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Regio- and Stereoselectivity in Propranolol Metabolism by Dog Liver Microsomes and the Expressed Dog CYP2D15[1]

Takafumi Tasaki,[2] Hisato Iwata, Akio Kazusaka, and Shoichi Fujita[3]

Laboratory of Toxicology, Department of Environmental Veterinary Medical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, N18, W9, Kita-ku, Sapporo 060

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We have studied the regio- and stereoselectivity of ring-hydroxylation and *N*-desisopropylation of *S*(−)- and *R*(+)-propranolol, using dog liver microsomes and the expressed dog CYP2D15 in insect cells. In dog liver microsomes, 4-hydroxylation was the preferred pathway in *S*(−)-propranolol oxidation, while *N*-desisopropylation was the preferred pathway in *R*(+)-propranolol oxidation. *S*(−)-Propranolol was preferred over *R*(+)-propranolol as substrate for 4- and 5-hydroxylations, while *R*(+)-propranolol was the preferred substrate for *N*-desisopropylation at higher substrate concentrations. The expressed CYP2D15 had high catalytic activities toward 4-, 5-hydroxylation, as well as *N*-desisopropylation of both enantiomers. At the substrate concentrations used, 4-hydroxylation was the preferred pathway for the metabolism of both enantiomers, and *S*(−)-propranolol was the preferred substrate over *R*(+)-propranolol for all three monooxygenations catalyzed by CYP2D15. Anti-CYP2D15 peptide antibody strongly inhibited 4- and 5-hydroxylation of both enantiomers in dog liver microsomes, while it did not inhibit their *N*-desisopropylation. These findings suggest that CYP2D15 is highly responsible for the stereoselective 4- and 5-hydroxylations of propranolol in dog liver microsomes.

Key words: CYP2D, cytochrome P450, dog, microsomes, propranolol.

Propranolol (PL) is a nonselective *beta* adrenergic blocker commercially available as a racemic mixture of *S*(−) and *R*(+) enantiomers. The *S*(−) enantiomer is about 100 times more effective as a *beta* blocker than the *R*(+) enantiomer (*2*). Propranolol is metabolized to a large number of products (*3*). There are species differences in metabolic pathways and stereoselectivity in propranolol metabolism among human, dog, and rat liver microsomes (*4, 5*).

In dog, *in vivo* studies showed that the plasma concentrations of *S*(−)-PL were lower than those of *R*(+)-PL after oral administration of racemic propranolol (*6*), whereas Silber and Riegelman (*7*) found the opposite phenomenon in human. von Bahr *et al.* (*4*) showed that although *R*(+)-PL was the preferred substrate over *S*(−)-PL for 4-hydroxylation (4-OH) and *N*-desisopropylation (DIP) in human liver microsomes, *S*(−)-PL was the preferred substrate over *R*(+)-PL for 4-OH in dog liver microsomes. They suggested that the difference in the *in vivo* disposition of PL was caused by the interspecies difference in the stereoselective catalytic property of PL-metabolizing enzymes. PL has been shown to be metabolized by a number of CYP isozymes (*8*). Nakamura *et al.* (*9*) showed that PL 4-OH and 5-hydroxylation (5-OH) in dog liver microsomes are largely mediated by dog CYP2D subfamily. However, since they did not use individual enantiomers of PL, it is not clear whether stereoselectivity in PL metabolism in dog liver microsomes is due to the catalytic nature of dog CYP2D (CYP2D15). Recently we obtained a high level of expression of dog CYP2D15 using a baculovirus, which allowed us to study drug metabolism at a sufficiently measurable level (*10*). In our previous study, recombinant CYP2D15 showed high catalytic activity for the hydroxylations of bunitrolol and imipramine, which are typical substrates of the CYP2D subfamily (*10*). In this article, we investigated the regio- and stereoselective propranolol 4-OH, 5-OH, and DIP by the dog liver microsomes and the expressed dog CYP2D15, and carried out immunoinhibition study using highly specific anti-CYP2D15 peptide antibody. We discuss the relation between the dog CYP2D subfamily and the stereoselective metabolism of PL enantiomers in dog liver microsomes.

MATERIALS AND METHODS

Chemicals—*R*-(+)-, *S*-(−)-propranolol hydrochlorides were purchased from Sigma Chemical (St. Louis, MO). *N*-Desisopropyl, 5-, 4-, and 7-hydroxypropranolol hydrochlorides were gifts from the Sankyo (Tokyo). Glucose-6-phosphate (G-6-P), G-6-P dehydrogenase and NADPH

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[3] To whom correspondence should be addressed. Tel: +81-11-706-6948, Fax: +81-11-706-5105, E-mail: fujita@vetmed.hokudai.ac.jp

were purchased from Oriental Yeast (Tokyo). All other chemicals and solvents were of analytical grade.

Preparation of Dog Liver Microsomes—Male beagle dogs (1.8 years old) were gifts from the Research Laboratories of New Drug Development (Iwamizawa). Microsomes from the livers of dogs were prepared according to Omura and Sato (*11*). Protein concentration was determined by the method of Lowry *et al.* (*12*).

Recombinant Dog CYP2D15 Expression in Insect Sf9 Cells—Dog CYP2D15 was expressed in Sf9 (*Spodoptera frugiperda*) cells and the microsomal membrane from Sf9 cells was prepared as described previously (*10*).

Purification of NADPH-P450 Reductase from Rat Liver Microsomes—NADPH-P450 reductase was purified from rat liver microsomes by the method of Ardies *et al.* (*13*).

Assay of Propranolol-Metabolizing Activity Catalyzed by the Expressed CYP2D15 or Dog Liver Microsomes—The reaction mixture contained 100 mM potassium phosphate buffer, pH 7.4, 3 mM $MgCl_2$, 10 mM G-6-P, various concentrations of substrate, and the desired amount of microsomal membrane from Sf9 insect cells or liver microsomes. After preincubation at 37°C for 5 min, the reaction was initiated with NADPH (500 μM) and G-6-P dehydrogenase (2 units). The addition of purified rat liver NADPH P450 oxidoreductase to the assay system was carried out by preincubating the proteins with the microsomal membrane from Sf9 insect cells at room temperature for 10 min.

Propranolol metabolites were assayed by the HPLC methods previously described (*8*). The reaction was performed at 37°C for 30 s in 1 ml of reaction mixture (0.5 mg of dog liver microsomes) or for 5 min in 0.5 ml of reaction mixture (10 pmol P450 of microsomal membrane from insect cells, 1.33 units of rat NADPH P450 oxidoreductase). 4-, 5-, and 7-hydroxylated metabolites, *N*-desisopropylated metabolite, 4-hydroxybunitrolol as an internal standard, and PL were detected by use of an HPLC spectrofluorometer (821-FP, Japan Spectroscopic, Tokyo) at the excitation wavelength of 310 nm and the emission wavelength of 380 nm.

Immunoinhibition Study—Synthesis of the CYP2D15-specific peptide (EMIQEHRKTRDPTQPPRH), corresponding to the amino acid residues 256-273 of CYP2D15, and preparation of an anti-peptide antibody were carried out as previously described (*9*). Western blot analysis with anti-CYP2D15 peptide antibody against dog liver microsomes and microsomes from Sf9 cells infected with the CYP2D15 recombinant virus showed co-migrated single bands (data not shown). In the immunoinhibition study, dog liver microsomes (0.05 mg) were preincubated in the presence of anti-CYP2D15 peptide IgG or preimmune IgG for 30 min at room temperature. The reaction was performed at 37°C for 5 min in 1 ml of reaction mixture. The substrate concentration was 5 or 100 μM. Other experimental conditions were as described above.

Analysis of Kinetic Data—Kinetic data obtained were analyzed by computer-assisted curve-fitting according to the Michaelis-Menten equation using a non-linear regression program (Simplex).

RESULTS AND DISCUSSION

Propranolol Monooxygenation by Dog Liver Microsomes—Figure 1 shows PL ring-hydroxylase and side-chain *N*-desisopropylase activities in male dog liver microsomes. Dog liver microsomes catalyzed 4-OH, 5-OH, and DIP of *S*(−)- and *R*(+)-propranolol. No detectable 7-hydroxylation (7-OH) of either enantiomer was observed.

The present study revealed that the regioselectivities for both PL enantiomers in dog liver microsomes were influenced by the concentrations of substrate. For the *S*(−)-PL enantiomer, although 4-OH was preferred over 5-OH and DIP at the three substrate concentrations tested, the ratio of DIP to 4-OH increased at the higher concentration (Fig. 1). This can be explained by the fact that the DIP/4-OH ratio of the V_{max} was greater than the DIP/4-OH ratio of the V_{max}/K_m for *S*(−)-PL (Table I). For the *R*(+)-PL enantiomer, the activity of 4-OH is similar to that of DIP at a substrate concentration of 5 μM, but smaller than that of DIP at 100 μM (Fig. 1). This tendency increased with increasing substrate concentration. This observation can be explained by the fact that, for *R*(+)-PL, the V_{max}/K_m value of 4-OH is slightly larger than that of DIP, while the V_{max} value of DIP is larger than that of 4-OH (Table I).

Although the K_m value of 5-OH is either smaller than or similar to that of 4-OH, activities of 5-OH are 8 to 10 times lower than activities of 4-OH (Fig. 1). These findings are consistent with the fact that both the V_{max}/K_m and the V_{max} value of 5-OH are extremely low compared to those of 4-OH for both enantiomers (Table I). We therefore con-

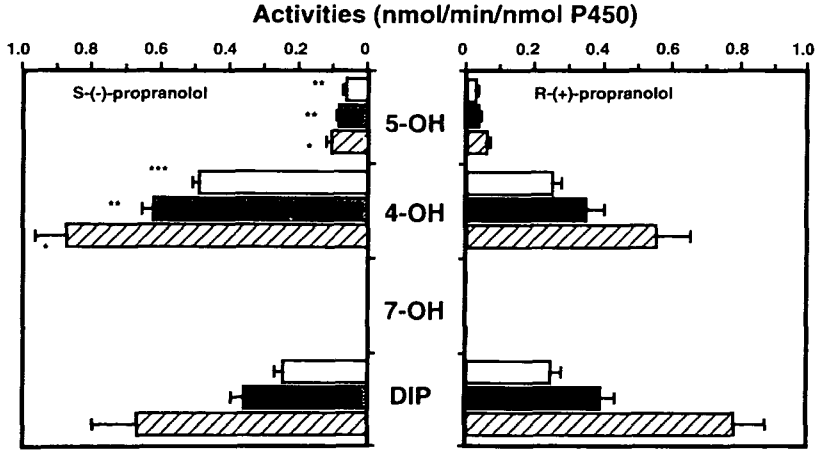

Fig. 1. **Activities of propranolol monooxygenation by dog liver microsomes.** Activities of 5-hydroxylation (5-OH), 4-hydroxylation (4-OH), 7-hydroxylation (7-OH), and *N*-desisopropylation (DIP) of *S*(−)-PL (left) and *R*(+)-PL (right) enantiomers by dog liver microsomes were determined as described in "MATERIALS AND METHODS." The substrate concentrations were 5 μM (□), 10 μM (▨), and 100 μM (▩). Each bar represents the standard deviation ($n=3$). Significantly different from *R*(+)-propranolol ($^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

clude that 4-OH is the preferred pathway of the metabolism of the $S(-)$-PL enantiomer in dog liver microsomes, while DIP is the preferred pathway of the metabolism of the $R(+)$-PL enantiomer, especially at high concentrations of $R(+)$-PL; and 5-hydroxylation is a minor pathway of the metabolism of both enantiomers.

In dog liver microsomes, $S(-)$-PL was preferred over $R(+)$-PL as substrate for both 4-OH and 5-OH at all substrate concentrations tested, while $R(+)$-PL was the preferred over $S(-)$-PL as substrate for DIP at higher substrate concentrations. These results are supported by the following: $S(-)/R(+)$ ratios of both the V_{max}/K_m and the V_{max} for the two hydroxylations are larger than 1.5, while the $S(-)/R(+)$ ratio of the V_{max} for DIP is smaller than 1.0 (Table I).

Propranolol Monooxygenation by the Expressed Dog CYP2D15—Figure 2 shows PL ring-hydroxylase and *N*-desisopropylase activities by the expressed dog CYP2D15. Incubation of a reaction mixture containing P450 reductase and substrate with the microsomal membrane from mock-infected Sf9 cells or Sf9 cells infected with wild-type baculovirus did not yield detectable quantities of metabolites (data not shown). The results of this study showed that the expressed CYP2D15 had high catalytic activities of 4-OH, 5-OH, and DIP of PL enantiomers, while 7-OH of both enantiomers, the preferred pathway of PL metabolism by rat CYP2D2 (*5*), was not detectable (Fig. 2). Although 4-OH was the preferred pathway for both enantiomers, the activity ratio of 4-OH relative to DIP tended to decrease at higher substrate concentrations (Fig. 2). This tendency was observed more strongly for $R(+)$-PL than $S(-)$-PL, and the V_{max} value of DIP of $R(+)$-PL suggests that the DIP and 4-OH pathways become equally preferred for metabolism of $R(+)$-PL by the expressed CYP2D15 at substrate concentrations of more than 100 μM (Table I). Although the activity ratio of 4-OH relative to 5-OH also tended to decrease at higher concentrations of substrate, the V_{max} values of 5-OH of both enantiomers were very low compared to those of 4-OH. This indicates that the 5-OH pathway is a minor pathway for both enantiomers in the expressed CYP2D15.

$S(-)$-PL was the preferred substrate for all three pathways catalyzed by the CYP2D15 at all substrate concentrations tested, while $S(-)/R(+)$ ratios of activities for both 4-OH and DIP decreased at higher substrate concentrations (Fig. 2). The $S(-)/R(+)$ ratios of V_{max} for 4-OH, 5-OH, and DIP suggest that PL ring-hydroxylations will be catalyzed stereoselectively toward $S(-)$-PL, whereas DIP will be catalyze stereoselectively toward $R(+)$-PL by the expressed CYP2D15 at substrate concentrations of more than 100 μM (Table I).

Earlier studies showed that human CYP2D6 and rat CYP2D2 both catalyze propranolol monooxygenation (*8, 14*). The expressed dog CYP2D15, as well as CYP2D6,

TABLE I. **The apparent kinetic parameters and $S(-)/R(+)$ ratios of propranolol monooxygenation by dog liver microsomes and the expressed CYP2D15.**

	K_m (μM)		$S(-)/R(+)$	V_{max} (nmol/min/nmol P450)		$S(-)/R(+)$	V_{max}/K_m (ml/min/nmol P450)		$S(-)/R(+)$
	$S(-)$-PL	$R(+)$-PL	ratio	$S(-)$-PL	$R(+)$-PL	ratio	$S(-)$-PL	$R(+)$-PL	ratio
Dog liver microsomes[a]									
4-OH	4.26 (1.18)	6.39 (1.03)	0.67	0.90 (0.11)	0.58 (0.12)	1.55	0.22 (0.03)	0.091 (0.004)	2.39
5-OH	3.75 (1.41)	6.982 (1.99)	0.54	0.11 (0.02)	0.06 (0.01)	1.88	0.03 (0.01)	0.009 (0.004)	3.56
DIP	9.80 (2.47)	14.10 (7.22)	0.69	0.72 (0.14)	0.92 (0.26)	0.78	0.08 (0.01)	0.07 (0.02)	1.07
The expressed CYP2D15[b]									
4-OH	9.34	12.02	0.78	14.63	11.84	1.24	1.57	0.99	1.58
5-OH	11.88	21.28	0.56	5.42	3.38	1.60	0.46	0.16	2.88
DIP	16.50	44.23	0.37	9.45	11.37	0.83	0.57	0.26	2.19

[a]Values for dog liver microsomes are means of three determinations performed with microsomes from three different animals. The values in parentheses indicate standard deviations ($n=3$). [b]Values for CYP2D15 are means of duplicate determinations.

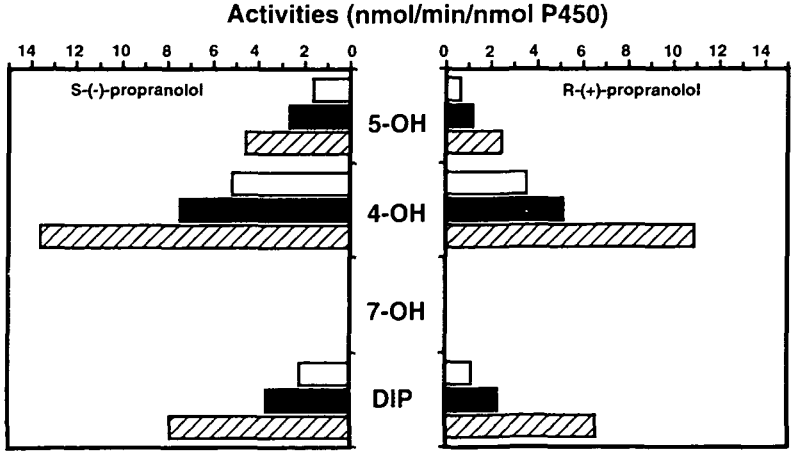

Fig. 2. **Activities of propranolol monooxygenation by the expressed dog CYP2D15.** Activities of 5-hydroxylation (5-OH), 4-hydroxylation (4-OH), 7-hydroxylation (7-OH), and *N*-desisopropylation (DIP) of both $S(-)$-PL (left) and $R(+)$-PL (right) enantiomers by CYP2D15 were determined as described in "MATERIALS AND METHODS." The substrate concentrations were 5 μM (□), 10 μM (▨), and 100 μM (▩). Data represent the means of two duplicate determinations.

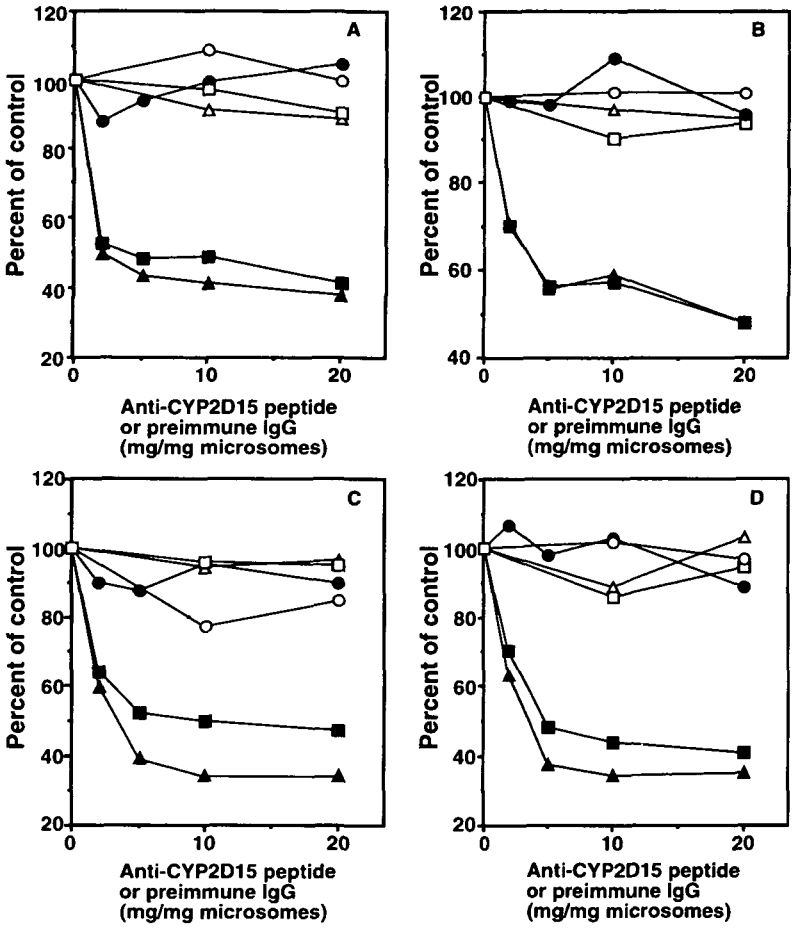

Fig. 3. **Immunoinhibition of propranolol 4,5-OH and DIP in dog liver microsomes.** Result are expressed as percent of control activities of 4-OH (■, □), 5-OH (▲, △), and DIP (●, ○) of *S*(−)-PL (A, C) or *R*(+)-PL (B, D) in dog liver microsomes at substrate concentrations of 5 μM (A, B) or 100 μM (C, D). Experimental conditions are described in "MATERIALS AND METHODS." Closed and open symbols represent the percent of control activities measured in the presence of anti-CYP2D15 peptide IgG and preimmune IgG, respectively. Control activities (nmol/min/mg), measured without IgG, at the substrate concentrations of 5 and 100 μM were respectively: 0.49 and 0.78 for 4-OH of *S*(−)-PL, 0.23 and 0.55 for 4-OH of *R*(+)-PL, 0.08 and 0.11 for 5-OH of *S*(−)-PL, 0.03 and 0.07 for 5-OH of *R*(+)-PL, 0.14 and 0.41 for DIP of *S*(−)-PL, 0.16 and 0.49 for DIP of *R*(+)-PL.

were unable to catalyze 7-OH, which was highly catalyzed by CYP2D2 (*8*). Although this might suggest that dog CYP2D15 is similar to human CYP2D6 rather than rat CYP2D2, CYP2D15, and CYP2D6 differ in both regio- and stereoselectivity. First, CYP2D6 had higher affinity (lower K_m) for *R*(+)-PL for 4-OH, 5-OH, and DIP (*14*), while the expressed CYP2D15 had higher affinity for *S*(−)-PL. Second, DIP was a minor pathway for both enantiomers in CYP2D6 (*14*), while the expressed CYP2D15 catalyzed DIP more strongly than it catalyzed 5-OH. We therefore conclude that the dog CYP2D15 has unique regio- and stereoselectivity compared to both the human CYP2D6 and rat CYP2D2.

It is known that CYP2D6 and CYP2D2 mainly catalyze ring-hydroxylations of both enantiomers in liver microsomes (*5, 15*). In our immunoinhibition study, an anti-CYP2D15 peptide antibody highly specific toward CYP2D15 strongly inhibited 4- and 5-OH of both enantiomers at substrate concentrations of 5 and 100 μM in dog liver microsomes (Fig. 3). These results indicate that CYP2D15 also mainly catalyzes 4- and 5-OH of both enantiomers in dog liver microsomes. Silber and Ringelman (*7*) showed that oral bioavailability of *S*(−)-PL was lower than *R*(+)-PL in dog, and von Bahr *et al.* (*4*) suggested that this stereoselectivity *in vivo* was largely governed by the fact that *S*(−)-PL was preferred over *R*(+)-PL as substrate for 4-hydroxylation in dog liver microsomes. Our findings are in accordance with their data, and we suggest that this feature of PL metabolism in dog liver microsomes is due to the stereoselective catalytic property of the dog CYP2D15.

Earlier studies showed that DIP was mainly catalyzed by CYP1A subfamilies in human (*15*) and rat (*8*) liver microsomes. Since the V_{max} values for DIP of both enantiomers by the expressed CYP2D15 are over 10-fold higher than those by the dog liver microsomes, the partial involvement of this isozyme in DIP at higher substrate concentrations was expected. However, the anti-CYP2D15 peptide antibody did not inhibit DIP of either enantiomer at substrate concentrations of 5 and 100 μM in dog liver microsomes (Fig. 3). Although the anti-CYP2D15 peptide antibody inhibited DIP, as well as 4- and 5-OH, of both enantiomers catalyzed by the expressed CYP2D15 (data not shown), these results of immunoinhibition study indicate that CYP2D15 does not play a major role in DIP of the enantiomers catalyzed by dog liver microsomes. Our previous study showed that the dog CYP2D15 was a minor subfamily in dog liver microsomes (*10*). DIP must be catalyzed by other P450 species than CYP2D15 in dog liver microsomes.

In summary, the regio- and stereoselectivity in PL metabolism by the dog CYP2D15 were different from those in PL metabolism by human and rat CYP2D subfamilies. We conclude that CYP2D15 is highly responsible for the unique stereoselectivity in 4- and 5-hydroxylations of propranolol in dog liver microsomes. These results will be

helpful in studying for interspecies differences in PL metabolism.

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