**Short Communication**

**Expression of Bama Minipig and Human CYP3A Enzymes: Comparison of the Catalytic Characteristics with Each Other and Their Liver Microsomes**

Received April 3, 2015; accepted June 12, 2015

**ABSTRACT**

Minipigs represent a good animal model because of the physiologic and anatomic similarities they share with humans. Three cytochrome P450 (CYP) 3A isoforms, CYP3A22, CYP3A29, and CYP3A46, have recently been reported to be expressed in Bama minipigs, which have limited data relating to their metabolic characteristics. In the present study, Bama minipig CYP3A22, CYP3A29, and CYP3A46 were recombinitely expressed and their metabolic manners were compared with those of human CYP3A4 and CYP3A5 and also human and Bama minipig liver microsomes. The results indicated Bama minipigs and human CYP3A enzymes showed similar metabolic kinetics and metabolite profiles using testosterone, midazolam, and nifedipine as substrates. However, the differences in amino acid sequences change the elimination velocity and metabolic preference of CYP3A enzymes to their substrates. It was demonstrated that CYP3A29, CYP3A4, and CYP3A5 were the most active enzymes for all reactions, whereas CYP3A46 was the least active enzyme. Substrate-dependent metabolism characteristics between human and Bama minipig CYP3A isoenzymes exist.

**Introduction**

Animal models are used in preclinical studies to predict the kinetics and toxicity of drugs in humans. However, the extrapolation of animal data to humans is often difficult because of species differences, which may lead to failure of candidate drugs during the clinical research process (Lin, 1995; Friedberg, 2000). An increasing number of studies suggest that minipigs may serve as suitable models for human pharmacological studies because of their physiologic and anatomic similarities to humans (Bode et al., 2010; Puccinelli et al., 2011). Previous studies have shown that all primary activities of human cytochrome P450 (CYP) enzymes are found in minipig CYP enzymes (Bode et al., 2010; Puccinelli et al., 2011). Similar CYP3A activities were observed in minipig and human liver microsomes when testing specific substrates (Anzenbacher et al., 1998; Bogaards et al., 2000; Zubert et al., 2002; Turpeinen et al., 2007). For this reason, the minipig is thought to be a good experimental model for drug metabolism mediated by human CYP3A. However, previous data are mostly from minipig microsomes, which represent a complex mixture of multiple enzymes. Data relating to pure minipig CYP3A isoforms are limited. Therefore, it is necessary to compare the catalytic specificities of minipig and human CYP3A enzymes.

CYP3A is considered the most important CYP subfamily, comprising approximately 30% of total human hepatic CYP and responsible for nearly 50% of clinical drug metabolisms (Wang et al., 2012). There are four CYP3A isoforms expressed in humans: CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A4 and CYP3A5 are the predominant CYP3A enzymes expressed in the human liver and are responsible for most CYP3A-mediated drug metabolism (Daly, 2006). CYP3A7 is mainly expressed in the fetal liver. Both CYP3A7 and CYP3A43 are expressed at low levels in the adult liver. In our recent study, we identified three CYP3A isoforms expressed in Bama minipigs: CYP3A22 (GenBank accession number KJ636082), CYP3A29 (GenBank accession number EU918131), and CYP3A46 (GenBank accession number NM_001134824.1) (Shang et al., 2009, 2013). CYP3A29 was the first sequenced and cloned porcine CYP3A and was previously thought to be the CYP3A4-like isoform found in minipigs (Nissen et al., 1998; Anzenbacherova et al., 2005; Yao et al., 2011). However, our results showed that CYP3A22 and CYP3A46 are the most highly expressed isoforms in the Bama minipig liver (Shang et al., 2013). Human and Bama minipig CYP3A show a high homology, sharing 73.8–76.8% identities and 83.3–85.7% positives (Shang et al., 2009, 2013).

In the present study, three pure Bama minipig CYP3A isoforms, CYP3A22, CYP3A29, and CYP3A46, as well as two human CYP3A isoforms, CYP3A4 and CYP3A5, were expressed using the Bac-to-Bac baculovirus expression system in Sf9 cells, a widely used expression system for enzyme protein expression of mammalian cells. A previous analysis has suggested that CYP3A4 has multiple probe substrates due to distinct CYP3A4-binding domains for each substrate subgroup (Kenworthy et al., 1999; Galetin et al., 2003). Usually, testosterone, midazolam, and nifedipine are selected as well known substrates of CYP3A4 (Katoh et al., 2001; Thörm et al., 2011). In this study, testosterone, midazolam, and nifedipine were selected as representative CYP3A4 substrates. However, previous studies have suggested that CYP3A5 is a poor metabolizer for testosterone (Ko et al., 2006) and midazolam (Toh et al., 2007) and that CYP3A46 is a poor metabolizer for nifedipine (Katoh et al., 2001) and testosterone (Ko et al., 2006). Thus, in this study, testosterone, midazolam, and nifedipine were also used as representative CYP3A4 substrates. Previous studies have suggested that CYP3A5 is a poor metabolizer for testosterone (Ko et al., 2006) and midazolam (Toh et al., 2007) and that CYP3A46 is a poor metabolizer for nifedipine (Katoh et al., 2001) and testosterone (Ko et al., 2006). Thus, in this study, testosterone, midazolam, and nifedipine were also used as representative CYP3A4 substrates.
study, we further investigated the metabolic similarities and differences between Bama minipig and human recombinant CYP3A enzymes as well as their liver microsomes.

Materials and Methods

Heterologous Expression of Human and Bama Minipig CYP3A Enzymes in Sf9 Cells. The polymerease chain reaction primers were designed for introducing restriction sites and a histidine tag (His6) (see Supplemental Materials and Methods). All polymerase chain reaction products were verified by DNA sequencing and subcloned into the donor vector pFastbac1. The recombinant CYP3A4, CYP3A5, CYP3A22, CYP3A29, and CYP3A46 baculovirus were generated using a Bac-to-Bac baculovirus expression system according to the manufacturer’s manual (Invitrogen, Carlsbad, CA). All steps for the production of recombinant enzymes were carried out as in our previous methods (Wang et al., 2011; Chen et al., 2012; Yu et al., 2013). Sf9 cells suspended in Gibco S900II medium were coinfectied with a virus encoding CYP3A enzymes, cytochrome P450 oxidoreductase and cytochrome b5 gene, at a ratio of 3:3:1. The total protein concentration was measured by bichinchoninic acid assay, and the content of cytochrome P450 was measured using the carbon monoxide (CO)-difference spectrum according to the method described previously (Guengerich et al., 2009).

Kinetic Assay. Testosterone 6β-hydroxylation, midazolam 1′-hydroxylation, and nifedipine oxidation are considered the specific reactions mediated by CYP3A in humans. Therefore, testosterone, midazolam, and nifedipine were chosen as substrates to compare the metabolic abilities between Bama minipig CYP3A22, CYP3A29, and CYP3A46 and human CYP3A4 and CYP3A5. Incubation mixtures of CYP3A enzymes with testosterone (20–1000 μM), midazolam (1–50 μM), or nifedipine (2–200 μM) were incubated in 100-μl final volumes (100 mM Tris-HCl, pH 7.4, 15 mM MgCl2, and NADPH generating system). The final concentration of organic solvent (methanol) in the incubation mixture was less than 0.5% (v/v). Pooled human liver microsomes (HLMs) with a protein concentration of 20 mg/ml (n = 3; Lot number DDND, YOBL, and YYIA) were purchased from the Research Institute for Liver Diseases (Shanghai, China). The pig liver microsomes (PLMs) were prepared with the ultracentrifugation methods from three Bama minipigs (Zhou et al., 2001). The P450 content of HLM and PLM was 110 and 88 pmol/mg protein, respectively. Substrates were incubated with 0.3 mg protein/ml HLM and PLM. All incubations were performed in triplicate under the linear conditions determined previously (data not shown) under optimized incubation times (15–60 minutes) and protein concentrations (0.1–0.5 mg/ml). The samples were preincubated for 2 minutes at 37°C in a shaking water bath. The reactions were initiated by the addition of 1 mM NADPH/NADP and terminated with 400 μl of methanol containing loratadine as an internal standard (final concentration of 10 μg/ml). Samples were centrifuged at 14,000g for 15 minutes to precipitate proteins and membranes. Ten microliter aliquots of the supernatants were injected into high-performance liquid chromatography (HPLC)–tandem mass spectrometry for analysis (the HPLC–tandem mass spectrometry method and the method validation results can be seen in the Supplemental Materials and Methods).

Metabolite Profiles by HPLC. Metabolite profiles of testosterone, midazolam, and nifedipine catalyzed by recombinant CYP3A enzymes and liver microsomes were determined by an Agilent 1200 HPLC system (Palo Alto, CA) equipped with a binary solvent delivery pump, column oven, UV detector, and autosampler. Chromatographic separation was performed on a ZORBAX BONUS-RP column (4.6 × 150 mm, 5 μm; Agilent) at 30°C. Metabolites of testosterone were analyzed by UV detection at 245 nm, with the isocratic mobile phase of 37% water and 63% methanol at a flow rate of 1 ml/min. Metabolites of midazolam were analyzed by UV detection at 220 nm, with the isocratic mobile phase of 65% water containing 0.05 mM ammonium acetate and 37% acetonitrile at a flow rate of 1 ml/min. Metabolites of nifedipine were analyzed by UV detection at 223 nm, with the isocratic mobile phase of 57% water and 43% acetonitrile at a flow rate of 1 ml/min.

Chemical Inhibition Assay. A range of concentrations of ketoconazole (0.02–5 μM for testosterone, 0.005–1 μM for midazolam, and 0.02–5 μM for nifedipine) were added in the incubation mixtures with 10 pmol/ml CYP3A enzymes. As for the liver microsomes assay, a concentration of ketoconazole (0.01–5 μM for testosterone, 0.005–5 μM for midazolam, and 0.01–5 μM for nifedipine) was adjusted in the mixtures with 0.3 mg protein/ml. All incubations were performed with optimized incubation times and substrate concentrations (near the S50 values of human and Bama minipig CYP3A enzymes and liver microsomes). The production of 6β-hydroxymidazolam, 1′-hydroxymidazolam, and oxidized nifedipine was used as the evaluation factor for the inhibitory effect.

Results and Discussion

Expression of Human and Bama Minipig CYP3A Enzymes in Sf9 Cells. All recombinant CYP3A enzymes registered a single band at approximately 55 kDa, according to a western blot assay (Supplemental Fig. 1). Total P450 was measured using the CO-difference spectrum, which produced a specificity spectrum of cytochrome P450, with a maximum wavelength of 450 nm. The P450 content of recombinant CYP3A4, CYP3A5, CYP3A22, CYP3A29, and CYP3A46 was 123, 181, 156, 142, and 241 pmol/mg protein, respectively.

Kinetic Properties and Chemical Inhibition Assay. Reaction rates were measured according to the production of metabolites (6β-hydroxysterosterone, 1′-hydroxymidazolam, and oxidized nifedipine). The categories of kinetic profiles of testosterone 6β-hydroxylation, midazolam 1′-hydroxylation, and nifedipine oxidation by recombinant Bama minipig and human CYP3A enzymes and liver microsomes were at first evaluated using the Eadie-Hofstee analysis, which indicated that not all of the reactions fitted the classic Michaelis-Menten model (Supplemental Fig. 2). Therefore, kinetic parameters were calculated by the Hill equation and the results are shown in Table 1 (Houston and Kenworthy, 2000). Both on pooled liver microsomes and recombinant enzymes levels, the kinetic parameters of the three reactions showed significantly differences. However, considering that most Vmax and S50 values had an order of magnitude, we also thought humans and minipigs had similarities in CYP3A metabolic characteristics to some extent. Kinetic analysis of testosterone 6β-hydroxylation revealed a nonlinear Eadie-Hofstee plot for CYP3A4, CYP3A5, and CYP3A46 and a linear plot for CYP3A22 and CYP3A29 (Supplemental Fig. 2A). Combined with the results of the microsomal incubation, human liver microsomes and recombinant enzymes have a similar metabolic behavior. Although the similarity between Bama minipig liver microsomes and CYP3A22 and CYP3A29 suggested testosterone might be mainly metabolized by CYP3A22 and CYP3A29 in the Bama minipig, the Hill coefficients were 1.23, 1.48, and 1.23 for CYP3A4, CYP3A5, and CYP3A46, respectively, indicating a positive cooperativity. This cooperativity, however, is abolished in CYP3A22 and CYP3A29. The intrinsic clearance (Clint) values of midazolam 1′-hydroxylation catalyzed by human CYP3A enzymes were dramatically higher than those catalyzed by the Bama minipig. Midazolam 1′-hydroxylation between CYP3A5 and CYP3A22 and nifedipine oxidation between CYP3A5 and CYP3A22 or CYP3A29 seemed to have no obvious differences in the S50 values. Since the liver microsomes are kind of mixture enzymes, it makes no sense to compare Vmax between liver microsomes and recombinant enzymes. However, the S50 values are relatively close.

All of the kinetic features for recombinant enzymes and liver microsomes cannot be explained with the classic Michaelis-Menten model, requiring characteristic curved Eadie-Hofstee plots to identify such behavior. Testosterone, metabolized by human recombinant CYP3A4/5 enzymes and human liver microsomes, and nifedipine, metabolized by human CYP3A5 and minipig CYP3A29 and 3A46, displayed autoactivation kinetics consistent with previous reports (Houston and Kenworthy, 2000; Hutler and Tracy, 2002). It was demonstrated that Bama minipig
TABLE 1

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Parameters</th>
<th>hCYP3A4</th>
<th>hCYP3A5</th>
<th>pCYP3A22</th>
<th>pCYP3A29</th>
<th>pCYP3A46</th>
<th>HLM</th>
<th>PLM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$ (mmol/min per nmol P450)</td>
<td>6.79 ± 0.16</td>
<td>1.33 ± 0.05</td>
<td>3.52 ± 0.11***</td>
<td>5.97 ± 0.12***</td>
<td>0.83 ± 0.03***</td>
<td>3.67 ± 0.07</td>
<td>2.96 ± 0.16**</td>
</tr>
<tr>
<td></td>
<td>$K_{i}$ (µM)</td>
<td>112.2 ± 6.4</td>
<td>136.2 ± 10.4</td>
<td>87.8 ± 7.4***</td>
<td>89.0 ± 4.6***</td>
<td>146.9 ± 11.9***</td>
<td>77.5 ± 3.5</td>
<td>107.5 ± 16.9*</td>
</tr>
<tr>
<td>Midazolam</td>
<td>$V_{\text{max}}$ (mmol/min per nmol P450)</td>
<td>0.20 ± 0.07</td>
<td>1.48 ± 0.13</td>
<td>0.98 ± 0.06</td>
<td>1.12 ± 0.05</td>
<td>1.23 ± 0.09</td>
<td>1.51 ± 0.10</td>
<td>0.82 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>$K_{i}$ (µM)</td>
<td>1.07 ± 0.01</td>
<td>2.84 ± 0.14</td>
<td>0.30 ± 0.06***</td>
<td>0.49 ± 0.02***</td>
<td>0.37 ± 0.01***</td>
<td>7.98 ± 0.21</td>
<td>3.19 ± 0.09***</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>$V_{\text{max}}$ (mmol/min per nmol P450)</td>
<td>3.0 ± 0.1</td>
<td>5.9 ± 0.2</td>
<td>5.7 ± 0.02***</td>
<td>16.2 ± 0.04***</td>
<td>19.4 ± 0.07***</td>
<td>9.0 ± 0.7</td>
<td>22.4 ± 1.6***</td>
</tr>
<tr>
<td></td>
<td>$K_{i}$ (µM)</td>
<td>360.9</td>
<td>480.3</td>
<td>52.3</td>
<td>30.6</td>
<td>18.9</td>
<td>89.1</td>
<td>142.4</td>
</tr>
</tbody>
</table>
| CYP3A29 and human CYP3A4 and CYP3A5 were the most active enzymes for testosterone 6β-hydroxylation, midazolam 1'-hydroxylation, and nifedipine oxidation, respectively, whereas Bama minipig CYP3A46 was the least active for all reactions.

The results of the present study had some differences as compared with a previous study, which compared the substrate kinetics of pig CYP3A29 and human CYP3A4 (Yao et al., 2011). The differences in kinetic parameters are likely due to differences between pig breeds, the source of recombinant enzymes, and the cytochrome P450 assay used. In the present study, each recombinant CYP3A was expressed in our laboratory under optimized conditions and coexpressed with the same source of recombinant enzymes, and the cytochrome P450 assay used.

The kinetic values for testosterone 6β-hydroxylation, midazolam 1'-hydroxylation, and nifedipine oxidation derived from our recombinant CYP3A4/5 are in accordance with previously published data (Krauser et al., 2004). The production of M2 and M3 was much less than that of 6β-hydroxytestosterone when catalyzed by both Bama minipig and human CYP3A enzymes. The peak of M2 was comparable with M3 in the samples catalyzed by human CYP3A4 and CYP3A5 and liver microsomes. The production of M2, however, was greater than that of M3 when metabolized by Bama minipig CYP3A29 and CYP3A46, but less than that of M3 catalyzed by Bama minipig CYP3A22 and liver microsomes.

**TABLE 2**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Testosterone</th>
<th>Midazolam</th>
<th>Nifedipine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu M$</td>
<td>$\mu M$</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>hCYP3A4</td>
<td>0.053 ± 0.001</td>
<td>0.048 ± 0.002</td>
<td>0.085 ± 0.003</td>
</tr>
<tr>
<td>hCYP3A5</td>
<td>0.643 ± 0.040</td>
<td>0.381 ± 0.018</td>
<td>1.247 ± 0.014</td>
</tr>
<tr>
<td>pCYP3A22</td>
<td>0.224 ± 0.006</td>
<td>0.068 ± 0.000</td>
<td>0.091 ± 0.004</td>
</tr>
<tr>
<td>pCYP3A29</td>
<td>0.354 ± 0.009</td>
<td>0.032 ± 0.001</td>
<td>0.098 ± 0.002</td>
</tr>
<tr>
<td>pCYP3A46</td>
<td>0.491 ± 0.084</td>
<td>0.022 ± 0.000</td>
<td>0.618 ± 0.080</td>
</tr>
<tr>
<td>HLM</td>
<td>0.031 ± 0.001</td>
<td>0.048 ± 0.003</td>
<td>0.068 ± 0.001</td>
</tr>
<tr>
<td>PLM</td>
<td>0.141 ± 0.002</td>
<td>0.044 ± 0.001</td>
<td>0.123 ± 0.002</td>
</tr>
</tbody>
</table>
indicating Bama minipig CYP3A22 might play a major role in the metabolism of testosterone. The major metabolites of midazolam, catalyzed by Bama minipig and human CYP3A enzymes, were 4-hydroxymidazolam (M4) (inferred according to published data) (Elbarbry et al., 2009) and 1'-hydroxymidazolam, as shown in Fig. 1B. The production of 1'-hydroxymidazolam was greater than that of M4 during incubation with the CYP3A enzymes, except in the case of Bama minipig CYP3A22 and its liver microsomes. These results show that midazolam metabolized by Bama minipig CYP3A22 is prone to generate M4. As shown in Fig. 1C, only one observed metabolite,
oxidized nifedipine, was produced during incubation with any of the Bama minipig or human CYP3A enzymes. The similar metabolite profiles were observed in the probe reactions mediated by Bama minipig and human CYP3A enzymes, which mean their catalytic centers are probably the same.

Authorship Contributions

Participated in research design: Yu, Wei, Zeng.
Conducted experiments: Bian, Shang, Yao, Hu, Guo, Zeng.
Contributed new reagents or analytic tools: Lei, Jiang.
Performed data analysis: Bian, Shang, Yao.
Wrote or contributed to the writing of the manuscript: Bian, Yu, Wei, Zeng.

References


Address correspondence to: Lushan Yu, Associate Professor, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China. E-mail: yulis@zju.edu.cn; or Hong Wei, Professor, Department of Laboratory Animal Science, College of Basic Medical Sciences, Third Military Medical University, Chongqing, 400038 China. E-mail: weihong63528@163.com

}