

Short communication

Regio- and stereo-selective oxidation of a cardiovascular drug metoprolol mediated by cytochrome P450 2D and 3A enzymes in marmoset livers

Shotaro Uehara, Sakura Ishii, Yasuhiro Uno, Takashi Inoue, Erika Sasaki, and Hiroshi Yamazaki

Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan (SU, SI, and HY); Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories, Ltd., Kainan, Wakayama, Japan (YU); Department of Applied Developmental Biology (TI) and Center of Applied Developmental Biology (ES), Central Institute for Experimental Animals, Kawasaki, Japan; and Keio Advanced Research Center, Keio University, Minato-ku, Tokyo, Japan (ES)

Running Title Page

Running title: Metoprolol oxidation in marmoset livers

Correspondence author: Hiroshi Yamazaki, PhD, Professor

Showa Pharmaceutical University, 3-3165 Higashi-tamagawa Gakuen, Machida, Tokyo 194-8543, Japan. Phone: +81-42-721-1406; Fax: +81-42-721-1406. E-mail: hyamazak@ac.shoyaku.ac.jp

Number of Text Pages :	16
Number of Tables :	1
Number of Figures :	1
Number of References :	19
Number of Words in Abstract :	239
Number of Words in Introduction :	282
Number of Words in Discussion :	790

Abbreviations: P450, general term for cytochrome P450.

Abstract

A β -blocker metoprolol is one of the *in vivo* probes for human cytochrome P450 (P450) 2D6. Investigation of non-human primate P450 enzymes helps improve accuracy of the extrapolation of pharmacokinetic data from animals into humans. Common marmosets (*Callithrix jacchus*) are a potential primate model for preclinical research, but detailed roles of marmoset P450 enzymes in metoprolol oxidations remained unknown. In this study, regio- and stereo-selectivity of metoprolol oxidations by a variety of P450 enzymes in marmoset and human livers were investigated *in vitro*. Although liver microsomes from cynomolgus monkeys and rats preferentially mediated *S*-metoprolol *O*-demethylation and *R*-metoprolol α -hydroxylation, respectively, those from humans, marmosets, minipigs, and dogs preferentially mediated *R*-metoprolol *O*-demethylation, in contrast to slow rates of *R*- and *S*-metoprolol oxidations in mouse liver microsomes. *R*- and *S*-metoprolol *O*-demethylation activities in marmoset livers were strongly inhibited by quinidine and ketoconazole, and were significantly correlated with bufuralol 1'-hydroxylation and midazolam 1'-hydroxylation activities and also with P450 2D and 3A4 contents, different from the cases in human livers which did not have any correlations with P450 3A-mediated midazolam 1'-hydroxylation. Recombinant human P450 2D6 enzyme and marmoset P450 2D6/3A4 enzymes effectively catalyzed *R*-metoprolol *O*-demethylation, comparable to the activities of human and marmoset liver microsomes, respectively. These results indicated that the major roles of P450 2D enzymes for the regio- and stereo-selectivity of metoprolol oxidations were similar between human and marmoset livers, but the minor roles of P450 3A enzymes were unique to marmosets.

Introduction

Species similarities and differences for metabolic clearance of drug candidates are important issues for drug development. For predicting human toxicokinetic and pharmacokinetic profiles of drug candidates, non-human primates have been used because of their physiological and genetic similarity to humans (Orsi et al., 2011; Sasaki, 2015). Common marmosets (*Callithrix jacchus*), small New World Monkeys, are useful non-human primate species for preclinical testing due to their functional similarity of cytochrome P450 (P450) enzymes to those of humans (Uno et al., 2016). Recently, our studies demonstrated similar enzymatic properties of P450 3A and 2D enzymes (Uehara et al., 2015a; 2017b).

Metoprolol is a β -adrenergic blocking drug used in cardiovascular medicine. Racemic mixtures of metoprolol have been used for treating hypertension, angina pectoris, and arrhythmia, but the pharmacological effects reside in the *S*-enantiomer of metoprolol (Lennard et al., 1986). Metoprolol is metabolized through *O*-demethylation (65% of dose), α -hydroxylation (10% of dose), and *N*-dealkylation (10% of dose) in humans *in vivo* (Borg et al., 1975). The α -hydroxylation of metoprolol is catalyzed almost entirely, and *O*-demethylation of metoprolol catalyzed partially, through human P450 2D6 (Otton et al., 1988). Reportedly, human P450 2D6 preferentially catalyzed the *R*-enantiomer, faster than the *S*-enantiomer (Mautz et al., 1995). In our recent studies, marmoset P450 2D6 and 2D8 enzymes catalyzed metoprolol *O*-demethylation (Uehara et al., 2015a) indicating that the roles of marmoset P450 enzymes in metoprolol metabolism need to be further elucidated for preclinical studies. In this study, the role of P450s for regio- and stereo-selective metoprolol oxidations were evaluated in marmosets and humans. We report herein the high affinity of marmoset P450 2D enzymes with *R*-metoprolol *O*-demethylation and the high capacity of marmoset P450 3A enzymes for *R*- and *S*-metoprolol *O*-demethylation.

Materials and Methods

Detailed methods are shown separately in the Supplemental Data. Pooled liver microsomes from mice (CD-1, 8 males, aged 11 weeks), rats (Sprague–Dawley, 3 males), minipigs (Gottingen, 2 males, aged 7 months), dogs (10 males, aged >12 months), marmosets (5 males, sexually mature), and humans (74 males and 76 females, aged 18–82 years) were purchased from Corning Life Sciences (Woburn, MA). Pooled liver microsomes from cynomolgus monkeys (5 males, sexually mature) were purchased from Xenotech (Lenexa, KS). Liver microsomes from 17 individual human subjects were obtained from Corning Life Sciences. Individual liver microsomes were prepared from 23 marmosets (14 males and 9 females, aged >2 years) at the Central Institution for Experimental Animals (Kawasaki, Japan) in our laboratory as described previously (Uehara et al., 2017a). This study was reviewed and approved by the Institutional Animal Care and Use Committee (Central Institution for Experimental Animals).

Activities of metoprolol *O*-demethylation and α -hydroxylation by liver microsomes and recombinant P450s were measured as described previously (Uehara et al., 2015a) with some minor modifications. Pearson's product-moment correlation were performed with GraphPad Prism (La Jolla, CA) The kinetic parameters V_{\max} , K_m , and V_{\max}/K_m values were determined by fitting to an integrated form of the Michaelis-Menten equation using Kaleidagraph (Synergy Software, Reading, PA).

Results and Discussion

R- and *S*-metoprolol oxidation activities by liver microsomes from humans, cynomolgus monkeys, marmosets, minipigs, dogs, rats, and mice were determined at a substrate concentration of 1.0 μM (Fig. 1). Rat liver microsomes preferentially catalyzed *R*- and *S*-metoprolol α -hydroxylations rather than *O*-demethylations, in contrast to slow rates of *R*- and *S*-metoprolol oxidations in mouse liver microsomes. On the other hand, cynomolgus monkey liver microsomes effectively catalyzed *R*- and *S*-metoprolol *O*-demethylations rather than α -hydroxylations. Human, marmoset, and minipig liver microsomes showed roughly similar regio- and stereo-selective *R*- and *S*-metoprolol oxidation activities under the present conditions.

R- and *S*-metoprolol oxidation activities by human and marmoset recombinant P450 enzymes were determined at the substrate concentration of 10 μM (Supplemental Fig. 1). High catalytic activities of *R*-metoprolol *O*-demethylation by human and marmoset recombinant P450 2D6 enzymes were observed. Rates of *R*- and *S*-metoprolol *O*-demethylations by marmoset P450 3A4 and 3A90 were higher than those of human P450 3A4 and 3A5. Human and marmoset P450 2D6 enzymes also catalyzed *R*- and *S*-metoprolol α -hydroxylations at slow rates. Roles of P450 enzymes for the metoprolol *O*-demethylation by liver microsomes from humans and marmosets were investigated.

Correlations between *R*- and *S*-metoprolol *O*-demethylation activities and P450 probe oxidation activities in individual human and marmoset liver microsomes were determined (Supplemental Fig. 2). *R*- and *S*-metoprolol *O*-demethylation activities in human liver microsomes at substrate concentrations of 1.0 and 100 μM were significantly correlated to those of bufuralol 1'-hydroxylations ($r > 0.67$). A high correlation coefficient ($r = 0.91$) was observed for *R*-metoprolol *O*-demethylation activities at the substrate concentration of 1.0

μM . *R*- and *S*-metoprolol *O*-demethylation activities in marmoset liver microsomes at substrate concentrations of 1.0, 10, and 100 μM were also significantly correlated to bufuralol 1'-hydroxylation ($r > 0.77$). High correlation coefficients ($r > 0.83$) were observed for *R*- and *S*-metoprolol *O*-demethylation activities at low substrate concentrations of 1.0 or 10 μM . *R*- and *S*-metoprolol *O*-demethylation activities in marmoset liver microsomes at substrate concentrations of 1.0, 10, and 100 μM were also significantly correlated to those of midazolam 1'-hydroxylation ($r > 0.71$), different from the cases in human liver microsomes. In addition, *R*- and *S*-metoprolol *O*-demethylation activities in marmoset liver microsomes at the low substrate concentration of 1.0 μM were significantly correlated to P450 2D contents ($r > 0.63$) and P450 3A4 contents ($r > 0.69$) in marmoset liver microsomes (Supplemental Fig. 3).

Effects of P450 inhibitors for *R*-metoprolol *O*-demethylation liver microsomes from humans and marmosets were determined at the substrate concentration of 10 μM (Supplemental Fig. 4). Dose-dependent suppression by quinidine (human P450 2D6 inhibitor, 2.0–20 μM) was observed for *R*-metoprolol *O*-demethylation in human liver microsomes (less than 23% of control activities) and for *R*- and *S*-metoprolol *O*-demethylation in marmoset liver microsomes (less than 10% of control activities). *R*- and *S*-metoprolol *O*-demethylations in marmoset liver microsomes were also dose-dependently suppressed by 1.0–10 μM of ticlopidine [human P450 2C19 and 2D6 inhibitor, Ko et al. (2000)] to ~26% of control activities. *R*- and *S*-metoprolol *O*-demethylations in marmoset liver microsomes were suppressed to less than 24% in the presence of 100 μM of ketoconazole (human P450 3A inhibitor), in contrast to results showing that ketoconazole (100 μM) inhibited *R*- and *S*-metoprolol *O*-demethylations in human liver microsomes to half of control activities (Supplemental Fig. 4). Taken together, liver microsomal marmoset P450 2D6 effectively mediated *R*- and *S*-metoprolol *O*-demethylations in a similar manner to human liver

microsomal P450 2D6, but the roles of P450 3A enzymes for *R*- and *S*-metoprolol *O*-demethylations in liver microsomes were partially different between human and marmoset livers.

Kinetic analyses for *O*-demethylations and α -hydroxylations of *R*- and *S*-metoprolol were performed using liver microsomes from humans and marmosets and recombinant P450 enzymes (Supplemental Fig. 5 and Table 1). Liver microsomes from humans and marmosets showed low and high K_m values (humans, 15 and 190 μM ; marmosets, 14 and 120 μM) for *R*-metoprolol *O*-demethylations with similar V_{max} values (humans, 0.15 and 0.48 nmol/min/mg protein; marmosets, 0.63 and 0.59 nmol/min/mg protein), respectively; those two component K_m values for *R*-metoprolol *O*-demethylations were lower than K_m values (humans, 32 and 920 μM ; marmosets, 21 and 150 μM) for *S*-metoprolol *O*-demethylations. Liver microsomes from humans and marmosets indicated low K_m values (37 and 27 μM) for *R*-metoprolol α -hydroxylations with low V_{max} values (0.054 and 0.047 nmol/min/mg protein), compared with those for *S*-metoprolol α -hydroxylations. Liver microsomes from humans and marmosets showed high V_{max}/K_m values (humans, 0.010 and 0.0025 mL/min/mg protein; marmosets, 0.045 and 0.0049 mL/min/mg protein) for *R*-metoprolol *O*-demethylation, in both the high and low affinity components. High affinity for *R*-metoprolol *O*-demethylations in human liver microsomes and *R*- and *S*-metoprolol *O*-demethylations in marmoset liver microsomes suggested to be accounted for by P450 2D6 enzymes because the corresponding recombinant P450 2D6 enzymes had a K_m value of 11 μM for *R*-metoprolol *O*-demethylation and K_m values of 62 and 61 μM for *R*- and *S*-metoprolol *O*-demethylations, respectively. Recombinant human and marmoset P450 2D6 enzymes showed high V_{max}/K_m values (3.3 and 0.48 mL/min/nmol) for *R*-metoprolol *O*-demethylation compared with *S*-metoprolol *O*-demethylation. Marmoset P450 2D8 enzyme showed low V_{max}/K_m values (0.028 and 0.024 mL/min/nmol) for *R*- and *S*-metoprolol *O*-demethylations compared with marmoset P450

2D6 enzyme. Marmoset P450 3A4 showed high K_m values (280 and 290 μM) and low V_{max} values (0.43 and 0.46 mL/min/ nmol of P450) for *R*- and *S*-metoprolol *O*-demethylations. In contrast, K_m values for *R*- and *S*-metoprolol *O*-demethylations in human P450 3A enzymes and other enzyme sources were high (over $\sim 500 \mu\text{M}$) under the present conditions.

Oral administrations of metoprolol in cynomolgus monkeys and minipigs yielded plasma concentrations similar to their quantitative detection limits (Mogi et al., 2012; Shida, et al., 2015), suggesting rapid oxidative clearance of metoprolol *in vivo* in a similar manner to the *in vitro* oxidations in cynomolgus monkeys (Fig. 1). At a high concentration of 100 μM (not at 1.0 μM in Fig. 1), metoprolol oxidations by minipig liver microsomal P450 2D25 were faster than human liver microsomal P450 2D6 (Mogi et al., 2012; Yamazaki, 2014), resulting in low bioavailability of metoprolol after oral administration in minipigs. On the other hand, human plasma concentrations of P450 probes containing metoprolol can be reportedly extrapolated from the corresponding data in marmosets after oral administration using simplified physiologically based pharmacokinetic modeling with *in vitro* metabolic clearance data (Utoh et al., 2016).

In this study, liver microsomes from humans and marmosets preferentially mediated *R*-metoprolol *O*-demethylations rather than *S*-metoprolol *O*-demethylations (Fig. 1), different from cynomolgus monkeys. Stereoselectivity for *R*-metoprolol *O*-demethylation by marmoset liver microsomes was efficiently mediated by liver microsomal P450 2D6, as shown in this study (Supplemental Fig. 1 and Table 1), similar to reported human P450 2D-dependent *R*-metoprolol *O*-demethylation (Mautz et al., 1995). The calculated V_{max}/K_m values for *R*- and *S*-metoprolol *O*-demethylations in liver microsomes from humans and marmosets were comparable (Table 1), which showed stereoselective metoprolol *O*-demethylation in a similar manner to recombinant human and marmoset P450 2D6 enzymes (Supplemental Fig. 1). The

stereoselectivity for *R*-metoprolol *O*-demethylation by marmoset liver microsomal P450 2D enzymes was not as predominant as those of human liver microsomal P450 2D6 in the correlation analyses (Supplemental Figs. 2 and 3) and inhibition assays with quinidine (Supplemental Fig. 4). On the other hand, *R*- and *S*-metoprolol *O*-demethylation by marmoset liver microsomes was efficiently mediated by liver microsomal P450 3A enzymes significantly correlated to midazolam 1'-hydroxylation, and strongly suppressed by ketoconazole (different from the cases of human liver microsomes). It was suggested that the roles of liver microsomal P450 3A enzymes for *R*- and *S*-metoprolol *O*-demethylation were different between humans and marmosets. In terms of *R*- and *S*-metoprolol *O*-demethylation activities by marmoset liver microsomes, the metabolite formation rate per mg protein was high, compared with those of human liver microsomes (Fig. 1), possibly accounted for by marmoset P450 2D and 3A enzymes which equally contributed at low substrate concentrations as evident by the correlation analysis (Supplemental Figs. 2 and 3). In this study, metabolite formation from *R*- and *S*-metoprolol *in vitro* elucidated P450 2D- dependent enantioselective metabolism in marmoset livers (Table 1). Further study of plasma concentrations of metoprolol and the metabolites separately in marmosets *in vivo* would be of great interest.

Similarly, regio- and stereo-selective oxidation of a classical β -blocker propranolol in human, cynomolgus monkey, and marmoset livers have been investigated (Narimatsu et al., 2011). Propranolol enantiomers were *S*-enantiomer-stereoselectively oxidized by human P450 2D6, cynomolgus monkey P450 2D6 (2D17), and marmoset P450 2D6 enzymes mainly into 4-hydroxypropranolol, followed by 5-hydroxypropranolol and *N*-desisopropylpropranolol, but cynomolgus monkey P450 2D6 (2D17) had stronger high *N*-desisopropylpropranolol formation activity among these three primate P450 2D enzymes. These results, together with the results in the present study, suggest similar regio- and

stereo-selectivity of human and marmoset P450 2D6 enzymes.

Metoprolol pharmacokinetics in humans has been influenced by human *P450 2D6* genotype groups (Blake et al., 2013). There was a significant difference with nearly 40% lower *R*- than *S*-metoprolol in plasma concentrations between ultra-rapid and extensive metabolizers having two active *P450 2D6* alleles and two *P450 2D6* null alleles (Blake et al., 2013). Marmoset P450 enzymes show high sequence homology to their human counterparts (Uno et al., 2016) and genetic polymorphisms have recently been found in marmoset *P450 2C19* gene (Uehara et al., 2015b; 2016a). However, we have not found marmoset P450 2D6 non-synonymous variants even after screening the genomes of nearly 80 individual marmosets by direct sequence analysis (Uehara, unpublished data). If marmoset P450 2D6 variants might change the enzyme properties like marmoset P450 2C19 (Uehara et al., 2015b; 2016), marmosets harboring genetic variant(s) would be suitable models for enantioselective drug metabolism associated with polymorphic P450 2D enzyme in livers.

In conclusion, metoprolol oxidation activities were evaluated with respect to regio- and stereo-selective *R*- and *S*-metoprolol *O*-demethylations in humans and marmosets. The high affinities of human and marmoset P450 2D6 enzymes with respect to *R*-metoprolol *O*-demethylations and the capacities of marmoset liver microsomal P450 3A enzymes for *R*- and *S*-metoprolol *O*-demethylation were observed. The present results demonstrated that the major roles of marmoset liver microsomal P450 2D6 enzymes for the regio- and stereo-selective metoprolol oxidation were similar to human P450 2D6, but the minor roles of P450 3A enzymes were unique to marmosets. Therefore, marmosets may be a good model for P450 2D-dependent regio- and enantio-selective drug metabolism in preclinical studies.

Authorship contribution

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

Conducted experiments: Uehara and Ishii.

Contributed new reagents or analytic tools: Inoue and Sasaki.

Performed data analysis: Uehara, Ishii, and Yamazaki.

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

References

- Blake CM, Kharasch ED, Schwab M, and Nagele P (2013) A meta-analysis of CYP2D6 metabolizer phenotype and metoprolol pharmacokinetics. *Clin Pharmacol Ther* **94**:394-399.
- Borg KO, Carlsson E, Hoffmann KJ, Jonsson TE, Thorin H, and Wallin B (1975) Metabolism of metoprolol-(3-h) in man, the dog and the rat. *Acta Pharmacol Toxicol (Copenh)* **36**:125-135.
- Ko JW, Desta Z, Soukhova NV, Tracy T, and Flockhart DA (2000) In vitro inhibition of the cytochrome P450 (CYP450) system by the antiplatelet drug ticlopidine: potent effect on CYP2C19 and CYP2D6. *Br J Clin Pharmacol* **49**:343-351.
- Lennard MS, Tucker GT, and Woods HF (1986) The polymorphic oxidation of beta-adrenoceptor antagonists. Clinical pharmacokinetic considerations. *Clin Pharmacokinet* **11**:1-17.
- Mautz DS, Nelson WL, and Shen DD (1995) Regioselective and stereoselective oxidation of metoprolol and bufuralol catalyzed by microsomes containing cDNA-expressed human P4502D6. *Drug Metab Dispos* **23**:513-517.
- Mogi M, Toda A, Iwasaki K, Kusumoto S, Takahara H, Shimizu M, Murayama N, Izumi H, Utoh M and Yamazaki H (2012) Simultaneous pharmacokinetics assessment of caffeine, warfarin, omeprazole, metoprolol, and midazolam intravenously or orally administered to Microminipigs. *J Toxicol Sci* **37**:1157-1164.
- Narimatsu S, Nakata T, Shimizudani T, Nagaoka K, Nakura H, Masuda K, Katsu T, Koeda A, Naito S, Yamano S, Miyata A, and Hanioka N (2011) Regio- and stereoselective oxidation of propranolol enantiomers by human CYP2D6, cynomolgus monkey CYP2D17 and marmoset CYP2D19. *Chem Biol Interact* **189**:146-152.
- Orsi A, Rees D, Andreini I, Venturella S, Cinelli S, and Oberto G (2011) Overview of the marmoset as a model in nonclinical development of pharmaceutical products. *Regul Toxicol Pharmacol* **59**:19-27.
- Otton SV, Crewe HK, Lennard MS, Tucker GT, and Woods HF (1988) Use of quinidine inhibition to define the role of the sparteine/debrisoquine cytochrome P450 in metoprolol oxidation by human liver microsomes. *J Pharmacol Exp Ther* **247**:242-247.
- Sasaki E (2015) Prospects for genetically modified non-human primate models, including the common marmoset. *Neurosci Res* **93**:110-115.
- Shida S, Utoh M, Murayama N, Shimizu M, Uno Y and Yamazaki H (2015) Human plasma concentrations of cytochrome P450 probes extrapolated from pharmacokinetics in cynomolgus monkeys using physiologically based pharmacokinetic modeling. *Xenobiotica* **45**:881-886.

- Uehara S, Uno Y, Hagihira Y, Murayama N, Shimizu M, Inoue T, Sasaki E, and Yamazaki H (2015a) Marmoset cytochrome P450 2D8 in livers and small intestines metabolizes typical human P450 2D6 substrates, metoprolol, bufuralol and dextromethorphan. *Xenobiotica* **45**:766-772.
- Uehara S, Uno Y, Inoue T, Kawano M, Shimizu M, Toda A, Utoh M, Sasaki E, and Yamazaki H (2015b) Novel Marmoset Cytochrome P450 2C19 in Livers Efficiently Metabolizes Human P450 2C9 and 2C19 Substrates, S-Warfarin, Tolbutamide, Flurbiprofen, and Omeprazole. *Drug Metab Dispos* **43**:1408-1416.
- Uehara S, Kawano M, Murayama N, Uno Y, Utoh M, Inoue T, Sasaki E, and Yamazaki H (2016) Oxidation of R- and S-omeprazole stereoselectively mediated by liver microsomal cytochrome P450 2C19 enzymes from cynomolgus monkeys and common marmosets. *Biochem Pharmacol* **120**:56-62.
- Uehara S, Uno Y, Ishii S, Inoue T, Sasaki E, and Yamazaki H (2017a) Marmoset cytochrome P450 4A11, a novel arachidonic acid and lauric acid omega-hydroxylase expressed in liver and kidney tissues. *Xenobiotica*, in press.
- Uehara S, Uno Y, Ishii S, Inoue T, Sasaki E, and Yamazaki H (2017b) Marmoset cytochrome P450 3A4 orthologue expressed in liver and small intestine tissues efficiently metabolizes midazolam, alprazolam, nifedipine, and testosterone. *Drug Metab Dispos*, *Drug Metab Dispos* **45**:457-467.
- Uno Y, Uehara S, and Yamazaki H (2016) Utility of non-human primates in drug development: Comparison of non-human primate and human drug-metabolizing cytochrome P450 enzymes. *Biochem Pharmacol* **121**:1-7.
- Utoh M, Suemizu H, Mitsui M, Kawao M, Toda A, Uehara S, Uno Y, Shimizu M, Sasaki E and Yamazaki H (2016) Human plasma concentrations of cytochrome P450 probe cocktails extrapolated from pharmacokinetics in mice transplanted with human hepatocytes and from pharmacokinetics in common marmosets using physiologically based pharmacokinetic modeling. *Xenobiotica* **46**:1049-1055.
- Yamazaki H (2014) Species, Ethnic, and Individual Differences in Human Drug-Metabolizing Cytochrome P450 Enzymes, in *Fifty Years of CYtochrome P450 Research* (Yamazaki H ed) pp 293-305, Springer, Tokyo.

Footnotes

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].

Legends for figures

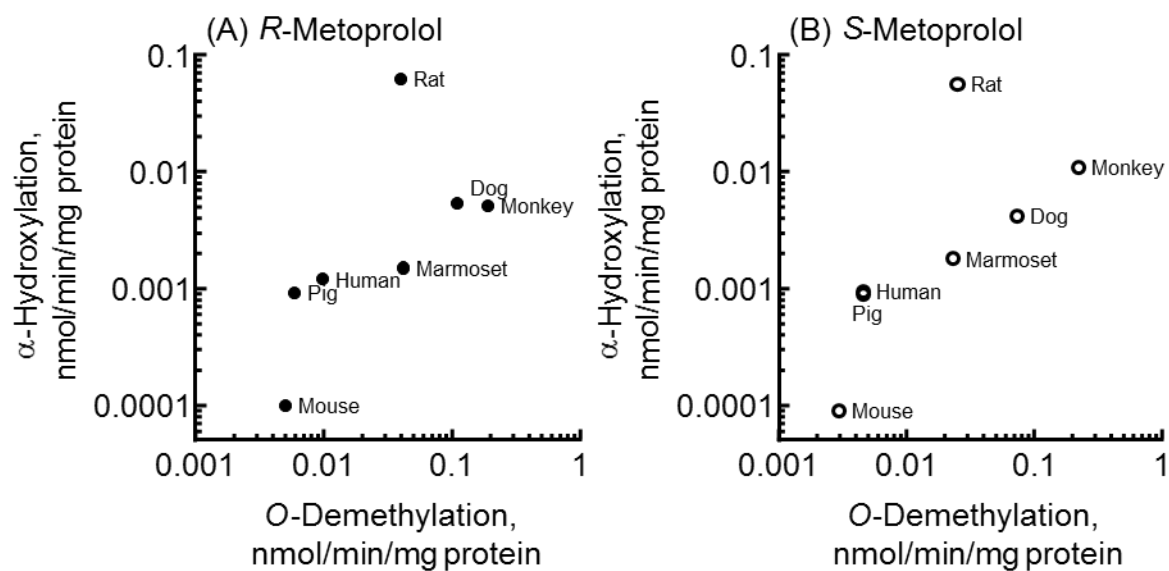
Fig. 1. *R*- and *S*-metoprolol oxidation activities by liver microsomes from humans, cynomolgus monkeys, marmosets, minipigs, dogs, rats, and mice. *R*- (A) and *S*- (B) metoprolol (1.0 μ M) were incubated with pooled liver microsomes (0.20 mg/mL) at 37°C for 15 min in the presence of an NADPH-generating system in triplicate determinations.

Table 1. Kinetic analyses for rates of *O*-demethylation and α -hydroxylation of *R*- and *S*-metoprolol by liver microsomes and recombinant P450s.

Enzymes	Reaction	<i>R</i> -metoprolol			<i>S</i> -metoprolol		
		K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Liver microsomes							
Human	<i>O</i> -Demethylation	15 ± 7 (K_{m1})	0.15 ± 0.06 (V_{max1})	0.010 (V_{max1}/K_{m1})	32 ± 27 (K_{m1})	0.12 ± 0.09 (V_{max1})	0.0038 (V_{max1}/K_{m1})
		190 ± 70 (K_{m2})	0.48 ± 0.04 (V_{max2})	0.0025 (V_{max2}/K_{m2})	920 ± 520 (K_{m2})	1.7 ± 0.4 (V_{max2})	0.0018 (V_{max2}/K_{m2})
	α -Hydroxylation	37 ± 3	0.054 ± 0.001	0.0015	40 ± 1	0.077 ± 0.001	0.0019
Marmoset	<i>O</i> -Demethylation	14 ± 6 (K_{m1})	0.63 ± 0.28 (V_{max1})	0.045 (V_{max1}/K_{m1})	21 ± 14 (K_{m1})	0.44 ± 0.33 (V_{max1})	0.021 (V_{max1}/K_{m1})
		120 ± 99 (K_{m2})	0.59 ± 0.24 (V_{max2})	0.0049 (V_{max2}/K_{m2})	150 ± 110 (K_{m2})	0.69 ± 0.28 (V_{max2})	0.0046 (V_{max2}/K_{m2})
	α -Hydroxylation	27 ± 2	0.047 ± 0.001	0.0017	46 ± 5	0.072 ± 0.003	0.0016
Recombinant P450							
Human P450 2D6	<i>O</i> -Demethylation	11 ± 1	36 ± 1	3.3	20 ± 1	26 ± 1	1.3
		α -Hydroxylation	14 ± 1	9.4 ± 0.2	0.67	9.3 ± 1.2	5.0 ± 0.1
Human P450 3A4	<i>O</i> -Demethylation	> 500	0.12 ^a	NA	> 500	0.045 ^a	NA
		α -Hydroxylation	> 500	0.084 ^a	NA	> 500	0.097 ^a
Human P450 3A5	<i>O</i> -Demethylation	> 500	0.11 ^a	NA	470 ± 180	0.13 ± 0.03	0.00028
		α -Hydroxylation	> 500	0.11 ^a	NA	> 500	0.12 ^a
Marmoset P450 2D6	<i>O</i> -Demethylation	62 ± 16	30 ± 3	0.48	61 ± 19	24 ± 2	0.39
		α -Hydroxylation	86 ± 23	1.5 ± 0.1	0.017	78 ± 24	1.9 ± 0.2
Marmoset P450 2D8	<i>O</i> -Demethylation	30 ± 9	0.84 ± 0.07	0.028	25 ± 5	0.59 ± 0.03	0.024
		α -Hydroxylation	> 500	0.024 ^a	NA	> 500	0.087 ^a
Marmoset P450 3A4	<i>O</i> -Demethylation	280 ± 110	0.43 ± 0.09	0.0015	290 ± 130	0.46 ± 0.10	0.0016
		α -Hydroxylation	> 500	1.1 ^a	NA	> 500	1.2 ^a
Marmoset P450 3A90	<i>O</i> -Demethylation	> 500	0.71 ^a	NA	> 500	0.57 ^a	NA
		α -Hydroxylation	370 ± 40	2.3 ± 0.2	0.0062	> 500	1.1 ^a

R- and *S*-metoprolol (1.0-500 μ M) were incubated with pooled liver microsomes (0.20 mg/mL) at 37°C for 15 minutes. Kinetic parameters were calculated from a fitted curve by non-linear regression (mean ± S.E.) with Michaelis-Menten equations: $v = V_{max} [S]/(K_m + [S])$. Units of enzyme activities for liver 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. ^a Catalytic activity at a substrate concentration of 500 μ M. NA: not available.

Figure 1



Supplemental Data

Regio- and stereo-selective oxidation of a cardiovascular drug metoprolol mediated by cytochrome P450 2D and 3A enzymes in marmoset livers

Shotaro Uehara, Sakura Ishii, Yasuhiro Uno, Takashi Inoue, Erika Sasaki, and Hiroshi Yamazaki

Drug Metabolism and Disposition

Supplemental Methods

Chemicals and enzymes

R- and *S*-metoprolol and α -hydroxymetoprolol were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); bufuralol and *O*-desmethylnmetoprolol from Toronto Research Chemicals (Toronto, ON, Canada); and ketoconazole, ticlopidine, quinidine, and N,N,N'-triethylenethiophosphoramidate (ThioTEPA) from Sigma-Aldrich (St. Louis, MO). Montelukast and α -naphthoflavone were purchased from Tokyo Kasei (Tokyo, Japan) and Wako Pure Chemicals (Osaka, Japan), respectively. Recombinant marmoset P450s were prepared by heterologous expression systems in *Escherichia coli* as described previously (Uehara et al., 2016a; Uehara et al., 2016b; Uehara et al., 2017). Recombinant human P450s expressed in baculovirus-infected insect cells were purchased from Corning Life Sciences. All other reagents used were the highest quality commercially available.

Analysis of *R*- and *S*-metoprolol oxidation

Activities of metoprolol *O*-demethylation and α -hydroxylation by liver microsomes and recombinant P450s were measured as described previously (Uehara et al., 2015a) with some minor

modifications. Briefly, a typical incubation mixture (0.25 mL) contained recombinant P450 protein (20 pmol equivalents/mL) or liver microsomes (0.20 mg/mL), an NADPH-generating system (0.25 mM NADP⁺, 2.5 mM glucose 6-phosphate, and 0.25 U/mL dehydrogenase), and 1.0-500 μ M *R*- and *S*-metoprolol in 100 mM potassium phosphate buffer (pH7.4). Effects of α -naphthoflavone (human P450 1A inhibitor), ThioTEPA (human P450 2B6 inhibitor), montelukast (human P450 2C8 inhibitor), ticlopidine (human P450 2C19 inhibitor), quinidine (human P450 2D6 inhibitor), and ketoconazole (human P450 3A inhibitor) were investigated at a substrate concentration of 10 μ M metoprolol. The reaction was started by adding the NADPH generating system, incubated at 37°C for 15 minutes, and terminated by adding 25 μ L of 60% HClO₄ (w/v). Reaction mixtures were centrifuged at 10,000g for 5 minutes; supernatants were analyzed directly by a Prominence-I LC-2030C high-performance liquid chromatography system with fluorescence (Shimadzu, Kyoto, Japan) at an excitation wavelength of 222 nm and an emission wavelength of 300 nm. Samples were injected into a reversed-phase C₁₈ column (Mightysil RP-18 GP, 5 μ m, 250 \times 4.6 mm; Kanto Chemical, Tokyo, Japan) with elution by 20% acetonitrile in 20 mM NaClO₄ (pH2.5) at 1.0 mL/minute. The retention times of α -hydroxymetoprolol, *O*-desmethylnmetoprolol, and metoprolol were 5.1, 5.5, and 17.1 min, respectively, under the present conditions. Bufuralol 1'-hydroxylation and midazolam 1'-hydroxylation activities by liver microsomes were measured as described previously (Uehara et al., 2015b). The quantitation of liver microsomal P450 2D and 3A proteins was performed by immunoblotting in a previous study (Uehara et al., 2017).

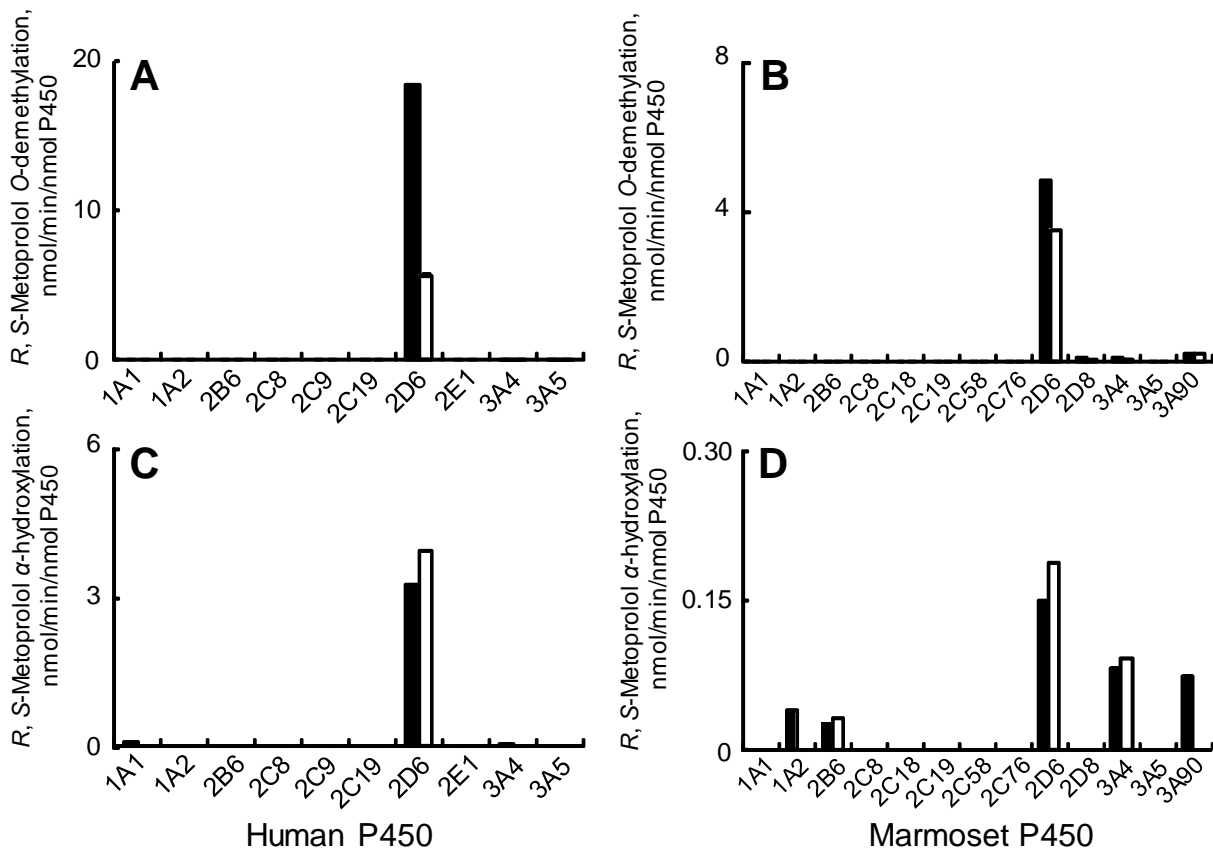
References

- Uehara S, Uno Y, Hagihira Y, Murayama N, Shimizu M, Inoue T, Sasaki E, and Yamazaki H (2015a) Marmoset cytochrome P450 2D8 in livers and small intestines metabolizes typical human P450 2D6 substrates, metoprolol, bufuralol and dextromethorphan. *Xenobiotica* **45**:766-772.
- Uehara S, Uno Y, Inoue T, Kawano M, Shimizu M, Toda A, Utoh M, Sasaki E, and Yamazaki H (2015b) Novel Marmoset Cytochrome P450 2C19 in Livers Efficiently Metabolizes Human P450 2C9 and 2C19 Substrates, S-Warfarin, Tolbutamide, Flurbiprofen, and Omeprazole. *Drug Metab Dispos* **43**:1408-1416.

- Uehara S, Kawano M, Murayama N, Uno Y, Utoh M, Inoue T, Sasaki E, and Yamazaki H (2016a) Oxidation of R- and S-omeprazole stereoselectively mediated by liver microsomal cytochrome P450 2C19 enzymes from cynomolgus monkeys and common marmosets. *Biochem Pharmacol* **120**:56-62.
- Uehara S, Uno Y, Inoue T, Sasaki E, and Yamazaki H (2016b) Molecular Cloning, Tissue Distribution, and Functional Characterization of Marmoset Cytochrome P450 1A1, 1A2, and 1B1. *Drug Metab Dispos* **44**:8-15.
- Uehara S, Uno Y, Ishii S, Inoue T, Sasaki E, and Yamazaki H (2017) Marmoset cytochrome P450 3A4 orthologue expressed in liver and small intestine tissues efficiently metabolizes midazolam, alprazolam, nifedipine, and testosterone, *Drug Metab Dispos* **45**:457-467.

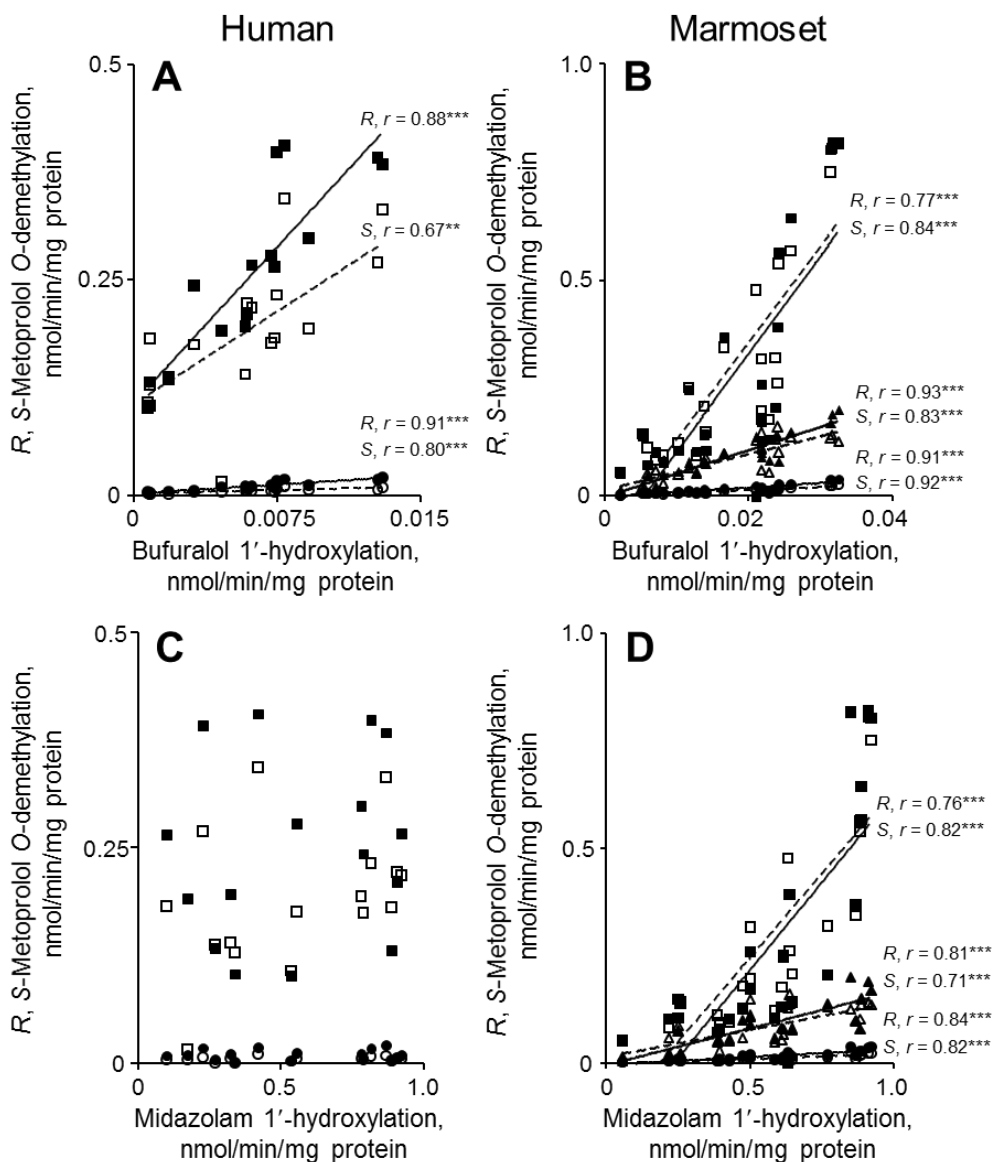
Supplemental Fig. 1.

R- and S-metoprolol O-demethylation (A, B) and α -hydroxylation (C, D) activities by recombinant P450s. R- (closed) and S- (open) metoprolol (at substrate concentrations of 10 μ M) were incubated with human recombinant P450s (expressed in insect cell microsomes; A, C) and marmoset P450s (expressed in *E. coli* membranes; B, D) in triplicate determinations.



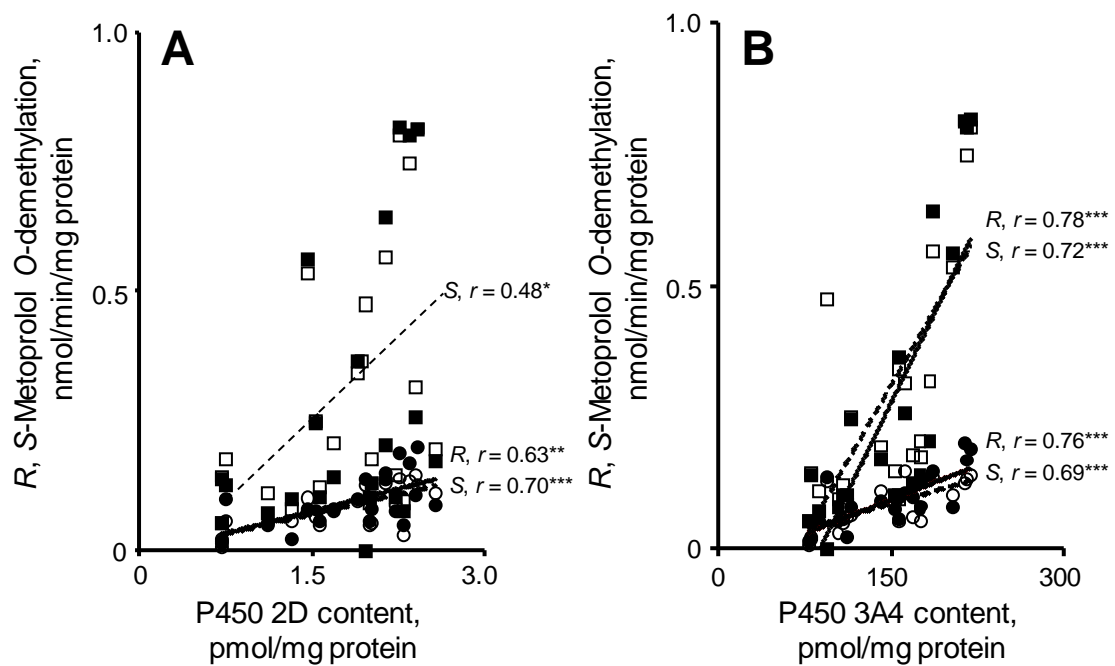
Supplemental Fig. 2.

Relationship between *R*- and *S*-metoprolol *O*-demethylation activities and P450 2D/3A probe activities in individual liver microsomes from humans (A, C) and marmosets (B, D). *R*- (closed, solid line) and *S*- (open, dashed line) metoprolol (at substrate concentrations of 1.0 μ M, circles; 10 μ M, triangles; and 100 μ M, squares) were incubated with liver microsomes from 17 individual humans and 23 individual marmosets. Bufuralol and midazolam hydroxylation activities were determined at substrate concentrations of 1.0 and 5.0 μ M, respectively. Pearson's product moment correlation coefficients were evaluated by *t*-test; * and **, $p < 0.001$ and $p < 0.01$, respectively.**



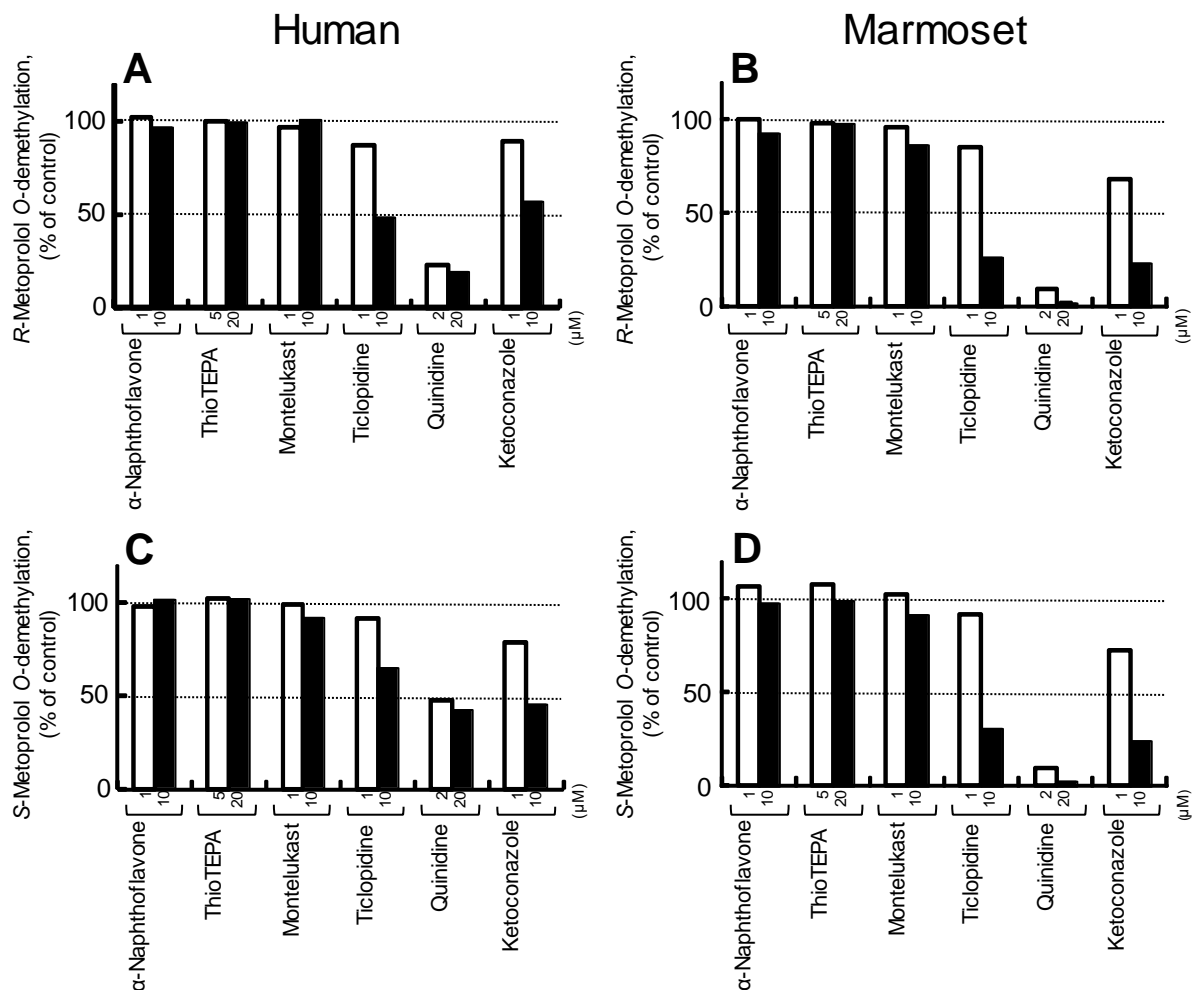
Supplemental Fig. 3.

Relationship between *R*- and *S*-metoprolol *O*-demethylation activities and P450 2D (A) and 3A (B) contents in individual liver microsomes from marmosets. *R*- (closed, solid line) and *S*- (open, dashed line) metoprolol (at substrate concentrations of 1.0 μ M, circles; and 100 μ M, squares) were incubated with liver microsomes from 23 individual marmosets. Pearson's product moment correlation coefficients were evaluated by *t*-test; *, **, and *, $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively.**



Supplemental Fig. 4.

Effects of P450 effectors on *R*- and *S*-metoprolol *O*-demethylation activities in liver microsomes from humans (A, C) and marmosets (B, D). Pooled liver microsomes (0.20 mg protein/mL) were incubated with metoprolol (10 μ M) in the absence (control) or presence of P450 effectors. *R*- and *S*-metoprolol *O*-demethylation activities in pooled liver microsomes from humans (74 males and 76 females) without effectors (control) were 0.039 and 0.024 nmol/min/mg protein, respectively, whereas *R*- and *S*-metoprolol *O*-demethylation activities in pooled liver microsomes from marmosets (14 males and 9 females) without effectors (control) was 0.14 and 0.12 nmol/min/mg protein, respectively. The numbers (1, 2, 10, and 20) indicate P450 effector concentrations co-incubated with the substrate for 15 min. Each bar represents the mean in triplicate determinations.



Supplemental Fig. 5.

Enzyme kinetics for *R*- and *S*-metoprolol *O*-demethylation by recombinant marmoset and human P450 2D/3A enzymes and liver microsomes from marmosets and humans. Kinetic analyses were performed for *R*- (closed) and *S*- (open) metoprolol *O*-demethylation by pooled liver microsomes from humans (A) and marmosets (B) and recombinant human P450 2D6 (C), marmoset P450 2D enzymes (D; P450 2D6, circles; P450 2D8, triangles), human P450 3A enzymes (E; P450 3A4, circles; P450 3A5, triangles), and marmoset P450 3A enzymes (F; P450 3A4, circles; P450 3A90, triangles). Each data represents the mean of duplicate determinations.

