— Regular Article —

Species Difference between Cynomolgus Monkeys and Humans on Cytochromes P450 2D and 3A-Dependent Drug Oxidation Activities in Liver Microsomes

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Cynomolgus monkeys [*Macaca fascicularis* (mf)] are widely used to determine pharmacokinetics and toxicological potential of many drug candidates as human models in the drug discovery and development. Cytochrome P450s (P450, CYP), one of the most important enzymes in drug metabolism, in monkey livers are generally similar to corresponding human P450s exhibiting high degrees of homologies in cDNAs and amino acid sequences. Species differences regarding important liver P450 3A and 2D function were examined between cynomolgus monkeys and humans using typical human P450 probe reactions using midazolam (a P450 3A marker), dextromethorphan and bufuralol (P450 2D markers). P450 3A-mediated midazolam 1′-hydroxylation activities in liver microsomes from individual monkey were highly correlated with midazolam 4′-hydroxylation activities but not correlated with dextromethorphan *N*-demethylation activities. Recombinant monkey CYP2D17 and CYP2D44 catalyzed dextromethorphan *O*- and *N*-demethylations as well as monkey mfCYP3A4 and mfCYP3A5 did. On the other hand, contributions of corresponding P450 2D6 and P450 3A4/5 to dextromethorphan *N*- and *O*-demethylations, in human liver microsomes were negligible under the present conditions. From these results, monkey P450 2D and 3A enzymes might have broader substrate specificity toward dextromethorphan oxidation than those in human livers. Special attention should be paid when enzymatic and pharmacokinetic data are extrapolated from monkeys to humans.

Key words —— cynomolgus monkey, human, cytochrome P450, cytochrome P450 2D, cytochrome P450 3A

INTRODUCTION

Cytochrome P450 (P450, CYP) enzymes consist of a superfamily of heme-containing monooxygenases, and multiple forms of P450 enzymes exist in mammals.[1)] These P450 enzymes are responsible for the oxidation of many drugs, environmental chemicals, and endogenous substrates. The total contribution of P450 enzymes to drug metabolism is reported to be more than two-third of the top 200 prescribed drugs in the United States in 2002.[2)] In humans, xenobiotics are metabolized primarily by CYP1, CYP2, and CYP3 family member enzymes.[3)] P450 3A enzymes have broad substrate specificities and are responsible for the oxidative metabolism of more than 50% of clinically used drugs.[4)] In addition, P450 2D6 metabolized approximately 20–25% of prescribed drugs.[5)] P450 2D6 is one of the most clinically important drug-metabolizing enzymes, in addition to P450 3A4/5.

Non-human primates are recognized as the secondary closest species to the human on the evolutionary tree. Cynomolgus monkeys (*Macaca fascicularis*, mf) and rhesus monkeys (*Macaca mulatta*) are widely used as experimental animals in the drug discovery and development stages to determine the efficacy, safety/toxicology, and absorption, distribution, metabolism, and excretion of drug candi-

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dates. Homologies of monkey mfCYP3A4 with human P450 3A4 were 96% and 93% in cDNA and amino acid sequences, respectively.[6] The corresponding homologies of monkey mfCYP3A5 with human P450 3A5 were 94% and 91%, respectively. Regarding P450 2D subfamily, Western blot analysis revealed two P450 2D protein bands derived from cynomolgus monkey liver microsomes.[7,8] P450 2D17 in cynomolgus monkey was reported as a human P450 2D6 homolog.[6] The homologies of cDNA and amino acid sequences between monkey P450 2D17 and human P450 2D6 were 94% and 93%, respectively. Recently, Uno *et al.*[9] successfully identified a novel P450 2D44 cDNA in cynomolgus monkey. The homologies of cDNA and amino acid sequences between P450 2D44 and P450 2D6 were 93% and 91%, respectively. In cynomolgus monkey P450 2D subfamily, the homologies of cDNA and amino acid sequences between P450 2D17 and P450 2D44 were 94% and 92%, respectively.

Monkey P450 function is not investigated in detail in spite of the high similarities to human regarding amino acid sequences. As mentioned above, both P450 2D6 and P450 3A are clinically important human drug-metabolizing enzymes. Therefore, the species difference in P450 2D- and P450 3A-dependent drug oxidations between cynomolgus monkeys and humans were investigated in this study. We report herein that monkey P450 2D and 3A enzymes might have broader substrate specificity toward dextromethorphan oxidation than those in human livers.

MATERIALS AND METHODS

Chemicals —— Midazolam was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Dextromethorphan and bufuralol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other regents used in this study were commercially available with the highest quality.

Enzyme Preparations —— Livers from individual cynomolgus monkeys were obtained from Shin Nippon Biomedical Laboratories (Wakayama, Japan). Ages, sex and origins of the monkeys used in this study are summarized in Table 1. Liver microsomes were prepared in 10 mM Tris-HCl buffer (pH 7.4) containing 0.10 mM EDTA and 20% (v/v) glycerol according to the reported method.[10] Pooled liver microsomes from humans (H150) and monkeys were obtained from BD Biosciences (Woburn, MA, U.S.A.). The membrane preparation of each recombinant human or monkey P450 coexpressed with human NADPH-P450 reductase (EC 1.6.2.4) in *Escherichia coli* membranes was prepared as described previously.[9,11] Microsomal protein concentrations were estimated using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, U.S.A.). Concentration of total P450 in membranes was estimated as described previously by Omura and Sato.[12]

Table 1. Information for Monkeys Used in this Study

Monkey No.	Origin	Sex	Age (years-old)	Liver microsomal total P450 (nmol/mg protein)
1	Indochina	Male	7	0.98
2			5	0.84
3			6	0.82
4			6	1.10
5			6	0.69
6			6	0.95
7			9	0.97
8			8	1.04
9		Female	7	0.98
10			7	0.97
11			7	1.06
12			7	0.25
13			6	0.46
14			5	0.92
15	Indonesia	Female	5	0.96
16			5	0.79
17			5	1.01
18			5	0.56
19			5	0.71
20			5	1.06
21			5	0.55
22			5	0.90

Enzyme Assays —— Bufuralol 1′-hydroxylation, dextromethorphan *N*- and *O*-demethylations, and midazolam 1′- and 4-hydroxylations were determined according to the methods described previously.[13,14] Briefly, typical incubation mixtures consisted of 50–100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, and 0.25 units/ml glucose 6-phosphate dehydrogenase), a substrate (10–400 μM), and liver microsomes or recombinant P450 enzymes in a final volume of 0.20 ml. Incubations were carried out at 37°C for 10 min. The reaction was terminated by the addition of 0.40 ml of ice-cold methanol or by the addition of 10 μl of 60% perchloric acid. After the

centrifugation at 1500 g for 10 min, the supernatant was subjected into HPLC system consisted of a LC-10AD$_{vp}$ pump, SPD-20A UV and RF-10A$_{XL}$ fluorescence detectors (Shimadzu, Kyoto, Japan) using an analytical C_{18} reversed-phase column (5 μm, 4.6 × 150 mm, Mightysil RP-18 GP, Kanto Chemicals, Tokyo, Japan).

Statistical Analysis —— The correlation analyses were carried out using statistical program Instat (GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

Drug Oxidation Activities in Liver Microsomes from Cynomolgus Monkeys

Activities of midazolam 1′- and 4-hydroxylations, dextromethorphan *N*- and *O*-demethylations, and bufuralol 1′-hydroxylation in liver microsomes from male and female cynomolgus monkeys from Indochina and female cynomolgus monkeys from Indonesia were investigated. Mean activities of both midazolam 1′- and 4-hydroxylations in monkey liver microsomes were similar (approximately 1.5 nmol/min/mg protein, Fig. 1A and 1B). Mean dextromethorphan *N*-demethylation activities were low (approximately 0.1 nmol/min/mg protein, Fig. 1C) in contrast to 0.3–1.0 nmol/min/mg protein of dextromethorphan *O*-demethylation activities (Fig. 1D). Bufuralol 1′-hydroxylation activities were approximately 0.8 nmol/min/mg protein (Fig. 1E).

Correlation among these five drug oxidation activities in individual monkeys was analyzed (Fig. 2). Midazolam 1′-hydroxylation activity was correlated with midazolam 4-hydroxylation activity in monkey liver microsomes (Fig. 2A, $r = 0.95$, $n = 22$, $p < 0.0001$). A significant correlation coefficient was seen between bufuralol 1′-hydroxylation and dextromethorphan *O*-demethylation in monkey liver microsomes (Fig. 2B, $r = 0.59$, $n = 22$, $p < 0.005$). In contrast, dextromethorphan *N*-demethylation activity did not correlate with midazolam 1′- and 4-hydroxylations in monkey liver microsomes (Fig. 2C and 2D).

Drug Oxidative Activities Catalyzed by Recombinant CYP2D and CYP3A Enzymes and Pooled Liver Microsomes from Monkey and Human

Table 2 shows drug oxidation activities cat-

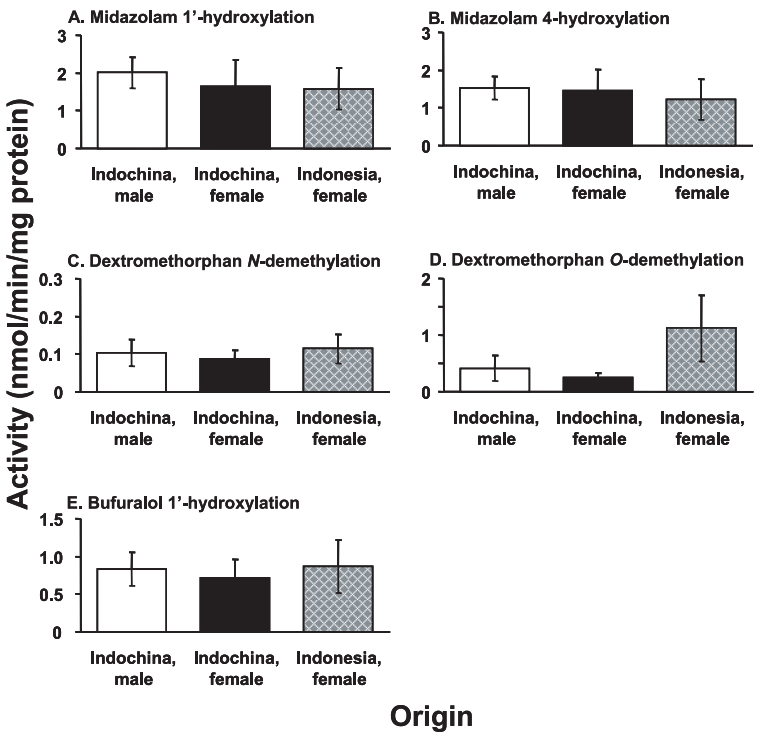

Fig. 1. Mean Drug Oxidation Activities in Liver Microsomes from Monkeys

Midazolam (100 μM), dextromethorphan (400 μM), and bufuralol (20 μM) were incubated with liver microsomes from male ($n = 8$, open) and female ($n = 6$, closed) Indochinese monkeys or female Indonesian monkeys ($n = 8$, cross-hatched). Data represent the means with S.D. (bar) for individual monkeys. There were no statically differences among these mean values.

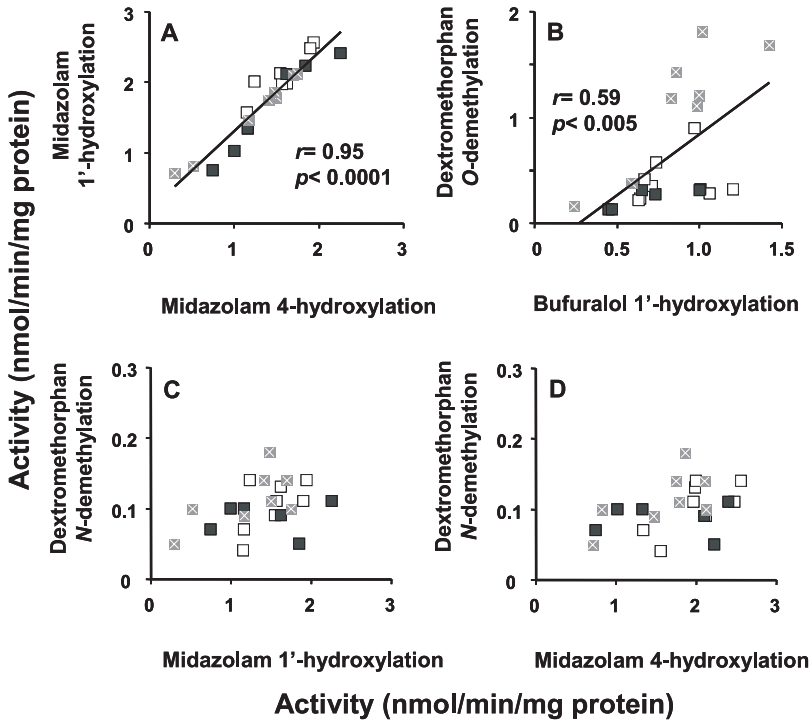

Fig. 2. Relationship between Activities of Midazolam 1′- and 4-hydroxylations, dextromethorphan *N*- and *O*-demethylations, and Bufuralol 1′-hydroxylation in liver Microsomes from Individual Monkeys

Experimental details regarding substrates, enzyme sources, and symbols are shown in the legend of Fig. 1. Solid lines represent significant correlations by the linear regression analysis ($n = 22$, $p < 0.05$).

Table 2. Drug Oxidation Activities Catalyzed by Recombinant CYP2D and 3A Enzymes and Liver Microsomes from Monkeys and Humans

Drug oxidation	Substrate (μM)	Recombinant P450 (nmol/min per nmol P450)							Liver microsomes (nmol/min per mg protein)	
		Monkey				Human				
		CYP2D17	CYP2D44	mfCYP3A4[a)]	mfCYP3A5[a)]	CYP2D6	CYP3A4[a)]	CYP3A5[a)]	Monkey[a)]	Pooled human[a)]
Midazolam	10	< 0.005	< 0.005	10	17	< 0.005	7	12	1.3	0.47
1′-hydroxylation	100	< 0.005	< 0.005	18	31	< 0.005	6	17	2.5	0.94
Midazolam	10	< 0.005	< 0.005	6	2	< 0.005	5	1	0.4	0.02
4-hydroxylation	100	< 0.005	< 0.005	25	16	< 0.005	13	4	1.8	0.14
Dextromethorphan	10	0.6	0.7	0.05	0.05	< 0.001	0.01	< 0.001	0.03	0.03
N-demethylation	200	3.2	3.6	0.99	1.1	< 0.001	0.33	0.14	0.17	0.15
Dextromethorphan	10	8	6	0.02	0.07	3.3	< 0.001	< 0.001	0.4	0.13
O-demethylation	200	15	10	0.07	0.33	5.4	< 0.001	< 0.001	1.2	0.31
Bufuralol	20	5.1	2.6	0.19	0.73	4.0	0.14	0.20	0.83	0.11
1′-hydroxylation	200	8.9	4.9	0.99	2.4	6.1	0.41	0.33	1.7	0.21

mf; *Macaca fascicularis*. *a*) Activities of liver microsomes from monkeys and humans are taken form Iwasaki *et al.*[11] using pooled livers from 150 individuals (H150) and 5 cynomolgus monkeys commercially available. Data are means of duplicate determinations.

alyzed by recombinant CYP2D and CYP3A enzymes, along with those of pooled liver microsomes from cynomolgus monkeys and humans. Recombinant human CYP2D6 and monkey CYP2D17 and CYP2D44 did not catalyze midazolam hydroxylations under the present conditions. Recombinant human CYP2D6 and CYP3A did not mediate dextromethorphan *N*- and *O*-demethylations, respectively. In contrast, recombinant monkey CYP2D17 and CYP2D44 effectively catalyzed dextromethorphan *O*- and *N*-demethylations, as mfCYP3A4 and mfCYP3A5 did (Table 2). Bufuralol 1′-hydroxy-

lation was catalyzed extensively by human and monkey CYP2D enzymes and slowly by human and monkey CYP3A enzymes (Table 2).

DISCUSSION

Cynomolgus monkeys are frequently used to determine pharmacokinetics and toxicological potential of many drug candidates. These results in monkey studies are extrapolated to humans in the drug discovery and development. Therefore, it is important to investigate if there are marked species-related differences in the catalytic roles of individual P450 enzymes between cynomolgus monkeys and humans. Known typical P450 3A4/5 (Fig. 2A) and 2D6 (Fig. 2B) maker reaction activities for humans were also correlated in a total of 22 monkey liver microsomes. In contrast, dextromethorphan *N*-demethylation activity could be mediated both by monkey P450 2D and 3A enzymes in some extents, judging from no significant correlation coefficient with liver microsomal markers, midazolam 1′- and 4-hydroxylations (Fig. 2C and 2D) and recombinant CYP2D17 and CYP2D44 activities (Table 2). The present study suggests that P450 2D and 3A enzymes in monkey livers might have broader substrate specificity than those in human livers with midazolam 1′- and 4-hydroxylations, dextromethorphan *N*- and *O*-demethylations, and bufuralol 1′-hydroxylation.

The expression levels of P450s in cynomolgus monkey livers have been shown to decrease with ages (12–32 years) as revealed in humans.[15, 16] Barter *et al.*[17] have also reported that the microsomal protein per gram of liver values increase from birth to a maximum of 40 mg/g at 28 years followed by a gradual decrease in older ages in humans. Jacqz *et al.*[18] have reported that cynomolgus monkeys exhibit a polymorphic phenotype with a frequency of poor metabolizers of 14% (95% confidence limits, 6.5–25%) on debrisoquine/4-hydroxydebrisoquine ratios obtained from urine samples collected during 0–8 hr after administration. Individual variability probably caused by the age-dependent and polymorphic differences should be considered in the study using cynomolgus monkeys. The little regional differences (Philippine, Malaysia or Indonesia) were reported in terms of total P450 contents and major P450s by Western blot analysis.[16] This was consistent with the present study that regional or gender differences were not observed in the activities of midazolam 1′- and 4-hydroxylations, dextromethorphan *N*- and *O*-demethylations, and bufuralol 1′-hydroxylation of liver microsomes from male and female cynomolgus monkeys aged from 5–9 years (Fig. 1 and Table 1).

There were similarities in catalytic properties of P450 enzymes in liver microsomes from monkeys and humans.[19] Monkey P450 3A enzymes have similar substrate selectivity to that of human P450 3A enzymes, but exhibit wider substrate selectivity toward P450 2D substrates.[11] Monkey mfCYP3A4 and mfCYP3A5 efficiently catalyzed human P450 2D substrates, although human CYP3A4 and CYP3A5 showed negligible or mostly low activities against these substrates (Table 2). Drug metabolizing activities for several oxidative reactions in monkey liver microsomes were higher than those in human liver microsomes in the present study (Table 2). Cynomolgus monkeys have occasionally been found to have a poorer bioavailability than human for some drugs after oral administrations.[20] In small intestines of cynomolgus monkeys, mRNA expression of CYP2D17 was much lower than those of mfCYP3A4 and mfCYP3A5.[21] Therefore, the low bioavailability in monkeys might be caused by highly active P450 enzymes and/or broader substrate specificity of P450 3A. In addition, CYP3A5 mRNA levels were comparable between the liver and jejunum.[21] The monkey mfCYP3A5 has a possibility to play a major role in the first-pass metabolism of P450 3A substrates.

CYP2D17 was cloned as the cynomolgus monkey homolog of human CYP2D6.[22] The amino acid residues known to interact with substrates and inhibitors of CYP2D6, including phenylalanine (Phe) 120, glutamic acid (Glu) 216, aspartic acid (Asp) 301, and Phe481, were conserved across both CYP2D17 and human CYP2D6. Subsequently, CYP2D44 was newly identified in cynomolgus monkey liver.[9] Both CYP2D17 and CYP2D44 mRNA were expressed predominantly in the liver, and CYP2D44 mRNA expression level was 4.4-fold lower than that of CYP2D17.[9] The functional characteristics of both CYP2D17 and CYP2D44 enzymes were similar to those of human CYP2D6 but measurably differed in dextromethorphan *N*-demethylation.[9] In this study, recombinant monkey CYP2D17 and CYP2D44 effectively catalyzed dextromethorphan *O*- and *N*-demethylation, as monkey mfCYP3A4 and mfCYP3A5 did, but recombinant human CYP2D6 did not mediate dextromethorphan

N-demethylation.

Recently revised nomenclature for some monkey P450s is reported to better reflect their orthologous relationships to the corresponding human P450 s.[23] From the present results, monkey P450 2D and 3A enzymes might have broader substrate specificity toward dextromethorphan oxidation than those in human livers. Such data could prove useful in conducting drug metabolism studies in cynomolgus monkeys. In the stage of drug discovery and development, *in vivo* drug interrelation studies with human probe drugs have been carried out using whole body of monkeys.[24] Special attention should be required when enzymatic and pharmacokinetic data such as drug interactions are extrapolated from monkeys to humans.

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