. 2. Multiple alignment of P450 3A amino acid sequences from marmosets, cynomolgus monkeys, and humans.

P450 3A amino acid sequences from marmosets, cynomolgus monkeys, and humans were aligned using Genetyx. Asterisks and dots under amino acid alignment indicate regions conserved and roughly conserved among the three species, respectively. Solid and broken lines indicate substrate recognition sites and heme-binding domain, respectively.

Fig. 3. A phylogenetic tree of P450 3A amino acid sequences in various species.

Phylogenetic analysis was performed using P450 3A amino acid sequences of marmoset (cj), human (h), chimpanzee (chim), orangutan (ora), cynomolgus monkey (mf), rhesus monkey (mm), dog (d), pig (p), rabbit (rab), guinea pig (cp), rat (r), and mouse (m) by the neighbor-joining method. Human P450 2D6 was used as an outgroup. Three marmoset P450 3A forms are shown in bold. The scale bar indicates the evolutionary distance of 0.1 amino acid substitutions per site.

Fig. 4. Tissue distribution of P450 3A mRNAs in five marmoset tissues.

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Expression levels of marmoset P450 3A4 orthologue, 3A5 orthologue, and 3A90 mRNAs were normalized to 18S rRNA level in each tissue (a pool of twelve marmosets, six males and six females). For graphical presentation, P450 3A4 orthologue mRNA level was adjusted to 1, and the relative expression level of other P450 3A mRNAs to P450 3A4 orthologue mRNA are shown. Data are mean ± standard deviation values of triplicate determinations.

Fig. 5. Immunoblots of marmoset P450 3A proteins in marmoset tissues.

Recombinant marmoset P450 3A proteins (1.0 pmol) were selectively detected by immunoblotting using human P450 3A4 and 3A5 antibodies (A). Expression levels of P450 3A proteins in marmoset tissue microsomes (10 μg) (B) and individual liver microsomes (lanes 1, 2, and 5, males; lanes 3 and 4, females) (C) were investigated. Protein disulfide isomerase (PDI) was used as a loading control.

Fig. 6. Enzyme kinetics for midazolam hydroxylation and nifedipine oxidation by recombinant marmoset, cynomolgus monkey, and human P450 3A enzymes and liver and small intestine microsomes from marmosets, cynomolgus monkeys, and humans.

Kinetic analyses were performed for midazolam 1′- (closed) and 4- (open) hydroxylation and nifedipine oxidation by liver (A and B) and small intestine (C and D) microsomes from marmosets (triangles), cynomolgus monkeys (squares), and humans (circles). Kinetic analyses were performed for 1′- (closed) and 4- (open) midazolam hydroxylation and nifedipine oxidation by recombinant P450 3A of marmosets (E and F; P450 3A4 orthologue, circles; P450 3A5 orthologue, squares; P450 3A90, triangles), cynomolgus monkeys (G and H; P450 3A4, circles; P450 3A5, squares), and humans (I and J; P450 3A4, circles; P450 3A5, squares).

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Fig. 7. Correlations between activities of midazolam and bufuralol 1´-hydroxylation and P450 3A and 2D contents in liver microsomes from 23 marmosets.

P450 3A4 orthologue (B), P450 3A5 orthologue /90 (C), and P450 2D (E) contents were estimated based on the immunochemically determined data. Sum of P450 3A (P450 3A4 orthologue + P450 3A5 orthologue/3A90) (A, F) in 23 individual marmoset liver microsomes were also calculated. Midazolam and bufuralol 1´-hydroxylation activities in individual marmoset liver microsomes were measured in duplicates at substrate concentrations of 5.0 and 1.0 μM, respectively.

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Table 1

Similarity of amino acid sequences of three marmoset P450 3A forms compared with P450 3As from other species

Species	P450 form	Marmoset P450		
		3A4 orthologue	3A5 orthologue	3A90
			%	
Human	3A4	90	83	82
	3A5	84	89	88
	3A7	86	79	78
	3A43	76	73	74
Chimpanzee	3A4	90	83	82
	3A5	85	89	88
	3A7	87	80	79
Orangutan	3A67	87	80	80
	3A43	76	74	74
Cynomolgus monkey	3A4	90	82	81
	3A5	83	89	88
	3A7	87	79	79
	3A43	76	73	74
Dog	3A12	78	77	77
	3A26	76	75	75
Pig	3A22	75	73	73
	3A29	75	75	73
	3A39	74	75	73
	3A46	75	74	72
Rabbit	3A6	75	75	74
Rat	3A2	71	70	70
	3A9	77	76	75
	3A18	69	69	68
	3A23	71	72	71
	3A62	71	72	71
Mouse	3A11	73	73	72
	3A13	75	75	74
	3A16	69	69	69
	3A25	71	70	69
	3A41	70	71	70
	3A44	69	69	69
	3A57	69	67	67
	3A59	70	69	68
Marmoset	3A4 orthologue	-	84	82
	3A5 orthologue	84	-	96
	3A90	82	96	-

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Table 2.

Drug-metabolizing activities by recombinant P450 enzymes and tissue microsomes from marmosets, cynomolgus monkeys, and humans

Enzyme source	Midazolam 1´-hydroxylation	Midazolam 4-hydroxylation	Alprazolam 4-hydroxylation	Nifedipine oxidation	Testosterone 6β-hydroxylation	Bufuralol 1´-hydroxylation	Bufuralol 1´-hydroxylation#
Microsomes							
Marmoset liver	3.6 ± 0.2	0.35 ± 0.03	0.11 ± 0.01	3.3 ± 0.2	1.6 ± 0.1	0.72 ± 0.02	-
Cynomolgus monkey liver	1.9 ± 0.1	0.49 ± 0.01	0.058 ± 0.002	3.8 ± 0.2	1.8 ± 0.3	-	1.7
Human liver	1.5 ± 0.1	0.13 ± 0.01	0.016 ± 0.002	2.2 ± 0.1	0.99 ± 0.01	0.24 ± 0.01	0.21
Marmoset small intestine	0.95 ± 0.02	0.075 ± 0.004	0.0080 ± 0.0001	0.32 ± 0.03	0.15 ± 0.02	-	-
Cynomolgus monkey small intestine	1.2 ± 0.1	0.29 ± 0.01	0.015 ± 0.001	1.1 ± 0.1	0.47 ± 0.01	-	-
Human small intestine	0.77 ± 0.01	0.082 ± 0.005	0.0085 ± 0.0002	1.2 ± 0.1	0.58 ± 0.01	-	-
Recombinant P450							
Marmoset 3A4 orthologue	29 ± 1	9.1 ± 0.6	1.4± 0.1	25 ± 1	58 ± 2	1.9 ± 0.2	-
Marmoset 3A5 orthologue	1.2 ± 0.1	0.051 ± 0.015	0.022± 0.005	1.1 ± 0.2	5.4 ± 0.3	1.4 ± 0.1	-
Marmoset 3A90	3.4 ± 0.4	0.11 ± 0.01	0.054± 0.006	2.8 ± 0.1	4.9 ± 0.7	0.81 ± 0.01	-
Marmoset 2D6	-	-	-	-	-	8.5 ± 0.9	-
Marmoset 2D8	-	-	-	-	-	1.9 ± 0.3	-
Cynomolgus monkey 3A4	18 ± 1	15 ± 1	1.5± 0.1	33 ± 3	50 ± 2	-	0.99
Cynomolgus monkey 3A5	36 ± 1	2.7 ± 0.3	0.78± 0.01	34 ± 2	60 ± 2	-	2.4
Cynomolgus monkey 2D6	-	-	-	-	-	-	8.9
Cynomolgus monkey 2D44	-	-	-	-	-	-	4.9
Human 3A4	11 ± 1	2.3 ± 0.1	0.74± 0.02	22 ± 2	20 ± 1	0.13 ± 0.01	0.41
Human 3A5	36 ± 2	1.4 ± 0.1	0.46± 0.01	7.8 ± 0.4	19 ± 1	0.24 ± 0.01	0.33
Human 2D6	-	-	-	-	-	5.8 ± 0.7	6.1

Units of drug oxidation rates by tissue microsomes and recombinant P450 proteins are nmol/min/mg protein and nmol/min/nmol of P450, respectively. Each activity was measured at a substrate concentration of 10 µM (midazolam), 200 µM (alprazolam), 20 µM (nifedipine), 50 µM (testosterone), and 100 µM (bufuralol). Values represent mean ± standard deviations from triplicate measurements. #Enzymatic activities for bufuralol 1´-hydroxylation by liver microsomes and recombinant P450 2D and 3A proteins at a substrate concentration of 200 µM are taken from Emoto et al. (2011).

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Table 3

Kinetic parameters of midazolam 1´- and 4-hydroxylation by recombinant P450 3A enzymes and tissue microsomes from marmosets, cynomolgus monkeys, and humans

Enzyme source	Midazolam 1´- hydroxylation				Midazolam 4- hydroxylation			
	K_m	V_{max}	V_{max}/K_m	K_S	K_m	V_{max}	V_{max}/K_m	K_S
Microsomes								
Marmoset liver	8.1 ± 0.5	6.0 ± 0.1	0.74	640 ± 50	37 ± 2	1.5 ± 0.1	0.041	-
Cynomolgus monkey liver	15 ± 1	5.0 ± 0.1	0.33	2200 ± 60	67 ± 4	4.2 ± 0.1	0.063	-
Human liver	6.6 ± 0.4	2.5 ± 0.1	0.38	1300 ± 130	64 ± 3	1.1 ± 0.1	0.017	-
Marmoset small intestine	6.6 ± 0.5	1.7 ± 0.1	0.26	3200 ± 820	33 ± 6	0.35 ± 0.02	0.011	-
Cynomolgus monkey small intestine	7.0 ± 0.2	2.1 ± 0.1	0.30	-	39 ± 1	1.4 ± 0.1	0.036	-
Human small intestine	1.7 ± 0.1	0.88 ± 0.01	0.52	930 ± 68	27 ± 2	0.34 ± 0.01	0.013	-
Recombinant P450								
Marmoset 3A4 orthologue	2.5 ± 0.4	35 ± 1	14	710 ± 150	21 ± 2	25 ± 1	1.2	-
Marmoset 3A5 orthologue	170 ± 44	3.7 ± 0.4	0.020	-	190 ± 21	4.7 ± 0.2	0.025	-
Marmoset 3A90	34 ± 1	16 ± 1		2000 ± 100	310 ± 21	3.4 ± 0.1	0.011	-
Cynomolgus monkey 3A4	2.5 ± 0.2	21 ± 1	8.4	600 ± 79	16 ± 1	40 ± 1	2.5	1200 ± 100
Cynomolgus monkey 3A5	3.2 ± 0.5	28 ± 1	8.8	1900 ± 680	8.2 ± 0.1	24 ± 1	2.9	-
Human 3A4	1.7 ± 0.2	12 ± 1	7.1	660 ± 100	25 ± 3	8.6 ± 0.5	0.32	2600 ± 1100
Human 3A5	3.8 ± 0.5	47 ± 2	12	1900 ± 560	30 ± 2	5.9 ± 0.1	0.20	-

Kinetic parameters were determined by non-linear regression analysis (mean ± standard error, n = 17 points of substrate concentrations of 0.5–625 µM) employing the equation, $v = V_{max} \times [S]/(K_m + [S])$ for Michaelis-Menten or $v = V_{max} \times [S]/(K_m + [S] + [S]^2/K_s)$ for substrate inhibition (Shimizu et al., 2007; Okada et al., 2009). Units of enzyme activities for tissue microsomes and recombinant P450 proteins are nmol/min/mg protein and nmol/min/nmol of P450, respectively. Units of V_{max}/K_m for tissue microsomes and recombinant P450 proteins are mL/min/nmol and mL/min/mg protein, respectively. The units of K_m and K_s values are µM.

Table 4

Kinetic parameters of nifedipine oxidation by recombinant P450 3A enzymes and tissue microsomes from marmosets, cynomolgus monkeys, and humans

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Enzyme source	K_m or S_{50}	n[a]	V_{max}	V_{max}/K_m
Microsomes				
Marmoset liver	42 ± 1	-	10 ± 1	0.24
Cynomolgus monkey liver	27 ± 1	1.4 ± 0.1	9.5 ± 0.2	0.35
Human liver	17 ± 1	1.1 ± 0.1	4.2 ± 0.1	0.25
Marmoset small intestine	84 ± 6	1.3 ± 0.1	2.2 ± 0.1	0.026
Cynomolgus monkey small intestine	37 ± 1	1.1 ± 0.1	3.2 ± 0.1	0.086
Human small intestine	24 ± 2	1.1 ± 0.1	2.6 ± 0.1	0.11
P450				
Marmoset 3A4 orthologue	33 ± 4	-	77 ± 3	2.3
Marmoset 3A5 orthologue	37 ± 10	1.5 ± 0.4	3.3 ± 0.4	0.089
Marmoset 3A90	190 ± 40	1.3 ± 0.1	54 ± 7	0.28
Cynomolgus monkey 3A4	31 ± 5	-	94 ± 5	3.0
Cynomolgus monkey 3A5	35 ± 4	1.4 ± 0.1	138 ± 7	3.9
Human 3A4	52 ± 2	1.6 ± 0.1	123 ± 2	2.4
Human 3A5	78 ± 6	2.3 ± 0.2	121 ± 7	1.6

[a]n represents the Hill coefficient. Kinetic parameters were determined by non-linear regression analysis (mean ± standard error, n = 12 points of substrate concentrations of 1.1–200 μM) employing the equation, $v = V_{max} \times [S]/(K_m + [S])$ for Michaelis-Menten or $v = V_{max} \times [S]^n/(S_{50}{}^n + [S]^n)$ for Hill equation (Emoto et al., 2001; Okada et al., 2009). Units of enzyme activities for tissue microsomes and recombinant P450 proteins are nmol/min/mg protein and nmol/min/nmol of P450, respectively. Units of V_{max}/K_m for tissue microsomes and recombinant P450 proteins are mL/min/nmol and mL/min/mg protein, respectively. The units of K_m and S_{50} values are μM.

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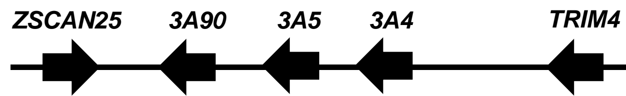
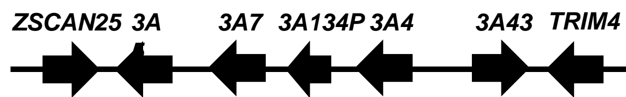
Fig. 1

Marmoset chromosome 2



Cynomolgus monkey chromosome 3



Human chromosome 7

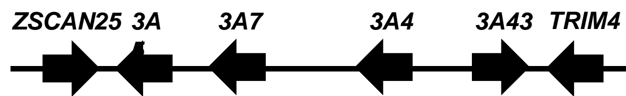


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Fig. 2

```
cjCYP3A4     1:MDFIPNLAVE TWLLLAVSLV LLYLYGTHSH GLFKKLGIPG PTPLPFLGTV LYYRQGFWKF DMECYKKYGK MWGIYDGRQP VLAITDPNII KTVLVKECYS 100
cjCYP3A5     1:MDLIPNLAVE TWLLLALSLV LLYLYGTRSH GIFKKLGIPG PAPLPFVGNI LSYRQGIWKF DSECHKKYGK MWGSYDGQLP VLAITDPDII KAVLVKECYS 100
cjCYP3A90    1:MDLIPNLAVE TWLLLALSLV LLYLYGTRSH GIFKKLGIPG PAPLPFVGNV LSYRQGIWKF DSECHKKYGK MWGSYDGQLP VLSITDPDMI KAVLVKECYS 100
mfCYP3A4     1:MDLIPDLAVE TWLLLAVTLV LLYLYGTHSH GLFKKLGIPG PTPLPLLGNI LSYRKGFWTF DMECYKKYGK VWGFYDGRQP VLAITDPNMI KTVLVKECYS 100
mfCYP3A5     1:MDLIPNLAME TWLLLAVSLV LLYLYGTRSY GLFKRQGIPG PTPLPFLGNI LSYRQGLWKF DTECYKKYGK MWRTQDGQLP VLTITDPDMI KTVLVKECYS 100
mfCYP3A7     1:MDLIPDLAVE TWLPLAVSLI LLYLYGTHSH GLFKKLGIPG PTPLPFFGNV LSFRKGFWTF DMECYKKYRK VWGFYDCRQP VLAITDPNMI KTVLVKECYS 100
mfCYP3A43    1:MDLIPNFAME TWVLVATSLV LLYIYGTHSH KLFKKVGIPG PTPLPFLGTI LFYLRGLWKF DRECNEKYGE MWGLYEGQQP MLVIMDPDMI KTVLVKECYS 100
hCYP3A4      1:MALIPDLAME TWLLLAVSLV LLYLYGTHSH GLFKKLGIPG PTPLPFLGNI LSYHKGFCMF DMECHKKYGK VWGFYDGQQP VLAITDPDMI KTVLVKECYS 100
hCYP3A5      1:MDLIPNLAVE TWLLLAVSLV LLYLYGTRTH GLFKRLGIPG PTPLPLLGNV LSYRQGLWKF DTECYKKYGK MWGTYEGQLP VLAITDPDVI RTVLVKECYS 100
hCYP3A7      1:MDLIPNLAVE TWLLLAVSLI LLYLYGTRTH GLFKKLGIPG PTPLPFLGNA LSFRKGYWTF DMECYKKYRK VWGIYDCQQP MLAITDPDMI KTVLVKECYS 100
hCYP3A43     1:MDLIPNFAME TWVLVATSLV LLYIYGTHSH KLFKKLGIPG PTPLPFLGTI LFYLRGLWNF DRECNEKYGE MWGLYEGQQP MLVIMDPDMI KTVLVKECYS 100
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               ______________________ SRS-1
cjCYP3A4   101:VFTNRRPFGP VGFMKSAISI AQDDEWKRIR SLLSPTFTSG KLKEMVPIIA QYGEVLVRNL RREAEKGKPI NMKDIFGAYS MDVITGTSFG VNIDSLNNPQ 200
cjCYP3A5   101:VFTNRRPFGP VGLMKSAISL AQDDEWKRIR SLLSPTFTSG KLKEMFPIIA QYGDVLVRNL RREAGKGKPV TMKDIFGAYS MDVITGTSFG VNIDSLNNPK 200
cjCYP3A90  101:IFTNRRPLGP VGFMKSAITV AQDDEWKRIR SLLSPTFTSG KLKEMFPIIA QYGDVLVRNL RREAEKGKLV TMKDIFGAYS MDVITGTSFG VNIDSLNNPK 200
mfCYP3A4   101:VFTNRRPFGP VGFMKNAISI AEDEEWKRIR SLLSPTFTSG KLKEMVPIIA KYGDVLVRNL RREAETGKPV TLKDVFGAYS MDVITSTSFG VNIDSLNNPQ 200
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mfCYP3A7   101:VFTNRRPLGP VGFMKSAITV AEDEEWKRIR SLLSPTFTSG KLKEMVPIIA QYGDVLVRNL RREAETGKPV TLKDVFGAYS MDVITSTSFG VNVDSLNNPQ 200
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hCYP3A5    101:VFTNRRSLGP VGFMKSAISL AEDEEWKRIR SLLSPTFTSG KLKEMFPIIA QYGDVLVRNL RREAEKGKPV TLKDIFGAYS MDVITGTSFG VNIDSLNNPQ 200
hCYP3A7    101:VFTNRRPFGP VGFMKNAISI AEDEEWKRIR SLLSPTFTSG KLKEMVPIIA QYGDVLVRNL RREAETGKPV TLKHVFGAYS MDVITSTSFG VSIDSLNNPQ 200
hCYP3A43   101:VFTNQMPLGP MGFLKSALSF AEDEEWKRIR TLLSPAFTSV KFKEMVPIIS QCGDMLVRSL RQEAENSKSI NLKDFFGAYT MDVITGTLFG VNLDSLNNPQ 200
               .***....** .*..*.*..  *.*.****.* .****.***. *.***.***. ..*..***.* *.**. .*.. ..*. ****. *****.*.** *..******.
                 _________ SRS-2                                    _______ SRS-3                                 _______
cjCYP3A4   201:DPFVESTKKL LRFDFLDPFF LSITIFPFLT PILEALNISM FPRDSTSFLR KSIKRIKESR LKDTHKHRVD FLQLMIDSQN SKETESDKAL SDLELVAQSI 300
cjCYP3A5   201:DPFVESVKKF LKFDFLDPLF LSTIFFPFLT PVFEALNFSL FPKDAINFLK QSVNRMKKSR LNDKQKHRVD FLQLMIDSQN SNETASHKAL SDLELLAQSI 300
cjCYP3A90  201:DPFVENVKKF LKFDVLAPLF LSVVLFPFLI PVFEALNFSL FPKDAINFLK QSVNRMKKSR LNDKQKHRVD FLQLMIDSQN SNETASHKAL SDLELVAQSI 300
mfCYP3A4   201:DPFVENTKKL LRFDFLDPFF LSITIFPFII PILEVLNISI FPREVTSFLR KSVKRIKESR LKDTQKHRVD FLQLMIDSQN SKETESHKAL SDLELVAQSI 300
mfCYP3A5   201:DPFVESVKKF LKFDFLDPLF LLTILFPFLI PAFEALNVSL FPKDAINFLN KSVNSMKKSR LNDKQKHRVD FLQLMIDSQN SKETESHKAL SDQELVAQSI 300
mfCYP3A7   201:DPFVENTKKL LRFNSLDPFF LSIKIFPFLT PIFEALNITV FPRKVTSFLR KSVKRIKEGR LKETQKRRVD FLQLMIDSQN SKDTASHKVL SDLELMAQSI 300
mfCYP3A43  201:DPFLKNMKKL LKLDFLDPFL LSISLFPFLT PVFEVLNIGL FPKDVTRFLK NSIERMKESR LKDKQKHRVD FFQQMIDSQN SKETKSHKAL SDLELVAQSI 300
hCYP3A4    201:DPFVENTKKL LRFDFLDPFF LSITVFPFLI PILEVLNICV FPREVTNFLR KSVKRMKESR LEDTQKHRVD FLQLMIDSQN SKETESHKAL SDLELVAQSI 300
hCYP3A5    201:DPFVESTKKF LKFGFLDPLF LSIILFPFLT PVFEALNVSL FPKDTINFLS KSVNRMKKSR LNDKQKHRLD FLQLMIDSQN SKETESHKAL SDLELAAQSI 300
hCYP3A7    201:DPFVENTKKL LRFNPLDPFV LSIKVFPFLT PILEALNITV FPRKVISFLT KSVKQIKEGR LKETQKHRVD FLQLMIDSQN SKDSETHKAL SDLELMAQSI 300
hCYP3A43   201:DPFLKNMKKL LKLDFLDPFL LLISLFPFLT PVFEALNIGL FPKDVTHFLK NSIERMKESR LKDKQKHRVD FFQQMIDSQN SKETKSHKAL SDLELVAQSI 300
               ***....**. *....*.*.. *..  ***.. * .*.**... **.... **  .*. ..*..* *....*.*.* *.*.****** *......*.* **.**.****
               ___________ SRS-4                                                       ________ SRS-5
cjCYP3A4   301:IFIFAGYETT SSTLSFIMYE LATHPDVQQK LQEEIDAVLP NKAPATYDTV LQMEYLDMVV NETLRLFPLA MRLERVCKKD VEINGVFIPK GVVVMIPSYA 400
cjCYP3A5   301:IFIFAGYETT SSVLSFTIYE LATNPDVQQK LQEEIDVVLP NKAPATYDAV VQMEYLDMVV NETLRLYPIA VRLERVCKKD VEINGVFIPK GALVVIPTYA 400
cjCYP3A90  301:IFIFAGYETT SSVLSFTIYE LATNPDVQQK LQEEIDVVLP NKAPATYDAV VQMEYLDMVV NETLRLYPIT VRHERVCKKD VEINGVFIPK GALVVIPTYA 400
mfCYP3A4   301:IFIFAGYETT SSVLSFIIYE LATHPDVQQK LQEEIDTVLP NKAPPTYDTV LQMEYLDMVV NETLRIFPIA MRLERVCKKD VEINGIFIPK GVVVMIPSYA 400
mfCYP3A5   301:IFIFAGYETT SSVLSFIIYE LATHPDVQQK LQKEIDAVLP NKAPATYDAM VQMEYLDMVV NETLRLFPIA IRLERACKKD VEINGVFIPK GAMVVIPTYA 400
mfCYP3A7   301:MFIFAGYETT SSVLSFIIYE LATHPDVQQK LQKEIDTVLP NKAPPTYDTM LQMEYLDMVV NETLRLFPVA MRLERVCKKD VEINGMSIPK GVVVMIPSYV 400
mfCYP3A43  301:IIIFAAYDTT STTLPFIMYE LATHPDVQQK LQEEIDAVLP NKATVTYDAL VQMEYLDIVV NETLRLFPVV SRVTRVCKKD IEINGVFIPK GLAVMVPIYA 400
hCYP3A4    301:IFIFAGYETT SSVLSFIMYE LATHPDVQQK LQEEIDAVLP NKAPPTYDTV LQMEYLDMVV NETLRLFPIA MRLERVCKKD VEINGMFIPK GVVVMIPSYA 400
hCYP3A5    301:IFIFAGYETT SSVLSFTLYE LATHPDVQQK LQKEIDAVLP NKAPPTYDAV VQMEYLDMVV NETLRLFPVA IRLERTCKKD VEINGVFIPK GSMVVIPTYA 400
hCYP3A7    301:IFIFAGYETT SSVLSFIIYE LATHPDVQQK VQKEIDTVLP NKAPPTYDTV LQLEYLDMVV NETLRLFPVA MRLERVCKKD VEINGMFIPK GVVVMIPSYV 400
hCYP3A43   301:IIIFAAYDTT STTLPFIMYE LATHPDVQQK LQEEIDAVLP NKAPVTYDAL VQMEYLDMVV NETLRLFPVV SRVTRVCKKD IEINGVFIPK GLAVMVPIYA 400
               ..***.*.** *..*.*..** ***.****** .*.***.*** ***. ***.. .*.****.** *****..* .  *..*.**** .****..*** *  *..* *.
                                                ---------                                      __________ SRS-6
cjCYP3A4   401:LHYDPKYWTE PEKFLPERFS K-NNKDNIDP YIYTPFGTGP RNCIGMRFAL MNMKLALIRV LQNFSFKPCK ETQIPLKLRL GGLLQTEKPI VLKVEPRDGT 499
cjCYP3A5   401:LHHDPKYWTE PKEFRPERFS K-KNKDSIDP YIYTPFGTGP RNCIGMRFAL MNMKLALIRV LQNFSFKPCK ETQIPLKLGV QGLLQAEKPI ILKVESRDGT 499
cjCYP3A90  401:LHHDPKYWTE PKEFRPERFS K-KNKDSIDP YIYTPFGTGP RNCIGMRFAL MNMKLALIRV LQNFSFKPCK ETQIPLKLGV QGILQAEKPI VLKVESRDGT 499
mfCYP3A4   401:LHHDPKYWPE PEKFLPERFS K-KNNDNIDP YIYTPFGSGP RNCIGMRFAL MNMKLAIIRV LQNFSFKPCK ETQIPLKLRL GGLLQTEKPI VLKIESRDGT 499
mfCYP3A5   401:LHHDPKYWTE PEEFRPERFS K-KNKDSIDP YIYTPFGSGP RNCIGMRFAL MNMKLAIIKV LQNFSFKPCK ETQIPLKLGN QGLLQSEKPI VLKVESRDGT 499
mfCYP3A7   401:LHHDPKYWTE PEKFLPERFS K-KNKDNIDP YIYTPFGNGP RNCIGMRFAL MNMKLALIRV LQNFSFKPCK ETQIPLKLSF GGLLKTEKPI VLTAESRDGT 499
mfCYP3A43  401:LHHDPKYWTE PEKFCPER-F SKKNKDSIDP YRYIPFGAGP RNCIGMRFAL TNIKLAVIKA LQNFSFEPCE ETQIPLKLNN LPILQPEKPI VLKVHLRDGI 499
hCYP3A4    401:LHRDPKYWTE PEKFLPERFS K-KNKDNIDP YIYTPFGSGP RNCIGMRFAL MNMKLALIRV LQNFSFKPCK ETQIPLKLSL GGLLQPEKPV VLKVESRDGT 499
hCYP3A5    401:LHHDPKYWTE PEEFRPERFS K-KKDS-IDP YIYTPFGTGP RNCIGMRFAL MNMKLALIRV LQNFSFKPCK ETQIPLKLDT QGLLQPEKPI VLKVDSRDGT 498
hCYP3A7    401:LHHDPKYWTE PEKFLPERFS K-KNKDNIDP YIYTPFGSGP RNCIGMRFAL VNMKLALVRV LQNFSFKPCK ETQIPLKLRF GGLLLTEKPI VLKAESRDET 499
hCYP3A43   401:LHHDPKYWTE PEKFCPESRF SKKNKDSIDL YRYIPFGAGP RNCIGMRFAL TNIKLAVIRA LQNFSFKPCK ETQIPLKLDN LPILQPEKPI VLKVHLRDGI 500
               **.*****.* *..* **... . .... **. *.*.*** ** ********** .*.***.... ******.**. ********     ..*. ***. .*....**..

cjCYP3A4   500:VSGA                                                                                                      503
cjCYP3A5   500:LSGE                                                                                                      503
cjCYP3A90  500:LSGE                                                                                                      503
mfCYP3A4   500:VSGA                                                                                                      503
mfCYP3A5   500:LSGE                                                                                                      503
mfCYP3A7   500:VSGA                                                                                                      503
mfCYP3A43  500:TSGP                                                                                                      503
hCYP3A4    500:VSGA                                                                                                      503
hCYP3A5    499:LSGE                                                                                                      502
hCYP3A7    500:VSGA                                                                                                      503
hCYP3A43   501:TSGP                                                                                                      504
                ** 
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Fig. 3

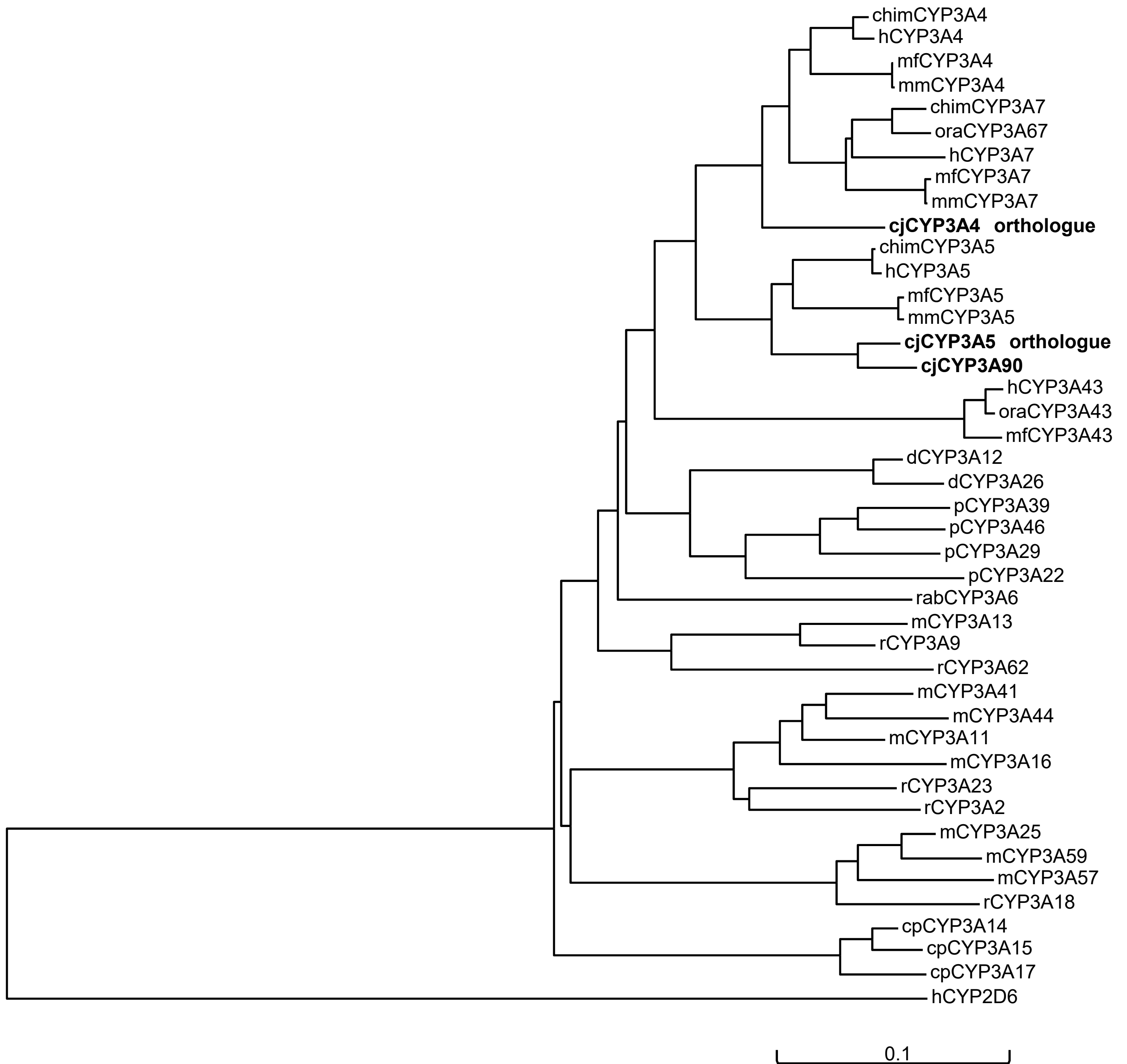

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Fig. 4

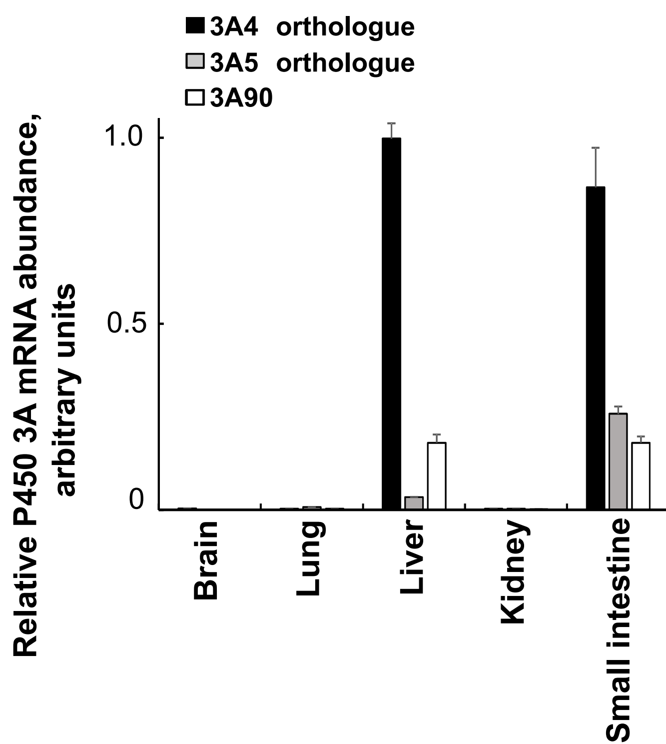

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Fig. 5

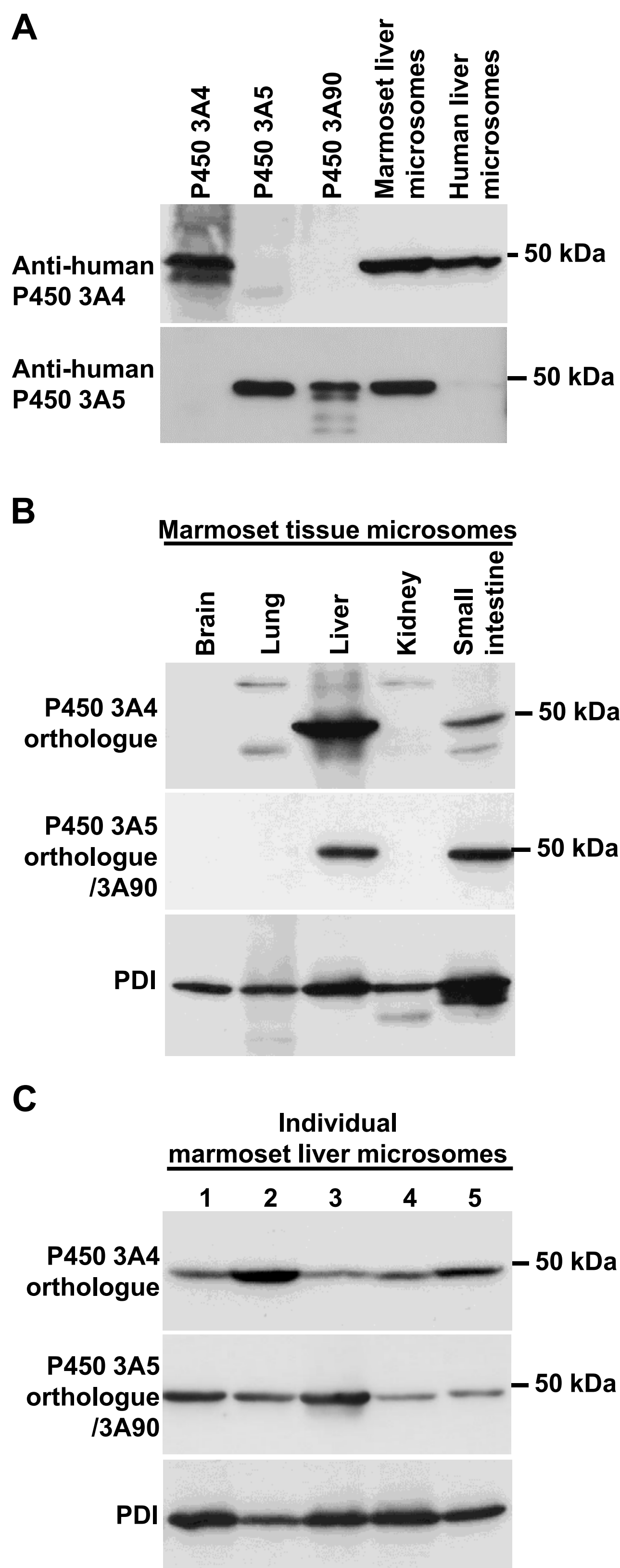

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Fig. 6

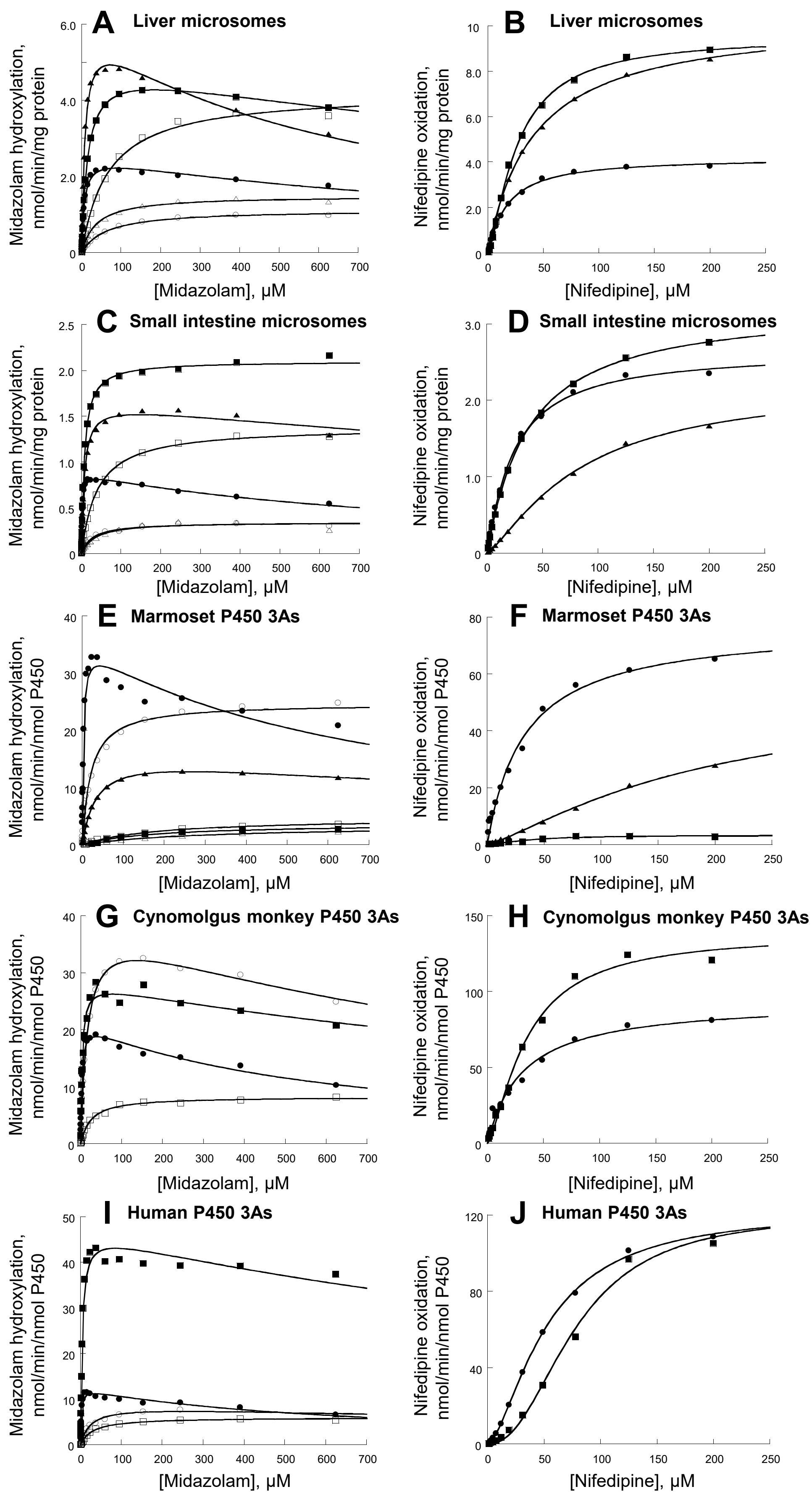

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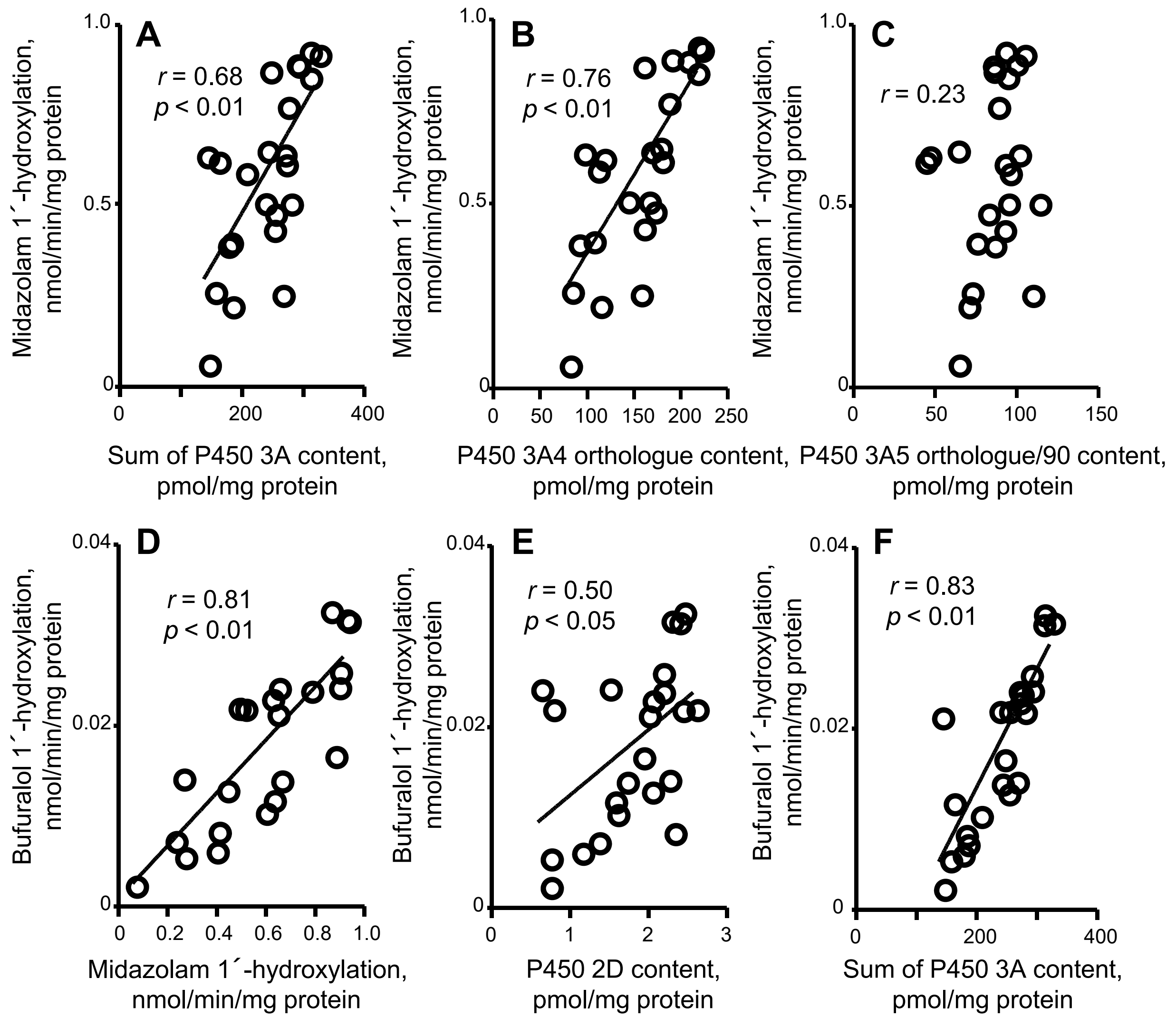
Fig. 7



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