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Short communication

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

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Running Title Page

Running title: Metoprolol oxidation in marmoset livers

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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´-hydroxylations. 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 ~500 μM) 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.

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Footnotes

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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 μM) 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	*R*-metoprolol			*S*-metoprolol		
		K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
Liver microsomes							
Human	*O-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	*O-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	*O-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	0.54
Human P450 3A4	*O-Demethylation*	> 500	0.12[a]	NA	> 500	0.045[a]	NA
	α-Hydroxylation	> 500	0.084[a]	NA	> 500	0.097[a]	NA
Human P450 3A5	*O-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]	NA
Marmoset P450 2D6	*O-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	0.024
Marmoset P450 2D8	*O-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]	NA
Marmoset P450 3A4	*O-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]	NA
Marmoset P450 3A90	*O-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]	NA

R- and *S*-metoprolol (1.0-500 μM) 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 μM. NA: not available.

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Figure 1