Predictability of Metabolism of Ibuprofen and Naproxen Using Chimeric Mice with Human Hepatocytes

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ABSTRACT:
Prediction of human drug metabolism is important for drug development. Recently, the number of new drug candidates metabolized by not only cytochrome P450 (P450) but also non-P450 has been increasing. It is necessary to consider species differences in drug metabolism between humans and experimental animals. We examined species differences of drug metabolism, especially between humans and rats, for ibuprofen and (S)-naproxen as nonsteroidal anti-inflammatory drugs, which are metabolized by P450 and UDP-glucuronosyltransferase, sulfotransferase, and amino acid N-acetyltransferase for taurine conjugation in liver, using human chimeric mice (h-PXB mice) repopulated with human hepatocytes and rat chimeric mice (r-PXB mice) transplanted with rat hepatocytes. We performed the direct comparison of excretory metabolites in urine between h-PXB mice and reported data for humans as well as between r-PXB mice and rats after administration of ibuprofen and (S)-naproxen. Good agreement for urinary metabolites (percentage of dose) was observed not only between humans and h-PXB mice but also between rats and r-PXB mice. Therefore, the metabolic profiles in humans and rats reflected those in h-PXB mice and r-PXB mice. Our results indicated that h-PXB mice should be helpful for predicting the quantitative metabolic profiles of drugs mediated by P450 and non-P450 in liver, and r-PXB mice should be helpful for evaluation of species differences in these metabolic enzymes.

Introduction

It is important to predict human drug metabolism and pharmacokinetics (PK) during the preclinical stage in the pharmaceutical industry because PK contributes to efficacy and toxicity, and the attrition rate during drug development has been decreasing as a result of improvement of predictability with regard to human metabolism (Kola and Landis, 2004). The number of new drug candidates metabolized by not only cytochrome P450 (P450) but also non-P450 has been increasing, and they show diverse chemical structures, including a carboxyl group to avoid metabolism by P450. Various approaches to predict human metabolism and PK using an in vitro metabolic system with human liver microsomes, S9 fraction, and hepatocytes have been reported (Obach et al., 1997; Nagilla et al., 2006; Brown et al., 2007; Fagerholm, 2007; Stringer et al., 2008; Anderson et al., 2009; Chiba et al., 2009; Dalvie et al., 2009; Hallifax et al., 2010). However, these methods have some limits for prediction. The above reports indicated that it was difficult to predict secondary metabolism owing to the complication of multiple drug metabolic enzymes such as P450 and non-P450 because the success rate corresponding to the observed metabolites using hepatocytes was low (Anderson et al., 2009; Dalvie et al., 2009).

Chimeric mice with humanized liver, generated using urokinase-type plasminogen activator [uPA (+/+)]/severe combined immunodeficiency (SCID) mice (h-PXB mice) repopulated with human hepatocytes (PhoenixBio Co., Ltd., Hiroshima, Japan) have been reported (Tateno et al., 2004). These mice are transplanted with approximately 80% of human hepatocytes, and the expression levels and activities of P450 and non-P450 in the liver of h-PXB mice are similar to those of humans (Katoh et al., 2004, 2005; Nishimura et al., 2005; Katoh and Yokoi, 2007; Kitamura et al., 2008).

Some specific metabolites were qualitatively detected in the urine and plasma of h-PXB mice (Inoue et al., 2009; Yamazaki et al., 2010; De Serres et al., 2011; Sanoh et al., 2012b). Thus, h-PXB mice could be a good in vivo model for predicting drug metabolism in humans. However, previous investigations for quantitative prediction as well as qualitative prediction of human metabolites involved in multiple metabolic pathways from data in h-PXB mice have been insufficient.

ABBREVIATIONS: PK, pharmacokinetics; P450, cytochrome P450; SCID, severe combined immunodeficiency; h-PXB mice, human chimeric mice; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; r-PXB mice, rat chimeric mice; RI, replacement index; LC, liquid chromatography; MS/MS, tandem mass spectrometry.
Racemic ibuprofen and (S)-naproxen have been widely used as nonsteroidal anti-inflammatory drugs, which are metabolized by certain metabolic enzymes such as P450 and UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), and amino acid N-acyltransferase for taurine conjugation in liver (Figs. 1 and 2). These metabolites are mainly excreted in urine. Furthermore, species differences in the metabolism of ibuprofen and (S)-naproxen between rats and humans have also been reported (Mills et al., 1973; Sugawara et al., 1978).

In this study, rat chimeric mice (r-PXB mice) containing rat hepatocytes were used to compare the metabolism and PK between rats and humans, as well as h-PXB mice, as an in vivo approach (Tateno et al., 2004; Emoto et al., 2005; Yamazaki et al., 2010; Sanoh et al., 2012b). The aim of this study was to assess the quantitative predictability of the metabolism by P450 and non-P450 by examining urinary excreted metabolites in h-PXB mice and r-PXB mice after administration of ibuprofen and (S)-naproxen.

Materials and Methods

Chemicals. 2-(4-Isobutylphenyl)-propionic acid (ibuprofen) and 2-(3-benzoylphenyl)-propionic acid (ketoprofen) were purchased from Wako Pure Chemicals (Osaka, Japan). (S)-(+)-2-(6-Methoxy-2-naphthyl)propionic acid [(S)-naproxen] was purchased from Cayman Chemical (Ann Arbor, Michigan). 2-[4-(2-Carboxypropyl)phenyl]-propionic acid (carboxy ibuprofen), 2-[4-(2-hydroxy-2-methylpropyl)phenyl]-propionic acid (2-hydroxyibuprofen), (S)-(+)-2-(6-hydroxy-2-naphthyl)-propionic acid [(S)-O-desmethylnaproxen], and (S)-naproxen acyl-β-o-glucuronide were obtained from Toronto Research Chemicals, Inc. (North York, ON, Canada). Ibuprofen taurine conjugate was synthesized in accordance with Shirley et al. (1994). All of the other reagents and solvents were commercial products of the highest available grade or analytical grade.

Animals. The present study was approved by the animal ethics committee and was conducted in accordance with the regulations on the use of living modified organisms of Hiroshima University. Sprague-Dawley rats (6 weeks of age) and SCID mice (10 weeks of age) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). h-PXB mice and r-PXB mice (10 weeks of age), transplanted with human and rat hepatocytes, respectively, were prepared by PhoenixBio Co., Ltd. (Hiroshima, Japan). All animals were housed in a temperature- and humidity-controlled environment under a 12-h light/dark cycle with free access to tap water and food.

Human hepatocytes of a donor (African-American boy, 5 years old) were obtained from BD Biosciences (San Jose, CA). Rat hepatocytes for the preparation of r-PXB mice were isolated from the liver of Sprague-Dawley rats (4

Fig. 1. Proposed metabolic pathways of ibuprofen in humans. This figure was drawn from the data of Shirley et al. (1994) and Kepp et al. (1997).

Fig. 2. Proposed metabolic pathways of (S)-naproxen in humans. This figure was drawn from the data of Sugawara et al. (1978).
weeks of age, male). The replacement ratio of host hepatocytes with human or rat hepatocytes, calculated as the replacement index (RI), was determined by measurement of the level of human or rat albumin in blood collected from the tail vein of each PXB mouse (Tateno et al., 2004; Emoto et al., 2005). Average RI values of h-PXB mice and r-PXB mice used in this study were 78 and nearly 100%, respectively.

Administration of Ibuprofen and (S)-Naproxen. Ibuprofen and (S)-naproxen solution (5 ml/kg) were administered orally to each animal at 20 and 10 mg/kg b.wt., respectively, which included 0.5% carboxymethylcellulose with a requisite minimum amount of potassium hydroxide for solution. After treatment of ibuprofen and (S)-naproxen, pooled urine samples were collected until 24 and 48 h, respectively.

Analysis and Quantitation of Ibuprofen, (S)-Naproxen, and Their Metabolites. Pooled urine (20 μl) was mixed with 0.1% formic acid (500 μl) and internal standard solution (30 μg/ml ketoprofen, 10 μl). These mixtures were absorbed to a MonoSpin C18 column (GL Sciences Inc., Tokyo, Japan) for solid-phase extraction. Samples purified by elution with 50% acetonitrile were subjected to liquid chromatography (LC)-tandem mass spectrometry (MS/MS). The concentrations of ibuprofen acyl glucuronide, carboxy ibuprofen acyl glucuronide and 2-hydroxyibuprofen acyl glucuronide were determined as increased amounts of ibuprofen, carboxy ibuprofen, and 2-hydroxyibuprofen by hydrolysis using 1 M sodium hydroxide before solid-phase extraction. (S)-Naproxen. Pooled urine (20 μl) was mixed with acetonitrile (30 μl). After centrifugation, the supernatants with 10 mM ammonium acetate were subjected to LC-MS/MS.

The concentrations of (S)-6-O-desmethyl naproxen glucuronide were determined as increased amounts of 6-O-desmethyl naproxen by incubation for 2 h at 37°C using β-glucuronidase (20 μl) in 1 M acetate buffer (100 μl) after the hepatocytes were thawed. The concentration of 6-O-desmethyl naproxen sulfate was estimated by subtracting the concentration of 6-O-desmethyl naproxen glucuronide from that of total hydrolyzed 6-O-desmethyl naproxen after enzyme deconjugation for 2 h at 37°C using β-glucuronidase-arylsulfatase (20 μl) in 1 M acetate buffer. Incubation mixtures were extracted with ethyl acetate (5 ml) and internal standard solution (ketoprofen). The organic layer (4 ml) was evaporated to dryness, and the residues were dissolved in aqueous acetonitrile (100 μl). Aliquots of 10 μl were applied to the LC-MS/MS system.

LC-MS/MS conditions. Aliquots (10 μl) of urine samples were introduced into the LC system (Agilent Technologies, Santa Clara, CA). The mobile phase condition for ibuprofen and (S)-naproxen consisted of 10 mM ammonium acetate (A) and acetonitrile (B) through an Inersil ODS-3 column (5 μm, GL Sciences Inc.) at 40°C. The flow rate was set at 0.2 ml/min. The starting condition for the LC gradient was 90:10 (A/B). From 0 to 5 min, the mobile phase composition was changed to 10:90 (A/B), and this was maintained until 8 min. The gradient was then returned to 90:10 (A/B) linearly from 8.1 to 8.1 min, and the column was reequilibrated to the initial condition from 8.1 to 15 min. The elution times of ibuprofen, ibuprofen taurine conjugate, carboxy ibuprofen, 2-hydroxyibuprofen, (S)-naproxen, naproxen acyl glucuronide, (S)-6-O-desmethylnaproxen, and ketoprofen as internal standard were 5.9, 5.8, 0.9, 4.2, 5.0, 4.9, 4.0, and 5.0 min, respectively.

The MS/MS experiments were conducted by using API2000 LC-MS/MS systems (Applied Biosystems, Foster, CA). Mass numbers of the ionization mode, molecular ion, and product ion for ibuprofen, (S)-naproxen, and their metabolites were as follows: ibuprofen m/z = 204.9 [M – H]− to 158.5, ibuprofen taurine conjugate m/z = 311.9 [M – H]− to 123.4, carboxy ibuprofen m/z = 235.1 [M – H]− to 72.6, 2-hydroxyibuprofen m/z = 221.3 [M – H]− to 176.9, (S)-naproxen m/z = 228.7 [M – H]− to 168.5, naproxen acyl glucuronide m/z = 404.8 [M – H]− to 169.1, (S)-6-O-desmethylnaproxen m/z = 214.7 [M – H]− to 170.4, and ketoprofen m/z = 253.2 [M – H]− to 208.7.

Results

Predictability of Metabolic Profiles of Ibuprofen in Humans. Proposed metabolic pathways of ibuprofen were reported previously from the urinary metabolic profile excreted in humans after oral administration of ibuprofen (Fig. 1). Six metabolites, ibuprofen acyl glucuronide, ibuprofen taurine conjugate, carboxy ibuprofen, carboxy ibuprofen glucuronide, 2-hydroxyibuprofen, and 2-hydroxyibuprofen acyl glucuronide were predominantly detected in urine (Shirley et al., 1994; Kepp et al., 1997).

The percentage values in Table 1 indicate urinary excreted metabolites in relation to the dose (percentage of dose) after oral administration of ibuprofen in humans (400 and 600 mg/person), h-PXB mice (20 mg/kg), rats (20 mg/kg), and r-PXB mice (20 mg/kg). These metabolites observed in humans were also identified in h-PXB mice, rats, and r-PXB mice. The amount of excreted unchanged form of ibuprofen in urine was negligible in all animals in this study (less than 2% of the dose). Amounts of excreted acyl glucuronide conjugates in human urine were higher than those of rats, whereas the amount of 2-hydroxyibuprofen in humans was lower than that of rats. We directly compared six urinary metabolites (percentage of dose) between humans and rats (Fig. 3A). There were weak correlations (r^2 = 0.471, p = 0.132) The correlations reflect species differences in the excretory metabolic profile between humans and rats. To investigate whether these differences reflect each chimeric mice, we directly compared the excreted metabolites between humans and h-PXB mice. This result showed good correlation (r^2 = 0.863, p = 0.007) (Fig. 3B). In addition, good correlation was also found between rats and r-PXB mice (r^2 = 0.928, p = 0.002) (Fig. 3C), whereas the relationship between h-PXB mice and r-PXB mice was weaker (r^2 = 0.286, p = 0.274) (Fig. 3D). These data suggested that the excretory metabolic profiles in humans and rats qualitatively reflected those of h-PXB mice and r-PXB mice, respectively.

In a comparison with SCID mice, the host of chimeric mice, a low correlation was observed between humans and SCID mice (r^2 = 0.246, p = 0.317) and between h-PXB mice and SCID mice (r^2 = 0.129, p = 0.484) (Fig. 3, E and F).

Predictability of Metabolic Profiles of (S)-Naproxen in Humans. (S)-Naproxen is metabolized into four metabolites: (S)-naproxen acyl glucuronide, (S)-6-O-desmethylnaproxen, and the latter’s metabolites, (S)-6-O-desmethylnaproxen sulfite and (S)-6-O-desmethylnaproxen sulfite.

### Table 1
Cumulative urinary excretion of six metabolites of ibuprofen

<table>
<thead>
<tr>
<th>Species</th>
<th>Urinary Excreted Metabolites</th>
<th>% dose</th>
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<tbody>
<tr>
<td>Ibuprofen Acyl Glucuronide</td>
<td>Ibuprofen Taurine Conjugate</td>
<td>Carboxy Ibuprofen</td>
</tr>
<tr>
<td>h-PXB mice</td>
<td>11.3 ± 8.5</td>
<td>0.4 ± 0.4</td>
</tr>
<tr>
<td>Humans</td>
<td>11.6 ± 7.6</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>r-PXB mice</td>
<td>0.9 ± 0.7</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>SCID mice</td>
<td>2.5 ± 1.7</td>
<td>0.5 ± 0.3</td>
</tr>
</tbody>
</table>

Human data (mean ± S.D., n = 4) after oral administration of ibuprofen (600 mg/person) are from Kepp et al. (1997). Data on the amount of ibuprofen taurine conjugate (mean ± S.E., n = 4) in humans after oral administration (400 mg/person) are from Shirley et al. (1994). Each value for h-PXB mice, r-PXB mice, rats, and SCID mice after oral administration (20 mg/kg) is the mean ± S.D. of n = 8, 3, 3, and 3 respectively.
acyl glucuronide, which were reported to be mainly excreted in the urine of humans (Fig. 2) (Sugawara et al., 1978). (S)-Naproxen was also excreted at negligible levels. Table 2 shows the percentage of each urinary metabolite after oral administration of (S)-naproxen in humans (200 mg/person), h-PXB mice (10 mg/kg), rats (10 mg/kg), and r-PXB mice (10 mg/kg). Four metabolites reported in humans were also found in the urine of h-PXB mice, rats, and r-PXB mice. Amounts of excreted naproxen acyl glucuronide and 6-O-desmethyl-naproxen acyl glucuronide in human urine were higher than those of rats, whereas the amounts of 6-O-desmethyl-naproxen and its sulfate in human urine were lower than that of rats.

We compared the percentages of dose of these excretory metabolites with those of chimeric mice. The amounts of naproxen acyl glucuronide, which was mainly observed in human urine and that of 6-desmethyl-naproxen, the amount of which was low, corresponded to those of h-PXB mice. On the other hand, the amount of 6-O-desmethyl-naproxen sulfate, which was mainly observed in rats, and that of naproxen acyl glucuronide, which was lower, were in close agreement with those of r-PXB mice. Differences in excretory metabolic profiles between humans and rats were similar to those between h-PXB mice and r-PXB mice.

Discussion

Identification of primary metabolites contributes to drug design for stable metabolic analogs. Not only primary metabolites but also secondary metabolites could be involved in efficacy and toxicity via biotransformation.

It is also necessary to reflect on species differences in isofrom composition, expression, and activity of drug metabolic enzymes between humans and experimental animals (Martignoni et al., 2006). We considered that h-PXB mice with a high replacement of human hepatocytes may be useful for prediction of human metabolism because the expression levels and activities of both P450 and non-P450 enzymes reflect those of the donor hepatocytes (Yoshitsugu et al., 2006; Yamasaki et al., 2010). Sanoh et al. (2012a) demonstrated the predictability of human PK of 13 model compounds, including ibuprofen and (S)-naproxen, metabolized by P450 and non-P450, using h-PXB mice. For ibuprofen, the predictability of in vivo intrinsic clearance in h-PXB mice reflected that observed in humans (Sanoh et al., 2012a).

Ibuprofen was metabolized by CYP2C9 and UGT2B7 (Hamman et al., 1997; Buchheit et al., 2011). In addition, a taurine conjugate of ibuprofen was identified in the urine of humans as a minor metabolite (Shirley et al., 1994). (S)-Naproxen was metabolized by CYP2C9, CYP1A2, UGT2B7, and SULT1A1 (Rodrigues et al., 1996; Bovalgaha et al., 2005; Falany et al., 2005).

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Cumulative urinary excretion of four metabolites of (S)-naproxen</th>
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<tbody>
<tr>
<td>Species</td>
<td>Urinary Excreted Metabolites</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>(S)-Naproxen Acyl Glucuronide</td>
<td>(S)-6-O-Desmethyl Naproxen</td>
</tr>
<tr>
<td>h-PXB mice</td>
<td>26.5 ± 6.6</td>
</tr>
<tr>
<td>Humans</td>
<td>25.3 ± 6.7</td>
</tr>
<tr>
<td>r-PXB mice</td>
<td>2.9 ± 2.9</td>
</tr>
<tr>
<td>Rats</td>
<td>1.2 ± 0.7</td>
</tr>
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</table>

CYP2C9 is one of the most abundant P450 enzymes in human liver. CYP2C9 metabolizes approximately 20% of clinical drugs, including a number of drugs with a narrow therapeutic ranges. UGT2B7 also contributes to the metabolism of numerous clinical drugs (Williams et al., 2004).

Ibuprofen and (S)-naproxen are suitable as representative model compounds to elucidate the predictability of multiple metabolic pathways associated with P450 and non-P450 using h-PXB mice. Metabolites of ibuprofen and (S)-naproxen were reported to be excreted in urine, which suggested that the kidneys are the main excretion route (Sugawara et al., 1978; Shirley et al., 1994; Kepp et al., 1997). Furthermore, we used r-PXB mice as a control model in consideration of species differences between humans and rats in this study.

Six metabolites of ibuprofen, which were identified in humans, were also found in the urine of h-PXB mice, r-PXB mice, and rats. On the other hand, fecal excretion of these metabolites was low (data not shown). These findings suggested that h-PXB mice reflected species differences of the main excretory pathways of cefmetazole (Okumura et al., 2007). We could confirm species differences in the amounts of urinary excretion of these metabolites between humans and rats because a weak correlation (r² = 0.471, p = 0.132) was observed, as shown in Fig. 3A. There were good correlations between humans and h-PXB mice (r² = 0.863, p = 0.007), as well as between rats and r-PXB mice (r² = 0.928, p = 0.002) (Fig. 3, B and C). Therefore, species differences in urinary excretion of metabolites between humans and rats reflect the relationship between h-PXB mice and r-PXB mice.

(S)-Naproxen is metabolized in humans by acyl glucuronidation, O-demethylation, and further sulfation and glucuronidation. Four metabolites were found in urine after administration in h-PXB mice, r-PXB mice, and rats. Species differences in excretory metabolites between humans and rats reflect the levels in humans and rats because amounts of each urinary metabolite were similar between humans and h-PXB mice as well as rats and r-PXB mice, in common with the results for ibuprofen.

We used h-PXB mice for which the average RI values were approximately 80%. The contribution of the remaining 20% of host hepatocytes may have influenced the predictability. Direct comparison of excretory metabolites between humans and SCID mice as host gave a value of r² = 0.246 (p = 0.317) (Fig. 3E). In addition, there was no correlation (r² = 0.129, p = 0.484) between h-PXB mice and SCID mice (Fig. 3F), although in vitro intrinsic clearance of ibuprofen in SCID mouse hepatocytes was similar to that of h-PXB mouse hepatocytes (Sanoh et al., 2012a). This result suggested that the remaining host mouse hepatocytes did not affect the predictability using h-PXB mice despite species differences between humans and SCID mice being observed. For r-PXB mice, it is not necessary to consider the remaining host mouse hepatocytes because RI of rat hepatocytes in liver of r-PXB mice is approximately 100%.

In this study, analysis of the predictability using h-PXB mice and r-PXB mice was conducted by oral administration. It is also necessary to consider the effects of the intestine, which is not humanized in h-PXB mice, in cases of oral administration. We compared recovery metabolites in h-PXB mice after intravenous and oral administration of ibuprofen. Because these results showed good correlation (r² = 0.900, p = 0.004), metabolic activities of ibuprofen in mouse intestine may be negligible (data not shown).

Our results using ibuprofen and (S)-naproxen indicated that in vivo metabolic activities of P450 and non-P450, such as those involving UGT, SULT, and amino acid N-acetyltransferase in h-PXB mice and r-PXB mice, should be similar to those of humans and rats. In this study, r-PXB mice were used as the control animal for transplantation.
of hepatocytes. Predictability using h-PXB mice may improve when the metabolic profiles of r-PXB mice reflect those of rats.

In conclusion, our results suggest that the combined use of h-PXB mice and r-PXB mice may be helpful for quantitative prediction of species differences of drug metabolism during the early stages of drug development in the pharmaceutical industry.

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Authorship Contributions
Participated in research design: Sanoh, Sugihara, Kotake, Tayama, Horie, Kitamura, and Ohta.
Conducted experiments: Sanoh and Horiguchi.
Contributed new reagents or analytic tools: Sugihara, Kotake, Uramaru, Ohshita, and Tateno.
Performed data analysis: Sanoh and Horiguchi.
Wrote or contributed to the writing of the manuscript: Sanoh, Kotake, and Ohta.

References


Katoh M, Matsui T, Nakajima M, Tateno C, Kataoka M, Soeno Y, Horie T, Iwasaki K, Yoshizato FIG. 3. Cross-species comparison of ibuprofen metabolites excreted in urine after oral administration of ibuprofen in h-PXB mice, humans, r-PXB mice, rats, and SCID mice. Comparison of each urinary excreted metabolite (percentage of dose) between (A) humans and rats, (B) humans versus h-PXB mice, (C) rats versus r-PXB mice, (D) h-PXB mice versus r-PXB mice, (E) humans versus SCID mice, and (F) h-PXB mice versus SCID mice.


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