Immunochemical Detection of Cytochrome P450 Enzymes in Liver Microsomes of 27 Cynomolgus Monkeys

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ABSTRACT

The cynomolgus monkey is widely used as a primate model in preclinical studies because of its evolutionary closeness to humans. Despite their importance in drug metabolism, the content of each cytochrome P450 (P450) enzyme has not been systematically determined in cynomolgus monkey livers. In this study, liver microsomes of 27 cynomolgus monkeys were analyzed by immuno blotting using selective P450 antibodies. The specificity of each antibody was confirmed by analyzing the cross-reactivity against 19 CYP1–3 subfamily enzymes using recombinant proteins. CYP2A, CYP2B6, CYP2C9/19, CYP2C76, CYP2D, CYP2E, CYP3A4, and CYP3A5 were detected in all 27 animals. In contrast, CYP1A, CYP1D, and CYP2J were below detectable levels in all liver samples. The average content of each P450 showed that among the P450s analyzed CYP3A (3A4 and 3A5) was the most abundant (40% of total immunoquantified P450), followed by CYP2A (25%), CYP2C (14%), CYP2B6 (13%), CYP2E1 (11%), and CYP2D (3%). No apparent sex differences were found for any P450. Interanimal variations ranged from 2.6-fold (CYP3A) to 11-fold (CYP2C9/19), and most P450s (CYP2A, CYP2D, CYP2E, CYP3A4, and CYP3A5) varied 3- to 4-fold. To examine the correlations of P450 content with enzyme activities, metabolic assays were performed in 27 cynomolgus monkey livers using 7-ethoxyresorufin, coumarin, pentoxyresorufin, flurbiprofen, bufuralol, dexetromethorphan, and midazolam. CYP2D and CYP3A4 contents were significantly correlated with typical reactions of human CYP2D (bufuralol 1′-hydroxylation and dextromethorphan O-deethylation) and CYP3A (midazolam 1′-hydroxylation and 4-hydroxylation). The results presented in this study provide useful information for drug metabolism studies using cynomolgus monkeys.

Introduction

Cytochromes P450 (P450s) are a gene superfamily comprised of a large number of genes, 57 functional genes and 58 pseudogenes in humans (Nelson et al., 2004). P450s, especially the CYP1–3 family enzymes, play important roles in the metabolism of a variety of drugs and are responsible for approximately 80% of oxidative metabolism (Wilkinson, 2005). The major P450s involved in drug metabolism have been quantified in 60 human livers by immunoblotting (Shimada et al., 1994). That study found that CYP3A was most abundant in total hepatic P450 content, followed by CYP2C, CYP1A2, CYP2E1, CYP2A6, CYP2D6, and CYP2B6. A similar study conducted in human small intestine found that CYP3A was most abundant, followed by CYP2C, CYP2J2, and CYP2D6 (Paine et al., 2006). These studies provided useful information for understanding drug biotransformation in humans.

Cynomolgus monkey (Macaca fascicularis) is a primate species widely used in drug metabolism studies. More than 20 P450s have been identified in cynomolgus monkey, and these enzymes are highly identical to orthologous human P450s (Uno et al., 2011a). The only exception is CYP2C76 that is not orthologous to any human P450 and is expressed as a functional drug-metabolizing enzyme in liver (Uno et al., 2010a). In cynomolgus monkey liver, other CYP2C genes encoding functional drug-metabolizing enzymes are also expressed, including CYP2C8, CYP2C9, and CYP2C19 (Uno et al., 2006). In this article, cynomolgus P450s are designated as recommended by the P450 Nomenclature Committee (http://drnelson.uthsc.edu/cytochromeP450.html) (Uno et al., 2011a). The cynomolgus CYP3A subfamily includes CYP3A4 and CYP3A5, which are predominantly expressed in liver (Uno et al., 2007a) and encode...
enzymes involved in the metabolism of human CYP3A substrates, such as midazolam and nifedipine (Iwasaki et al., 2010; Uno et al., 2010c). Likewise, other cytochrome P450 subfamilies including CYP1A (CYP1A1 and CYP1A2), CYP2A (CYP2A23, CYP2A24, and CYP2A26), CYP2B (CYP2B6), CYP2D (CYP2D17 and CYP2D44), and CYP2E1 (CYP2E1), are also predominantly expressed in liver and encode the proteins specific contents of these P450s were calculated and pre-
using the recombinant proteins of 19 cynomolgus P450s. The specificity of the antibodies was assessed in liver microsomes of 27 cynomolgus monkeys by immuno-
microscopy, although its function remains to be characterized.

Despite the importance of cytochromes in the metabolism of drugs, the expression of the major P450 en-
yzymes has not been systematically examined in cynomolgus monkey liver. In this study, the major P450s were measured in liver microsomes of 27 cynomolgus monkeys by immuno-
blotting using selective antibodies. Analyzed P450s included CYP1A1(1/2), CYP1D1, CYP2A3(23/24/26), CYP2B6, CYP2C9/19, CYP2C76, CYP2D17(44), CYP2E1, CYP2J2, CYP3A4,
and CYP3A5. The specificity of the antibodies was assessed using the recombinant proteins of 19 cytochrome P450s. The specific contents of these P450s were calculated and pre-
sented as mean values and interanimal variations.

Materials and Methods

Chemicals and Materials. Polyclonal anti-human CYP1A1, anti-human CYP2A6, anti-human CYP2E1, anti-human CYP2C9, anti-human CYP2D6, and anti-human CYP3A4 antibodies were pur-
bought from Nason Corporation (Yokohama, Japan), and polyclonal anti-human CYP2B6 and anti-human CYP3A5 antibodies were pur-
bought from Bio-Rad Laboratories (Heracles, CA). All other chemicals were of analytical grade from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Animals, Tissues, and Microsomal Preparation. Liver sam-
plies were collected from 27 cynomolgus monkeys (14 males and 13 females from Indochina or Indonesia, 4–9 years of age). This study was reviewed and approved by the Institutional Animal Care and Use Committee at Shin Nippon Biomedical Laboratories, Ltd. (Kainan, Japan). Each liver sample was homogenized in a 9-fold volume of 0.25 M Tris-buffer sucrose solution, pH 7.4, under ice-cold conditions, followed by centrifugation at 9000 g for 30 min at 4°C. The resultant supernatants were centrifuged at 105,000 g for 1 h at 4°C, and the microsomal pellets were resuspended in 0.25 M Tris-buffer sucrose solution, pH 7.4. Protein concentrations of the prepared microsomes were measured by the Bradford method using Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) with serum albumin as the standard.

Heterologous Expression of P450s in Escherichia coli. The recombinant proteins of 19 cytochrome P450s (CYP1A1, CYP1A2, CYP1D1, CYP2A23, CYP2A24, CYP2A26, CYP2B6, CYP2C18, CYP2C9, CYP2C19, CYP2C76, CYP2D17, CYP2D44, CYP2E1, CYP2J2, CYP3A4, CYP3A5, or CYP3A43) were expressed in E. coli, and membrane preparations were performed as described previously (Uno et al., 2006, 2007a, 2009b, 2010b, 2011d; Uehara et al., 2010). For expression of cytochrome P452 recombinant protein, the N-terminus modification was conducted by polymerase chain reaction with the forward and reverse primers, 5’-GGAATTC-
CTATGGCTCTGTTATGACGCTGCCC
TCTGGG-3’ and 5’-GCTCTAGACAAATCACGCGAGAAC-
3’, respectively. The Ndfel and Xbal sites (underlined) in the forward and reverse primers, respectively, were used for subcloning of poly-
merase chain reaction products into pCW vectors that contained human NADPH-P450 reductase cDNA. The content of each P450 protein in the membrane preparation was determined by Fe2+ - CO versus Fe2+ difference spectra as described previously (Omura and Sato, 1964).

Immunoblotting. To measure the expression of P450 proteins in cytochrome P450 monkey liver, immunoblotting was performed as described previously (Uno et al., 2006). In brief, specificity of each antibody was assessed using recombinant proteins (1.0 pmol each) of the 19 cytochrome P450 proteins, which were fractionated in 10% SDS polyacrylamide gels and transferred to Hybond-P filters (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The filters were immunoblotted with primary antibody (1:200–1:100,000), including polyclonal anti-human CYP1A1, anti-human CYP2A6, anti-human CYP2B6, anti-human CYP2C9, anti-human CYP3A4, and anti-human CYP3A5 antibodies. The filters were then immunoblotted with secondary antibody (1:5000), and developed using an enhanced chemiluminescence Western blotting detection reagent (GE Healthcare) and autoradiography. The developed films were scanned with a desktop scanner, and the optical density of the bands was quantified using Image J software (National Institutes of Health, Bethesda, MD). Standard curves for quantification were generated using the recombinant P450. For CYP2A, CYP2C9/19, and CYP2D, the recombinant protein of CYP2A23, CYP2C9, and CYP2D17 was used. Pilot experiments for each antibody and five representative liver samples were conducted and it was decided to load 5 µg of microsomal proteins in gels to keep the densities of the protein bands within the linear range of the standard curves. Limits of detection are provided in Table 1. Each liver sample was analyzed in duplicate with each P450 antibody. The amount of each P450 protein per lane was calculated relative to the standard curve and was divided by the amount of total protein loaded to determine specific content.

Enzyme Assays. Drug-metabolizing enzyme activities were mea-
sured using typical human P450 substrates (bufuralol, coumarin, dextromethorphan, 7-ethoxyresorufin, midazolam, pentoxifylline, and progesterone) as described previously (Yamazaki and Shirako, 1997; Yamazaki et al., 2002; Emoto et al., 2009). In brief, each mixture (0.20 ml) contained liver microsomes (20 µg of protein), 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, 10 µM coumarin, 200 µM dextromethorphan, 10 µM 7-ethoxyresorufin, 100 µM midazolam, 10 µM pentoxifylline, or 100 µM progesterone) in 50 to 100 mM potassium phosphate buffer, pH 7.4. After incubation at 37°C for 10 min, reactions were terminated by adding 0.40 ml of ice-cold methanol, 10 µl of 60% perchloric acid, or 1.5 ml of ethyl acetate. After centrifuga-
tion at 1500 g for 10 min, the supernatant or extract was analyzed by reverse-phase high-performance liquid chromatography with a fluorescence or UV detector. Metabolic assays using diclofenac and testosterone as substrates were carried out as described previously (Nakanishi et al., 2011). To estimate a correlation between drug-
metabolizing enzyme activities and P450 amounts, linear regression analysis was performed using Origin7.5J software (OriginLab Corp., Northampton, MA).

Results

Specificity of the P450 Antibodies. Because most anti-
bodies used were originally raised against human P450 pro-

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TABLE 1
Individual P450 contents in cynomolgus monkey liver

<table>
<thead>
<tr>
<th>P450</th>
<th>Total P450</th>
<th>Male</th>
<th>Female</th>
<th>Range</th>
<th>Detection Limit</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(Mean ± S.D.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450</td>
<td>pmol/mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total P450*</td>
<td>724 ± 192</td>
<td>771 ± 156</td>
<td>674 ± 220</td>
<td>275–1141</td>
<td>N.A.</td>
</tr>
<tr>
<td>CYP1A</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>0.01</td>
</tr>
<tr>
<td>CYP1D</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>0.01</td>
</tr>
<tr>
<td>CYP2A</td>
<td>26 ± 7.2</td>
<td>24 ± 6.7</td>
<td>28 ± 7.2</td>
<td>14–41</td>
<td>0.025</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>14 ± 6.5</td>
<td>14 ± 6.3</td>
<td>14 ± 6.9</td>
<td>3.3–26</td>
<td>0.025</td>
</tr>
<tr>
<td>CYP2C9/19</td>
<td>11 ± 3.8</td>
<td>9.8 ± 4.6</td>
<td>12 ± 2.4</td>
<td>1.5–16</td>
<td>0.01</td>
</tr>
<tr>
<td>CYP2C76</td>
<td>4.3 ± 2.0</td>
<td>4.3 ± 1.7</td>
<td>4.3 ± 2.4</td>
<td>1.4–8.5</td>
<td>0.01</td>
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<tr>
<td>CYP2D</td>
<td>3.2 ± 0.7</td>
<td>3.3 ± 0.6</td>
<td>3.1 ± 0.8</td>
<td>1.4–4.6</td>
<td>0.00025</td>
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<tr>
<td>CYP2E1</td>
<td>12 ± 2.7</td>
<td>12 ± 3.1</td>
<td>11 ± 2.2</td>
<td>5.5–17</td>
<td>0.01</td>
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<tr>
<td>CYP2J2</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>0.01</td>
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<tr>
<td>CYP3A4</td>
<td>27 ± 5.3</td>
<td>29 ± 2.5</td>
<td>26 ± 6.9</td>
<td>10–34</td>
<td>0.01</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>9.0 ± 3.1</td>
<td>10 ± 3.5</td>
<td>7.9 ± 2.3</td>
<td>4.8–21</td>
<td>0.01</td>
</tr>
<tr>
<td>CYP3A4 + 3A5</td>
<td>36 ± 6.3</td>
<td>39 ± 4.6</td>
<td>34 ± 6.9</td>
<td>19–49</td>
<td>N.A.</td>
</tr>
<tr>
<td>Total*</td>
<td>106 ± 14</td>
<td>106 ± 10</td>
<td>106 ± 18</td>
<td>81–130</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

N.A., not available; BDL, below detection limit.

* Spectrally determined P450.

† Sum of the immunoquantified P450s.

The content of most P450 proteins varied 3- to 4-fold in the animals analyzed, including CYP2A, CYP2D, CYP2E1, CYP3A4, and CYP3A5 (Table 1). The variations were even less for total CYP3A content (2.6-fold). The larger interanimal variations were observed for CYP2B6 (7.9-fold), CYP2C9/19 (11-fold), and CYP2C76 (6.3-fold) (Table 1). The differences in the interanimal variations were remarkable between CYP3A4 and CYP2C9/19, which showed the smallest and largest degree of variations in 27 animals, respectively (Fig. 3). Because of the interanimal variations, CYP2A was the most abundant P450 in three animals, whereas CYP3A was the most abundant in the rest of the animals. Likewise, CYP2C9/19 was more abundant than CYP2C76 in most animals, but CYP2C76 was more abundant than
CYP2C76 in two animals. The CYP3A4 amount varied 3.3-fold in 27 animals, among which two animals expressed CYP3A4 approximately 2-fold less than others (Fig. 3). When these two animals were excluded, the amount of CYP3A4 varied only 1.4-fold in the 25 animals.

Total immunoquantified P450s averaged 106 pmol/mg protein, ranging from 81 to 130 pmol/mg protein, and were lower than spectrally determined P450s. Using this value, the content of each P450, expressed as a percentage of total P450s, ranged from 23 to 44, 11 to 34, 14 to 42, 4 to 24, 5 to 17, 2 to 5%, for CYP3A, CYP3A4, CYP2A, CYP2B6, CYP2E1, CYP2A23, CYP2C9, CYP2C76, and CYP2D, respectively, and the average values generally followed this trend (Fig. 4). These results indicated that CYP3A is the most abundant subfamily in cynomolgus monkey liver.

**Drug-Metabolizing Enzyme Activities.** To assess the correlation between quantified P450 amount and drug-metabolizing enzyme activities, enzyme activities were measured using liver microsomes of 27 cynomolgus monkeys. We examined bufuralol 1’-hydroxylation, coumarin 7-hydroxylation, dextromethorphan N- and O-deethylation, diclofenac 4’-hydroxylation, 7-ethoxyresorufin O-deethylation, midazolam 1’- and 4-hydroxylation, pentoxysorufin O-deethylation, progesterone 6β-hydroxylation, and testosterone 2α-, 6β-, 16α-, and 16β-hydroxylation. The correlation coefficients indicated that among the P450s analyzed CYP2D was highly correlated with bufuralol 1’-hydroxylation and dextromethorphan O-deethylation, whereas CYP3A and CYP3A4 were highly correlated with midazolam 1’-hydroxylation, midazolam 4-hydroxylation, and testosterone 6β-hydroxylation (Table 2). Significant correlation coefficients were also observed for CYP3A4 (dextromethorphan N-deethylation and progesterone 6β-hydroxylation), CYP2B6 (testosterone 16β-hydroxylation), and CYP2C9/19 (diclofenac 4-hydroxylation) (Table 2). Other occasional correlations were found for CYP2A (7-ethoxyresorufin O-deethylation), CYP2B6 (midazolam 1’- and 4-hydroxylation), CYP2C9/19 (midazolam 4-hydroxylation), CYP2C76 (bufuralol 1’-hydroxylation, 7-ethoxyresorufin O-deethylation, and pentoxysorufin O-deethylation), CYP2D (coumarin 7-hydroxylation), and CYP3A5 (bufuralol 1’-hydroxylation and testosterone 2α- and 16α-hydroxylation) (Table 2). No correlation was found for CYP2E1.

**Discussion**

In this study, the amount of P450 proteins was immunoquantified individually in 27 cynomolgus monkey livers using anti-P450 antibodies with their specificities confirmed on
cynomolgus P450 proteins. The P450 enzymes were selected based on gene and protein expression results that have been reported previously. We have analyzed the macaque genome to identify and characterize cynomolgus P450s orthologous to human P450s that are relevant to drug metabolism in the CYP1–3 family (Uno et al., 2011a), and all of the P450 proteins that were expressed at detectable levels by immunoblotting (Fig. 2) were quantified in this study.

As shown by average immunoquantified P450 values in cynomolgus monkey liver, CYP3A4 represented the most abundant P450, making CYP3A (CYP3A4 plus CYP3A5) the most abundant P450 subfamily in this species. In human liver, CYP3A is also the most abundant P450 subfamily and constitutes approximately 40% of total immunoquantified P450 content (Shimada et al., 1994), similar to that of cynomolgus monkey (35%) as shown in this study. Moreover, CYP3A4 and total CYP3A content varied 3.3- and 2.6-fold, respectively, and these contents (CYP3A4 and total CYP3A) varied even less (1.4-fold) when two animals showing low expression were excluded. In contrast, human CYP3A4 content seems to vary nearly 60-fold (Wrighton et al., 1990; Mimura et al., 1993; Stevens et al., 1993; Shimada et al., 1994), and the variation is approximately 6-fold even when the outliers from the data set are excluded. Therefore, the variation of hepatic CYP3A4 content is much smaller in cynomolgus monkeys than in humans.

In humans, the variation in CYP3A4 hepatic content seems to be accounted for by regulatory factors, including pregnane X receptor, but less likely by genetic variants (Stevens, 2006). Cynomolgus CYP3A4 is predominantly expressed in liver (Uno et al., 2007a) and can be substantially induced by P450 inducer such as rifampicin via pregnane X receptor, similar to human CYP3A4 (Kim et al., 2010), suggesting that cynomolgus monkeys and humans share transcriptional regulatory mechanisms for CYP3A4. Moreover, cynomolgus CYP3A4 metabolizes various human CYP3A4 substrates (e.g., midazolam, nifedipine, and dexamethasone), but not the substrates largely metabolized by other P450 subfamily enzymes (Iwasaki et al., 2010). This is further supported by high correlation coefficients observed between CYP3A4 content and catalytic activities for human CYP3A substrates, such as midazolam, indicating the similar substrate selectivity of CYP3A4 in cynomolgus monkeys and human. Based on similarities in hepatic content and substrate selectivity of CYP3A4, regulatory mechanism for CYP3A4, and small interanimal variations of cynomolgus CYP3A4, cynomolgus monkey would be a suitable animal species to investigate a CYP3A4-dependent drug metabolism in liver.

CYP2A was the second most abundant P450 subfamily in cynomolgus monkey liver, representing 25% of total immunoquantified P450 content. In human liver, CYP2A6 represents 6% (14 pmol/mg protein) of total immunoquantified P450 content, less than that (26 pmol/mg protein) of cynomolgus monkey. In human liver, CYP2A6 is the major CYP2A expressed, whereas CYP2A23, CYP2A24, and CYP2A26 are expressed in cynomolgus monkey liver and metabolize the human CYP2A substrate coumarin (Uehara et al., 2010). A larger number of CYP2A enzymes and their predominant expression in liver might partly account for more abundant CYP2A proteins and the higher rate of coumarin 7-hydroxylation in cynomolgus monkey liver than in human liver (Sharer et al., 1995; Bogaards et al., 2000). The abundance of CYP2A protein suggests the

### Table 2

<table>
<thead>
<tr>
<th>Activity</th>
<th>Total P450</th>
<th>2A</th>
<th>2B6</th>
<th>2C9/19</th>
<th>2C76</th>
<th>2D</th>
<th>2E1</th>
<th>3A4</th>
<th>3A5</th>
<th>3A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n mol/min/mg protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Bufuralol 1'-hydroxylation</td>
<td>0.79 ± 0.26</td>
<td>0.29</td>
<td>0.25</td>
<td>−0.12</td>
<td>0.15</td>
<td>0.43</td>
<td>0.65***</td>
<td>−0.09</td>
<td>−0.37</td>
<td>0.42*</td>
</tr>
<tr>
<td>Coumarin 7'-hydroxylation</td>
<td>0.097 ± 0.061</td>
<td>0.15</td>
<td>0.14</td>
<td>−0.11</td>
<td>−0.15</td>
<td>0.12</td>
<td>0.44*</td>
<td>0.08</td>
<td>−0.26</td>
<td>−0.02</td>
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<tr>
<td>Dextromethorphan N-deethylation</td>
<td>0.12 ± 0.047</td>
<td>0.11</td>
<td>0.37</td>
<td>−0.18</td>
<td>−0.31</td>
<td>0.01</td>
<td>0.08</td>
<td>0.13</td>
<td>0.46***</td>
<td>0.02</td>
</tr>
<tr>
<td>Dextromethorphan O-deethylation</td>
<td>0.63 ± 0.52</td>
<td>0.18</td>
<td>0.31</td>
<td>0.08</td>
<td>−0.01</td>
<td>0.33</td>
<td>0.66***</td>
<td>0.10</td>
<td>−0.51</td>
<td>0.16</td>
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<tr>
<td>Diclofenac 4-hydroxylation</td>
<td>0.13 ± 0.033</td>
<td>0.30</td>
<td>0.02</td>
<td>−0.18</td>
<td>0.38*</td>
<td>0.16</td>
<td>0.12</td>
<td>0.07</td>
<td>0.22</td>
<td>0.31</td>
</tr>
<tr>
<td>7-Ethoxyresorufin O-deethylation</td>
<td>0.092 ± 0.056</td>
<td>0.06</td>
<td>0.47*</td>
<td>−0.01</td>
<td>−0.22</td>
<td>0.39*</td>
<td>0.04</td>
<td>−0.22</td>
<td>−0.25</td>
<td>−0.21</td>
</tr>
<tr>
<td>Midazolam 1'-hydroxylation</td>
<td>1.78 ± 0.56</td>
<td>0.44*</td>
<td>0.08</td>
<td>0.43*</td>
<td>0.32</td>
<td>0.28</td>
<td>0.11</td>
<td>−0.16</td>
<td>0.51**</td>
<td>0.22</td>
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<tr>
<td>Midazolam 4-hydroxylation</td>
<td>1.33 ± 0.41</td>
<td>0.40*</td>
<td>0.12</td>
<td>0.44*</td>
<td>0.45*</td>
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<td>−0.25</td>
<td>0.69***</td>
<td>0.07</td>
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<tr>
<td>Pentoxysoruran O-deethylation</td>
<td>0.0031 ± 0.0092</td>
<td>0.43*</td>
<td>0.32</td>
<td>0.05</td>
<td>0.11</td>
<td>0.39*</td>
<td>−0.15</td>
<td>−0.11</td>
<td>0.32</td>
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<tr>
<td>Progestosterone 6β-hydroxylation</td>
<td>3.01 ± 0.94</td>
<td>0.32</td>
<td>0.29</td>
<td>0.16</td>
<td>0.11</td>
<td>0.16</td>
<td>−0.16</td>
<td>0.02</td>
<td>0.51**</td>
<td>0.04</td>
</tr>
<tr>
<td>Testosterone 2α-hydroxylation</td>
<td>0.16 ± 0.027</td>
<td>0.01</td>
<td>−0.02</td>
<td>0.08</td>
<td>0.02</td>
<td>−0.14</td>
<td>0.06</td>
<td>0.02</td>
<td>0.07</td>
<td>0.43*</td>
</tr>
<tr>
<td>Testosterone 6β-hydroxylation</td>
<td>4.87 ± 1.16</td>
<td>0.31</td>
<td>0.22</td>
<td>0.07</td>
<td>0.07</td>
<td>0.11</td>
<td>−0.08</td>
<td>−0.06</td>
<td>0.64***</td>
<td>0.06</td>
</tr>
<tr>
<td>Testosterone 16α-hydroxylation</td>
<td>0.11 ± 0.074</td>
<td>0.12</td>
<td>−0.01</td>
<td>0.39</td>
<td>−0.07</td>
<td>0.05</td>
<td>0.22</td>
<td>−0.07</td>
<td>−0.35</td>
<td>0.50**</td>
</tr>
<tr>
<td>Testosterone 16β-hydroxylation</td>
<td>0.29 ± 0.070</td>
<td>0.29</td>
<td>0.13</td>
<td>0.42*</td>
<td>−0.02</td>
<td>0.23</td>
<td>0.23</td>
<td>−0.08</td>
<td>0.25</td>
<td>0</td>
</tr>
</tbody>
</table>

Statistical significance was determined based on the P value (probability that r is zero) of the linear regression: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
possible critical role of CYP2A for drug metabolism in cynomolgus monkey liver.

CYP2C was the third most abundant P450 subfamily in cynomolgus monkey liver, representing 14% of total immunoquantified P450s, including CYP2C9/19 (10%) and CYP2C7 (4%). The CYP2C subfamily represents the second most abundant P450 subfamily in human liver, constituting 25% of total immunoquantified P450s (Shimada et al., 1994). In liver, human CYP2C content (60 pmol/mg protein) is more abundant than cynomolgus CYP2C content (15 pmol/mg protein). Less CYP2C protein might partly account for the lower rate of tolbutamide 4-hydroxylation (Weaver et al., 1999; Turpeinen et al., 2007), which cynomolgus CYP2C9 and CYP2C7 catalyze (Uno et al., 2006, 2007b). In this study, CYP2C8 was not analyzed, because specific antibody for cynomolgus CYP2C8 was not available. CYP2C8 is one of the major functional CYP2C enzymes in human liver. CYP2C8 content, if quantified, would provide more accurate CYP2C content in cynomolgus monkey liver.

CYP2C7/C9 protein content (4%) was less than CYP2C9/19 protein content (10%) in cynomolgus monkey liver. A previous study showed that CYP2C7/C9 mRNA was most abundantly expressed in cynomolgus monkey liver among the major CYP2C mRNAs (Uno et al., 2006). In this study, CYP2C7 was more abundant in CYP2C9/19 in only 2 of the 27 animals analyzed. This discrepancy is most likely accounted for by the interanimal variations in expression of CYP2C9/19 and CYP2C7 proteins, which varied 11- and 6.3-fold, respectively, in this study. Moreover, the content of CYP2C9 and CYP2C19 might also vary among animals, although their contents were not measured separately in this study. Therefore, the most abundant CYP2C enzyme might be different in each animal.

Cynomolgus CYP2B6, the only CYP2B enzyme in cynomolgus monkey, represented 13% of total immunoquantified P450s in liver. In human liver, previous reports indicated that CYP2B6 amount was 1 pmol/mg protein, constituting <1% of total P450 content (Shimada et al., 1994). However, studies using selective antibodies demonstrated that mean CYP2B6 content in human liver was higher, ranging from 2 to 82 pmol/mg protein (Stresser and Kupfer, 1999) and 0.7 to 71 pmol/mg protein (Ekins et al., 1998), making CYP2B6 content 6% of total hepatic P450 content (Stresser and Kupfer, 1999). In human liver, a large variation has been observed in CYP2B6 content. CYP2B6 content varied 108-fold (Shimada et al., 1994) and 100-fold (Ekins et al., 1998), representing the largest interindividual variations among the P450s analyzed (Shimada et al., 1994). In this study, relatively large interanimal differences (7.9-fold) among the P450s analyzed were observed in CYP2B6 content of cynomolgus monkey liver. These interindividual variations might account for variation in a CYP2B6-dependent drug metabolism in cynomolgus monkey as well as human.

Cynomolgus CYP2E1, the only cynomolgus CYP2E enzyme, represented 11% of total immunoquantified P450s in liver. In human, CYP2E1 content is 22 pmol/mg protein, representing 9% of total immunoquantified P450s (Shimada et al., 1994). Less CYP2E1 content (12 pmol/mg protein) of cynomolgus monkey might account for the lower rate of aniline p-hydroxylation (metabolized by human CYP2E1) in cynomolgus monkey liver than in human liver (Shimada et al., 1997).

CYP2D represented 3% of total immunoquantified P450 content in cynomolgus monkey liver. Likewise, human CYP2D6, orthologous to cynomolgus CYP2D17/44, constitutes 4% of total immunoquantified P450 content in liver (Shimada et al., 1994). The previous studies showed that the rate of the reactions catalyzed by CYP2D enzymes (i.e., bufuralol 1’-hydroxylation, dextromethorphan O-demethylation) was higher in cynomolgus monkey liver than in human liver (Sharer et al., 1995; Weaver et al., 1999). CYP2D17/44 content was 3.2 pmol/mg protein, similar to that of human CYP2D6 (5 pmol/mg protein) (Shimada et al., 1994). Thus, the higher rate of CYP2D-dependent reaction in cynomolgus monkey liver might be partly caused by the faster rate of cynomolgus CYP2D enzyme, as shown previously (Mankowski et al., 1999; Uno et al., 2010d). In human, CYP2D6, involved in the metabolism of approximately 25% of known drugs in the market, is highly polymorphic, leading to interindividual variations in response to drugs that are metabolized by CYP2D6 (Ingelman-Sundberg, 2005). Genetic polymorphisms have been also identified in cynomolgus P450 genes (Uno et al., 2009c, 2010c). Genetic polymorphisms in CYP2D genes, if any, might account for the higher rate of the CYP2D-dependent reaction in some animals.

In this study, CYP1A1/2 by the anti-human CYP1A1 antibody, was not detected in monkey liver (< 0.01 pmol/mg protein). In contrast, CYP1A2 is abundantly expressed and constitutes 18% of total immunoquantified P450 content in human liver (Shimada et al., 1994). Previous studies also reported that the proteins that reacted with anti-CYP1A antibody were not detected or barely detected in untreated cynomolgus monkey liver, but highly induced by P450 inducers such as β-naphthoflavone, 3-methylcholanthrene, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Edwards et al., 1994; Bullock et al., 1995; Sadrieh and Snyderwine, 1995). CYP1A1 mRNA is highly induced in cynomolgus monkey hepatocyte culture by the P450 inducer omeprazole (Nishimura et al., 2007; Ise et al., 2011), suggesting that CYP1A1 is induced to a sufficient level to play a functional role, upon exposure to exogenous compounds. Likewise, CYP2J2 was not detected in cynomolgus monkey liver using anti-human CYP2J2 antibody (< 0.01 pmol/mg protein). CYP2J2 mRNA is expressed in cynomolgus monkey liver (Uno et al., 2007a). The anti-human CYP2J2 antibody used might not be sensitive enough to detect CYP2J2 expression in cynomolgus monkey liver.

In this study, total immunoquantified P450s represented 15% of spectrally determined P450 content in 27 cynomolgus monkeys, lower than human liver where 72% of spectrally determined P450 content are total immunoquantified P450s (Shimada et al., 1994). This raises the possibility that other enzymes such as CYP4A and CYP4F enzymes are abundantly expressed in cynomolgus monkey liver. Indeed, CYP4A and CYP4F mRNAs are predominantly expressed in cynomolgus monkey liver (Uno et al., 2007a), and CYP4F enzymes are involved in the metabolism of drugs in cynomolgus monkey small intestine (Hashizume et al., 2001; Nishimuta et al., 2011). It is of great interest to measure the CYP4 family enzymes in cynomolgus monkey liver.

The correlation of P450 enzyme amounts to enzyme activities showed relatively high correlations for CYP2D with bufuralol 1’-hydroxylation and dextromethorphan O-deethylation, which are catalyzed by cynomolgus CYP2D (Uno et al., 2010d). Likewise, CYP3A4 was highly correlated with midazolam 1’-hydroxylation and midazolam 4-hydroxylation, which are catalyzed by cynomolgus CYP3A4 (Iwasaki et al., 2010). Other significant correlations found also coincided well with previous studies: testosterone 16β-hydroxylation by CYP2B6 (Uno et al., 2009b), diclofenac 4-hydroxylation by CYP2C9/19 (Uno et al., 2009c, 2010c). Genetic polymorphisms in CYP2D genes, if any, might account for the higher rate of the CYP2D-dependent reaction in some animals.
Development.

The distribution of the P450 enzymes for the metabolism of drugs in metabolism in cynomolgus monkey and estimating the contribution of each CYP2A enzyme might vary in animal livers, the apparent low correlation of cytochrome P450 CYP2A with covalin 7-hydroxylation might be accounted for by the variable amount of each CYP2A enzyme in the animal livers analyzed.

Significant correlation coefficients between metabolic activity and P450 content were generally smaller in cytochrome monoenzymes than in humans. For example, in cytochrome monoenzymes, correlation coefficients were 0.65 between CYP2B1 activity and bufuralol 1-hydroxylation and 0.64 between CYP3A4 activity and 6β-hydroxylation, which were 0.80 and 0.81 in humans, respectively (Shimada et al., 1994). Lower correlation coefficients in cytochrome monoenzymes might be simply attributable to the fact that these substrates were selected for human P450s, not cytochrome P450s. In addition, the involvement of other P450s in these reactions might also account for lower correlation coefficients in cytochrome monoenzymes; bufuralol 1-hydroxylation is also catalyzed by CYP2C76 (Uno et al., 2011b) and testosterone 6β-hydroxylation might be accounted for by the variable amount of each CYP2A enzyme in the animal livers analyzed.

In summary, immunoquantification of P450 enzymes revealed that CYP3A was the most abundant P450 subfamily in cynomolgus monkey liver, similar to human liver, representing also P450A6, which is partly responsible for differences in pi-tubulin metabolism between cytochrome monoenzymes and humans (Uno et al., 2010a). This information needs to be carefully considered when conducting drug metabolism studies using cytochrome monoenzymes.

In brief, comparison of P450 enzymes revealed that CYP3A was the most abundant P450 subfamily in cytochrome monkey liver, similar to human liver, representing 35% of total immunoquantified CYP450 content, followed by CYP2A (25%), CYP2B (14%), CYP2B6 (13%), CYP2E1 (11%), and CYP2D (3%). Interanimal variations were observed, generally 3- to 4-fold for most P450s including CYP3A4. This degree of variation is much less than that in humans. The results provide essential information for better understanding drug metabolism in cytochrome monoenzymes and estimating the contribution of the P450 enzymes for the metabolism of drugs in development.

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Authorship Contributions
Participated in research design: Yamazaki and Uno.
Conducted experiments: Uehara, Murayama, and Nakashiki.

Received new reagents or analytic tools: Zeldin.

Performed data analysis: Uehara, Murayama, and Uno.

Wrote or contributed to the writing of the manuscript: Uehara, Zeldin, Yamazaki, and Uno.

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