Note

Cytochrome P450-dependent Drug Oxidation Activity of Liver Microsomes from Microminipigs, A Possible New Animal Model for Humans in Non-clinical Studies

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Summary: Small minipigs (Bland name, Micromini Pig; registered as a novel variety of pig in the Japanese Ministry of Agriculture, Forestry and Fisheries) were developed with the aim of non-clinical pharmacological/toxicological use. They were principally mated with < 10 kg body weight at 7 months-old resulting in good handling. Cytochrome P450 (P450)-and flavin-containing monooxygenases (FMO)-dependent drug oxidation activity of liver microsomes prepared from male Microminipigs (8 months-old) was compared with that for pooled dogs, monkeys, and humans. High P450 2D-dependent bufuralol 1'-hydroxylation and FMO-dependent benzydamine N-oxygenation activity was observed in liver microsomes from Microminipigs. Typical P450 1A, 2B, 2C, 2E, and 3A-dependent drug oxidation activity was also seen in Microminipigs. However, occasional differences might give undetected low P450 2A-dependent coumarin 7-hydroxylation in Microminipigs at 8-months-old, in contrast to liver microsomes from one 10-days-old Microminipig and commercially available pooled minipigs which had low but detectable coumarin 7-hydroxylation activity. The present results suggest that there is some overlap in Microminipig and human P450 substrate specificity. These findings should provide important information for greater understanding of drug metabolism in Microminipigs, as an experimental animal model for non-clinical use.

Keywords: minipig; CYP2A; CYP2D; CYP3A; FMO; preclinical study

Introduction

In vivo preclinical pharmacokinetic studies are a key component of drug discovery and development. Typically for a given drug candidate, pharmacokinetic data are first available in at least two or three preclinical species, often including rat, dog and/or monkey, before clinical data emerge. In practice, however, species differences often complicate extrapolation of preclinical species data to the predicted pharmacokinetics of a molecule in humans. To choose an appropriate experimental model relevant to human metabolism, it is highly desirable to understand species differences in the metabolism of xenobiotics. Pigs or minipigs also have been used for a relatively long time but only rarely, apparently because of problems with handling, in spite of close physiological characteristics to those of humans.

It is widely accepted that the liver is the primary site of first-pass metabolism because of its size and high content of drug-metabolizing enzymes. One important source of interspecies variation is the difference in content and activity of hepatic cytochrome P450 (P450) enzymes, which comprise a superfamily of enzymes involved in oxidation of a large number of exogenous and endogenous compounds. Pig and minipig liver microsomal P450 enzymes have been studied. It is difficult to assess how new or old, approved or non-approved, drugs may show pharmacological and toxicological effects and/or drug interactions in animals without this enzyme information.

Recently Fuji Micra Inc. (Fujinomiya, Shizuoka, Japan) developed small minipigs (Bland name, Micromini Pig; registered as a novel variety of pig in the Japanese Ministry of Agriculture, Forestry and Fisheries) as a possible experimental animal model for humans for non-clinical...
pharmacological/toxicological use. The Microminipigs had been principally mated with less than approximately 10 kg body weight at 7 months-old resulting in good handling for experimental animals with fewer test compounds in vivo study. We first report data showing a panel of P450- and flavin-containing monoxygenases (FMO)-dependent drug oxidation activity of liver microsomes prepared from male Microminipigs. The present results suggest that there is some overlap in Microminipig and human P450 substrate specificity, with high FMO and low P450 2A activity in microminipig liver microsomes.

Materials and methods

Chemicals: Typical substrates, their reaction products and other reagents used in this study were obtained from sources described previously or were of the highest quality commercially available.8–11

Enzyme preparations: Liver microsomes from individual male Microminipigs (one 10-days-old and four 8-months-old produced in Fuji Micra Inc.) and pooled male Wistar rats (7-weeks-old) were prepared as described.9 These studies were approved by the local committee on the care and use of laboratory animals. Pooled liver microsomes from male monkeys, dogs and three strains of minipigs (Yucatan, Sinclair, and Gottingen) were obtained from BD Gentest (Woburn, MA, USA). According to the manufacturer’s data sheet the pool was comprised of livers from 3 male control Yucatan, Sinclair, and Gottingen minipigs of 12, 8, and 11 months of age, respectively.

Enzyme Assays: P450 content was determined spectrally by the original method.12 Microsomal protein concentrations were estimated using a bichinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Activities for O-dealkylation of ethoxyresorufin (20 μM, for P450 1A), 7-ethoxycoumarin (100 μM, P450 1A), and pentoxysorufin (100 μM, P450 2B), coumarin 7-hydroxylation (100 μM, P450 2A), testosterone 7α-hydroxylation (200 μM, P450 2A), tolbutaminode methyl hydroxylation (1,000 μM, P450 2C), S-mephenytoin 4′-hydroxylation (200 μM, P450 2C), bufuralol 1′-hydroxylation (20 μM, P450 2D), chlorozoxazine 6-hydroxylation (50 μM, P450 2E), and midazolam 1′- and 4-hydroxylation (100 μM, P450 3A) and oxygennation of benzodamine (1,000 μM, FMO) and methyl p-tolyl sulfide (1,000 μM, FMO) were assayed according to described methods6–10,13 using HPLC. Briefly, a typical incubation mixture consisted of 50–100 mM potassium phosphate buffer (pH 7.4 or 8.4), 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 (20 to 1,000 μM) and liver microsomes (0.10 to 0.50 mg protein/mL) at a final volume of 0.20 mL. Incubations were carried out at 37°C for 10–30 min. Assay linearity with respect to time and protein concentration and reproducibility (within <15%) was confirmed. Incubation was terminated by adding 0.40 mL ice cold methanol or 20 μL 50% phosphoric acid or 60% perchloric acid. For extraction with 1.5 mL ethyl acetate, the organic phase was transferred to a clean tube after centrifugation at 2,000 g for 10 min and evaporated to dryness at 40°C. The residue was dissolved in 0.20 mL mobile phase and introduced onto HPLC.8–10,13

Results and Discussion

P450 content and a panel of P450-dependent drug oxidation activity of liver microsomes prepared from individual Microminipigs were compared with those from pooled dogs, monkeys, and humans (Fig. 1). Total P450 content (~0.6 nmol P450/mg protein) in liver microsomes from Microminipigs was similar to that of monkeys (Fig. 1A). Typical P450 2B, 2C, and 3A-dependent drug oxidation activity of liver microsomes from Microminipigs roughly comparable to those of human liver microsomes was seen (Figs. 1B–1E, 1G, 1H). Human P450 1A and 2E activity appeared to be 4-times and 6-times higher, respectively, than that observed in the Microminipig whereas the human P450 3A activity appeared approximately 2-fold lower. In contrast, activity observed for P450 2B and 2C appeared to be of the same magnitude. High P450 2D-dependent bufuralol 1′-hydroxylation activity was observed in liver microsomes from Microminipigs and monkeys (Fig. 1F). Selective midazolam 1′-hydroxylation, dependent on P450 3A, was also detected in Microminipigs in a similar manner to humans (Fig. 1H, Table 1). Mean ratio of 1′-hydroxylation to 4-hydroxylation of midazolam in individual Microminipigs was 1.9, which was similar to the ratio of 2.1 in pooled human livers (Fig. 1H, Table 1). High FMO-dependent benzamidine N-oxygenation and methyl p-tolyl sulfide S-oxygenation activity in Microminipig liver microsomes was compared with that for humans, monkeys, and dogs (Fig. 2). These activities were consistent with reported proprieties of pig FMO.14

Although P450 2A-dependent coumarin 7-hydroxylation activity as low as experimental detection limits in Microminipigs at 8-months-old was seen (Table 1), liver microsomes from one 10-days old Microminipig had a detectable activity similar to pooled minipigs liver preparations commercially available with low coumarin 7-hydroxylation activity. Testosterone 7α-hydroxylation activity was observed only in rat liver microsomes which had undetectable coumarin 7-hydroxylation activity as reported previously.13 In contrast, there were apparently no developmental changes in P450 1A-dependent 7-ethoxycoumarin O-deethylation or P450 3A-dependent midazolam hydroxylation activity of Microminipig liver microsomes. Dynamic and substantial changes in some drug metabolizing enzyme expression in Microminipigs may have occurred. It would be interesting to note that
the same product 7-hydroxycoumarin was rapidly produced from 7-ethoxycoumarin but structurally similar coumarin was not effectively metabolized by Microminipig liver microsomes.

The present results for drug oxidation activity suggest that there is some overlap in Microminipig and human P450 substrate specificity. The differences may give unusually low P450 2A-dependent coumarin 7-hydroxylation profiles in Microminipigs at 8-months-old (Table 1). In our preliminary study, immunoblot analysis of liver microsomes from matured Microminipigs with anti-human P450 2A6 antibodies revealed that there were apparently no bands corresponded to human liver microsomes (results not shown). A deletion mutant of guanine

**Table 1. Comparison of P450 1A, 2A, and 3A activities of liver microsomes from pigs, humans, and rats**

<table>
<thead>
<tr>
<th>Liver microsomes</th>
<th>7-Ethoxycoumarin O-deethylation (pmol/min/mg protein)</th>
<th>Coumarin 7-hydroxylation (pmol/min/mg protein)</th>
<th>Testosterone 7α-hydroxylation (pmol/min/mg protein)</th>
<th>Midazolam 1′-hydroxylation (pmol/min/mg protein)</th>
<th>Midazolam 4-hydroxylation (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig 10-days-old</td>
<td>290</td>
<td>9.1</td>
<td>&lt;0.01</td>
<td>3800</td>
<td>2300</td>
</tr>
<tr>
<td>Pig 8-months-old</td>
<td>250 ± 30</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>2700 ± 1300</td>
<td>1800 ± 1300</td>
</tr>
<tr>
<td>Yucatan</td>
<td>320</td>
<td>32</td>
<td>&lt;0.01</td>
<td>5400</td>
<td>4600</td>
</tr>
<tr>
<td>Sinclair</td>
<td>200</td>
<td>4.5</td>
<td>&lt;0.01</td>
<td>8400</td>
<td>9600</td>
</tr>
<tr>
<td>Gottingen</td>
<td>200</td>
<td>1.7</td>
<td>&lt;0.01</td>
<td>5800</td>
<td>6300</td>
</tr>
<tr>
<td>Human</td>
<td>300</td>
<td>140</td>
<td>&lt;0.01</td>
<td>1600</td>
<td>750</td>
</tr>
<tr>
<td>Rat</td>
<td>240</td>
<td>&lt;0.01</td>
<td>200</td>
<td>2000</td>
<td>4800</td>
</tr>
</tbody>
</table>

* Data are mean and SD using liver microsomes prepared from individual four Microminipigs. 
at nucleotide position 421 resulting in a non-functional enzyme (204 residues of the original 495 amino acids) by a frame shift has been reported in Yorkshire minipigs.\(^5\) In our experiments, this guanine deletion mutation was not observed in liver genomic DNA samples from four Microminipigs. Minipig P450 2A has been shown reversibly inhibited by androgens on a transcriptional basis \(\text{in vivo}\).\(^6\) Recently, the regulation of hepatic P450 2A expression in pigs has also been reported by skatole (3-methylindole), indole and testicular steroids.\(^7\) These findings are consistent with the present undetectable P450 2A-dependent coumarin 7-hydroxylation activity or apparently little immunoreactive bands in liver microsomes from male matured Microminipigs.

Little information regarding drug oxidation enzymes affecting metabolic fate of pharmaceutical agents administered to pigs, dogs or monkeys, as expected animal models for humans, is available. The present study design may be limited for a quick assessment of specific activities of Microminipigs. In this context detailed kinetic analysis may make interspecies comparison more reliable and useful. However, the present results regarding drug oxidation activity in newly developed small minipigs suggest that this Microminipig can be used as a possible new animal model for humans in non-clinical pharmacological/toxicological research area, like predecessor minipigs. Microminipigs may be easy to handle as experimental animals with lesser amounts of test compounds in vivo study, because of reported principal disadvantage of requiring more test compounds for traditional minipigs.\(^8\) These findings should provide important information for greater understanding of drug metabolism in Microminipigs, as one of the possible new human models.

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**References**

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