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Note

Cytochrome P450 Metabolic Activities in the Small Intestine of Cynomolgus Macaques Bred in Cambodia, China, and Indonesia

Yasuharu Nakanishi[1], Hiroyuki Yamashita[2], Tsuyoshi Yoshikawa[2], Takeshi Tominaga[2], Koichiro Nojiri[2], Yoshiharu Sunaga[2], Atsunobu Muneoka[2], Kazuhide Iwasaki[1], Masahiro Utoh[1], Chika Nakamura[1], Hiroshi Yamazaki[3] and Yasuhiro Uno[1,*]

[1]*Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories, Ltd., Kainan, Japan*
[2]*Drug Safety Research Center, Shin Nippon Biomedical Laboratories, Ltd., Kagoshima, Japan*
[3]*Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, Machida, Japan*

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Summary: Cynomolgus macaques, used in drug metabolism studies due to their evolutionary closeness to humans, are mainly bred in Asian countries, including Cambodia, China, and Indonesia. Cytochromes P450 (P450s) are important drug-metabolizing enzymes, present in the liver and small intestine, major drug metabolizing organs. Previously, our investigation did not find statistically significant differences in hepatic P450 metabolic activities measured in cynomolgus macaques bred in Cambodia (MacfaCAM) and China (MacfaCHN). In the present study, P450 metabolic activity was investigated in the small intestine of MacfaCAM and MacfaCHN, and cynomolgus macaques bred in Indonesia (MacfaIDN) using P450 substrates, including 7-ethoxyresorufin, coumarin, bupropion, paclitaxel, diclofenac, *S*-mephenytoin, bufuralol, chlorzoxazone, and testosterone. The results indicated that P450 metabolic activity of the small intestine was not statistically significantly different (<2.0-fold) in MacfaCAM, MacfaCHN, and MacfaIDN. In addition, statistically significant sex differences were not observed (<2.0-fold) in any P450 metabolic activity in MacfaCAM as supported by mRNA expression results. These results suggest that P450 metabolic activity of the small intestine does not significantly differ statistically among MacfaCAM, MacfaCHN, and MacfaIDN.

Keywords: monkey; cytochrome P450; small intestine; gene expression; metabolic activity

Introduction

The cynomolgus macaque (*Macaca fascicularis*) is an important primate species for drug metabolism studies, since this species has molecular and functional properties of important drug-metabolizing enzymes similar to humans, including cytochromes P450 (P450s or CYPs).[1)] P450 is a gene family consisting of a large number of drug-metabolizing enzyme genes. In humans, the CYP3A subfamily enzymes are important due to their involvement in the biotransformation of >50% of prescription drugs[2)] and their abundant expression in the liver and small intestine.[3,4)] CYP3A enzymes catalyze the metabolism of some orally administered drugs, thus exerting a first-pass effect.[5)]

In cynomolgus macaques, more than 20 P450s have been identified to date, including CYP1A1, CYP1D1, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2C76, CYP2D17, CYP2E1, CYP3A4, and CYP3A5.[1)] In this paper, we have designated cynomolgus CYP2C20, CYP2C43, CYP2C75, and CYP3A8 as CYP2C8, CYP2C9, CYP2C19, and CYP3A4, respectively, after consulting with the P450 Nomenclature Committee (http://drnelson.uthsc.edu/cytochromeP450.html). Similar to humans, CYP3A enzymes predominate the total P450 content of the liver in cynomolgus macaques.[6)] Although the content of each P450 in the small intestine has not been reported for cynomolgus macaques, expression of CYP3A4 and CYP3A5 mRNAs is relatively abundant in the small intestine among the 14 P450 mRNAs analyzed.[7)] Moreover, first-pass metabolism of CYP3A substrates, including midazolam, fexofenadine, and simvastatin, has been observed in the cynomolgus macaque intestine.[8)] Therefore, CYP3A enzymes might also play important roles in first-pass metabolism in the cynomolgus macaque small intestine.

Cynomolgus macaques used for biomedical research are mainly bred in Asian countries, including Cambodia (MacfaCAM), China (MacfaCHN), and Indonesia (MacfaIDN). We previously did not find any statistically significant differences in the activities or mRNA expressions of hepatic P450 enzymes in MacfaCAM and

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To whom correspondence should be addressed: Yasuhiro Uno, D.V.M., Ph.D., *Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories, Ltd., 16-1 Minami Akasaka, Kainan 642-0017, Japan. Tel. +81-73-483-8881, Fax. +81-73-483-7377, E-mail: uno-yasuhiro@snbl.co.jp*

MacfaCHN.[9] However, such studies have not been done in the small intestine. In the present study, P450 metabolic activities were measured in the small intestine (jejunum) of MacfaCAM, MacfaCHN, and MacfaIDN, using nine P450 substrates, 7-ethoxyresorufin, coumarin, bupropion, paclitaxel, diclofenac, *S*-mephenytoin, bufuralol, chlorzoxazone, and testosterone. Sex differences and inter-animal variations of P450 metabolic activities were also assessed in the jejunum of MacfaCAM. Furthermore, the mRNA expression of the eleven P450s responsible for these metabolic reactions was also measured in the jejunum of males and females (MacfaCAM) by quantitative PCR (qPCR) analysis.

Materials and Methods

Materials: Bufuralol, chlorzoxazone, 7-ethoxyresorufin, and *S*-mephenytoin were purchased from Sigma-Aldrich (St. Louis, MO). Coumarin, diclofenac, paclitaxel, and testosterone were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bupropion and NADPH-regenerating system solutions were purchased from BD Gentest (Woburn, MA). All other reagents were purchased from Wako Pure Chemical Industries unless otherwise specified.

Tissue samples and preparation of RNA and microsomes: Small intestine (jejunum) samples were collected from 54 cynomolgus macaques (weighing 3–5 kg), including 40 MacfaCAM (3–4 years of age, 20 males, 20 females), 9 MacfaCHN (4–10 years of age, all males), and 5 MacfaIND (3–10 years of age, 4 males, 1 female). Animals were fed a standard animal diet (*i.e.*, Teklad Global Certified 25% Protein Primate Diet, Harlan Sprague-Dawley, Indianapolis, IN), and food was provided *ad libitum* except during the overnight fasting periods before sampling. The present study was reviewed and approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd. Total RNA was extracted from jejunum samples from 20 MacfaCAM (10 males and 10 females), as previously described.[7] For all animals, microsomes were prepared from jejunum samples as described previously.[10] Microsomal protein concentrations were determined by the Bradford method (Bio-Rad Protein Assay Kit, Bio-Rad, Hercules, CA) using bovine serum albumin as the standard.

Enzyme assays: Metabolic activity of cynomolgus P450s was measured using 7-ethoxyresorufin, coumarin, bupropion, paclitaxel, diclofenac, *S*-mephenytoin, bufuralol, chlorzoxazone, and testosterone as substrates, according to the previous method.[10] Briefly, a typical incubation mixture (0.5 mL) contained jejunum microsomes (0.025, 0.1, or 0.25 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 (20 μM bufuralol, 160 μM bupropion, 50 μM chlorzoxazone, 3 μM coumarin, 20 μM diclofenac, 0.8 μM 7-ethoxyresorufin, 60 μM *S*-mephenytoin, 15 μM paclitaxel, or 100 μM testosterone). Reactions were incubated at 37°C for 5, 10, or 20 min and terminated by addition of 0.25 mL of ice-cold acetonitrile. Metabolites in supernatants obtained by centrifugation at 7,500 × *g* (10 min) or 10,000 × *g* (15 min) were analyzed by liquid chromatography coupled with tandem mass spectrometry or radio high-performance liquid chromatography, as described previously.[10]

qPCR analysis: Expression of CYP1A1, CYP1D1, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2C76, CYP2D17, CYP2E1, CYP3A4, CYP3A5, and pregnane X receptor (PXR) mRNA was measured by real-time reverse transcription (RT)-PCR as described previously.[7,11,12] The relative expression levels were determined by normalizing the raw data to the 18S ribosomal RNA level, based on three independent amplifications. To estimate a correlation between P450 mRNA levels, linear regression analysis was performed using Microsoft Excel (Microsoft, Redmond, WA). Statistical significance was determined for metabolic activities and mRNA expressions of P450s using a two-tailed unpaired Student's *t*-test. Values were considered statistically significant if p was <0.05.

Results and Discussion

Metabolic activities of cynomolgus P450s were measured in 40 MacfaCAM (20 males, 20 females), 9 MacfaCHN (all males), and 5 MacfaIND (4 males, 1 female) using P450 substrates. The reactions analyzed included 7-ethoxyresorufin *O*-deethylation for CYP1A/1D; coumarin 7-hydroxylation for CYP2A; bupropion hydroxylation and testosterone 16β-hydroxylation for CYP2B; diclofenac 4′-hydroxylation, paclitaxel 6α-hydroxylation, and *S*-mephenytoin 4′-hydroxylation for CYP2C; bufuralol 1′-hydroxylation for CYP2D; chlorzoxazone 6-hydroxylation for CYP2E1; and testosterone 6β-hydroxylation for CYP3A. All reactions were detected in the small intestine except for coumarin 7-hydroxylation and testosterone 16α-hydroxylation (**Table 1**). *S*-Mephenytoin 4′-hydroxylation was not detected in 4 animals. Comparison of male samples revealed that the P450 metabolic activities analyzed were not statistically significantly different (<2.0-fold) among MacfaCAM, MacfaCHN, and MacfaIND (**Supplemental Fig. 1**). The largest differences were observed between MacfaCHN and MacfaIND testosterone 2α-hydroxylation (1.5-fold). These results indicated the similarities in the P450 metabolic activities among MacfaCAM, MacfaCHN, and MacfaIND.

The rate of testosterone 6β-hydroxylation in the small intestine (**Table 1**) was comparable with that in the liver, which we reported previously.[9] Since cynomolgus CYP3A is the most abundant P450 protein in the liver,[6] cynomolgus CYP3A might also be abundant in the small intestine. Indeed, our immuno-quantification of P450s revealed that CYP3A was the most abundant P450 in the cynomolgus small intestine, among the P450s analyzed, using antibodies raised against nine P450s of the CYP1-3 families (Uehara and Uno, unpublished data). In humans, CYP3A is also the most abundant P450 in the small intestine.[3] These results suggest that CYP3A enzymes play important roles for drug metabolism in the small intestine of cynomolgus macaques as well as humans.

The rates of 7-ehoxyresorufin *O*-deethylation and bufuralol 1′-hydroxylation in the small intestine (**Table 1**) were slower than those in the liver, which were reported previously.[9] Since cynomolgus CYP1A1 and CYP2D17 mainly catalyze 7-ehoxyresorufin *O*-deethylation and bufuralol 1′-hydroxylation,[1] respectively, the slower rates of these reactions might be partly accounted for by the lower expression of cynomolgus CYP1A1 and CYP2D17 in the small intestine than in the liver, as shown at the mRNA level.[7] Similarly, the rates of diclofenac 4′-hydroxylation and *S*-mephenytoin 4′-hydroxylation in the small intestine (**Table 1**) were slower than those in the liver, which we reported previously.[9] The slower rates of these reactions might be partly accounted for by the lower expression of cynomolgus CYP2C19 and CYP2C9 in the small intestine than in the liver, as shown at the mRNA level,[7] due to the involvement of cynomolgus CYP2C19