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Monkey liver cytochrome P450 2C19 is involved in *R*- and *S*-warfarin 7-hydroxylation

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ABSTRACT

Cynomolgus monkeys are widely used as primate models in preclinical studies. However, some differences are occasionally seen between monkeys and humans in the activities of cytochrome P450 enzymes. *R*- and *S*-warfarin are model substrates for stereoselective oxidation in humans. In this current research, the activities of monkey liver microsomes and 14 recombinantly expressed monkey cytochrome P450 enzymes were analyzed with respect to *R*- and *S*-warfarin 6- and 7-hydroxylation. Monkey liver microsomes efficiently mediated both *R*- and *S*-warfarin 7-hydroxylation, in contrast to human liver microsomes, which preferentially catalyzed *S*-warfarin 7-hydroxylation. *R*-Warfarin 7-hydroxylation activities in monkey liver microsomes were not inhibited by α-naphthoflavone or ketoconazole, and were roughly correlated with P450 2C19 levels and flurbiprofen 4-hydroxylation activities in microsomes from 20 monkey livers. In contrast, *S*-warfarin 7-hydroxylation activities were not correlated with the four marker drug oxidation activities used. Among the 14 recombinantly expressed monkey P450 enzymes tested, P450 2C19 had the highest activities for *R*- and *S*-warfarin 7-hydroxylations. Monkey P450 3A4 and 3A5 slowly mediated *R*- and *S*-warfarin 6-hydroxylations. Kinetic analysis revealed that monkey P450 2C19 had high V_{max} and low K_m values for *R*-warfarin 7-hydroxylation, comparable to those for monkey liver microsomes. Monkey P450 2C19 also mediated *S*-warfarin 7-hydroxylation with V_{max} and V_{max}/K_m values comparable to those for recombinant human P450 2C9. *R*-warfarin could dock favorably into monkey P450 2C19 modeled. These results collectively suggest high activities for monkey liver P450 2C19 toward *R*- and *S*-warfarin 6- and 7-hydroxylation in contrast to the saturation kinetics of human P450 2C9-mediated *S*-warfarin 7-hydroxylation.
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1. Introduction

Drug metabolizing forms of cytochromes P450 (P450) play important roles in the metabolism of endogenous and exogenous compounds in humans [1]. The P450 gene superfamily is made up of 57 functional genes and 58 pseudogenes in humans [2]. The cynomolgus monkey (*Macaca fascicularis*) is one of the most widely used primate species in preclinical drug metabolism studies because of its evolutionary closeness to humans [3]. More than 20 P450 enzymes in the cynomolgus monkey have been identified and were found to be highly identical to orthologous human P450s [4]. Although cynomolgus monkey P450 2C76 is not orthologous to any human P450 [5], monkey P450 2C8 (previously named P450 2C20) is orthologous to human P450 2C8. In addition, monkey P450 2C9 (previously P450 2C43) and 2C19 (previously P450 2C75) are 91–93% identical to human P450 2C9 and 2C19 [4], respectively. Cynomolgus monkey P450 2C76, 2C8, 2C9, and 2C19 are expressed as functional drug-metabolizing enzymes in monkey livers, just as the latter three are in human livers [6].

P450 2C enzymes have been shown to constitute the major forms in human and other mammalian livers [7] and to mediate the oxidation of a variety of clinically used drugs such as warfarin, diclofenac, and flurbiprofen [8,9]. Despite the importance of the cynomolgus monkey in drug metabolism studies, the roles and activities of its liver microsomal P450 2C enzymes have not been fully elucidated. Some P450 2C-mediated drug oxidation activities in monkey livers were found to be low in comparison with those of other species in our previous studies [10,11]. *R*- and *S*-warfarin are model substrates for stereoselective drug oxidations, and their metabolisms have been reported in a number of studies [9]. In

Abbreviations: P450, general term for cytochrome P450 (EC 1.14.14.1).

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addition, we reported that *R*- and *S*-warfarin 7-hydroxylations are catalyzed principally by P450 1A2 and P450 2C9, respectively, in human liver microsomes [12]. There is little information available on stereoselective drug oxidations mediated by P450 enzymes in monkeys.

In this study, the activities of 14 major P450 enzymes in monkey liver microsomes were measured with respect to *R*- and *S*-warfarin 6- and 7-hydroxylations. We report herein the high capacity of monkey liver P450 2C19 with respect to *R*- and *S*-warfarin 6- and 7-hydroxylations supported by molecular docking simulation.

2. Materials and methods

2.1. Chemicals

Racemic warfarin, diclofenac, and flurbiprofen were purchased from Sigma–Aldrich (St. Louis, MO). *R*- and *S*-warfarin and their 6- and 7-hydroxlated metabolites were obtained from Ultrafine Chemicals (Manchester, UK). The other chemicals and reagents used were obtained in the highest grade commercially available.

2.2. Enzyme preparations

Pooled human liver microsomes (H150) were obtained from BD Gentest (Franklin Lakes, NJ). Sample collections from monkeys were reviewed and approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd. Liver microsomes were prepared as described previously [13]. The P450 2C19 contents in liver microsomes were estimated using the immunochemically determined total P450 2C contents as described previously [13] multiplied by the ratios of mRNA levels of P450 2C19 over P450 2C9 and 2C19 detemined in individual monkey liver microsomes [6,14]. Human and monkey P450s with NADPH-P450 reductase were recombinantly expressed in *Escherichia coli*, as described previously [5,6,14–20]. The catalytic functions of these human and monkey P450 enzymes were determined using typical substrates.

2.3. Analysis of P450-mediated drug oxidation activities

The activities of monkey liver P450 enzymes with respect to *R*- and *S*-warfarin 6- and 7-hydroxylations were analyzed as described previously for human P450 enzymes [12] with slight modifications. Briefly, a typical incubation mixture (0.20 mL) contained recombinant P450 enzymes (5.0 pmol equivalent) or liver microsomes (0.50 mg protein/mL), an NADPH-generating system (0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, and 0.25 unit/mL glucose 6-phosphate dehydrogenase), and substrate in 50 mM potassium phosphate buffer (pH 7.4). Reactions were carried out at 37 °C for 15 min and were terminated by adding 0.025 mL of ice-cold 17% perchloric acid. After centrifugation at 900 *g* for 5 min, metabolites in the supernatant were determined by high-performance liquid chromatography with a fluorescence detector. For kinetic analyses, warfarin (0–400 μM) was incubated with recombinant human P450 2C9/2C19, recombinant monkey P450 2C19, or liver microsomes at 37 °C for 15 min in the presence of an NADPH-generating system as described above. Other drug oxidation activities in monkey liver microsomes were determined as reported previously, including ethoxyresorufin *O*-deethylation, diclofenac 4′-hydroxylation, flurbiprofen 4-hydroxylation, and midazolam 1′-hydroxylation [5,11].

2.4. Statistical analysis

Kinetic parameters were calculated from a fitted curve by nonlinear regression (mean ± SE). Linear regression analysis was performed with the program InStat (GraphPad Software, San Diego, CA, USA). Comparison of *R*- and *S*-warfarin hydroxylation activities in monkey livers was analyzed using the Mann–Whitney *U* test.

2.5. Docking simulation

Monkey P450 2C19 primary sequence was aligned with a crystal structure of human P450 2C9 (Protein Data Bank code 1R90) [21] using MOE software (ver. 2011.10, Computing Group, Montreal, Canada) for modeling of the three-dimensional structure. Prior to docking simulation, the energy of the P450 structures was minimized using the CHARMM22 force field. Docking simulation was carried out for *R*- and *S*-warfarin binding to P450 enzymes using the MMFF94x force field distributed in the MOE Dock software [22]. Twenty solutions were generated for each docking experiment and ranked according to the total interaction energy (*U* value).

3. Results

3.1. R- and S-warfarin 6- and 7-hydroxylations by monkey liver microsomes

Rates of 6- and 7-hydroxylations of *R*- and *S*-warfarin (10 and 100 μM) were determined using microsomes from pooled human livers and individual monkey livers (Fig. 1). In pooled human liver microsomes, *S*-warfarin 7-hydroxylation activity was high at a substrate concentration of 10 μM (Fig. 1A). Human *S*-warfarin 6-hydroxylation activity at 100 μM appeared to be higher than *R*-warfarin 6- or 7-hydroxylation activities under the present conditions. In liver microsomes from individual monkeys, *R*- and *S*-warfarin 6-hydroxylation activities at the low substrate concentration were roughly similar, but *R*-warfarin 7-hydroxylation activities were predominant at 100 μM (Fig. 1B). Mean *R*-warfarin 7-hydroxylation activities at the high substrate concentration were significantly higher ($p < 0.05$, $n = 20$) than mean *S*-warfarin 7-hydroxylation activities in monkey liver microsomes.

To investigate the major P450 isoforms involved in *R*- and *S*-warfarin 7-hydroxylation, correlations between *R*-/*S*-warfarin 7-hydroxylation and typical drug oxidation activities or P450 2C contents in individual monkey liver microsomes were investigated (Table 1 and Fig. 2). *R*-warfarin 7-hydroxylation activities in 20 monkey liver microsomes were significantly correlated with the

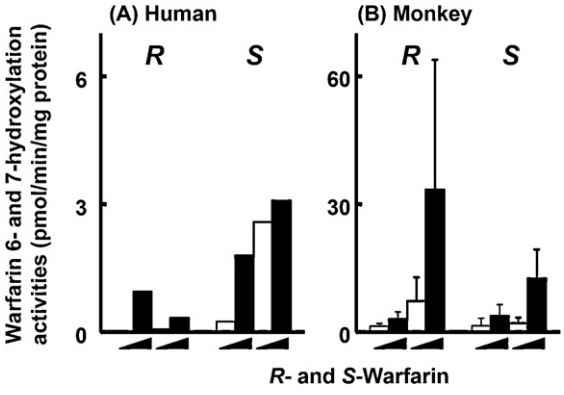

Fig. 1. *R*- and *S*-warfarin 6- and 7-hydroxylation activities of microsomes from pooled humans livers (A) and 20 individual monkey livers (B). *R*- and *S*-warfarin (10 and 100 μM) were incubated with microsomes from pooled human livers (A) and from individual monkey livers (B). Rates of warfarin 6- (open columns) and 7-hydroxylation (solid columns) are shown. Data are means (and SDs for the 20 individual monkey livers) of duplicate determinations. In panel (B), *R*-warfarin 7-hydroxylation activities at the high substrate concentration were significantly higher ($p < 0.05$, $n = 20$) than *S*-warfarin 7-hydroxylation activities in monkey liver microsomes.

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