Application of Chimeric Mice with Humanized Liver for Study of Human-Specific Drug Metabolism

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ABSTRACT

Human-specific or disproportionately abundant human metabolites of drug candidates that are not adequately formed and qualified in preclinical safety assessment species pose an important drug development challenge. Furthermore, the overall metabolic profile of drug candidates in humans is an important determinant of their drug-drug interaction susceptibility. These risks can be effectively assessed and/or mitigated if human metabolic profile of the drug candidate could reliably be determined in early development. However, currently available in vitro human models (e.g., liver microsomes, hepatocytes) are often inadequate in this regard. Furthermore, the conduct of definitive radiolabeled human ADME studies is an expensive and time-consuming endeavor that is more suited for later in development when the risk of failure has been reduced. We evaluated a recently developed chimeric mouse model with humanized liver on uPA/SCID background for its ability to predict human disposition of four model drugs (lamotrigine, diclofenac, MRK-A, and propafenone) that are known to exhibit human-specific metabolism. The results from these studies demonstrate that chimeric mice were able to reproduce the human-specific metabolite profile for lamotrigine, diclofenac, and MRK-A. In the case of propafenone, however, the human-specific metabolism was not detected as a predominant pathway, and the metabolite profiles in native and humanized mice were similar; this was attributed to the presence of residual highly active propafenone-metabolizing mouse enzymes in chimeric mice. Overall, the data indicate that the chimeric mice with humanized liver have the potential to be a useful tool for the prediction of human-specific metabolism of xenobiotics and warrant further investigation.

Introduction

Preclinical safety assessment of small molecule drug candidates typically entails evaluation of the parent molecule and metabolites at doses/exposures that are several folds above their anticipated human exposure and preferably achieved after administration of the parent drug only. Drug candidates that form human-specific or disproportionately more abundant human metabolites relative to the nonclinical safety species present an important development challenge in that supplemental preclinical safety studies in additional species, or those involving administration of synthesized metabolites may be necessary (see the Food and Drug Administration guidance on this subject at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079266.pdf). Thus, it is important to understand the anticipated human metabolic profile of a new chemical entity early in the development process so that necessary steps can be taken to characterize and mitigate the risk of inadequate assessment of toxicity of metabolites in animal studies and minimize unnecessary development delays.

An understanding of the clearance pathways for a drug candidate also helps determine its susceptibility to various drug-drug interactions via modulation of enzymes and/or transporters involved in these pathways. This knowledge is often used in the development candidate selection phase of drug discovery to minimize the risk of particular drug-drug interactions (e.g., via inhibition of CYP3A) and/or during early development to design appropriate clinical studies to characterize the interaction profile for clinical safety evaluation or aid proper labeling.

Although human ADME studies after administration of the radiolabeled drug candidate provide definitive identification of human metabolites and clearance pathways, these studies are costly and labor intensive and are typically performed during late development when the risk of attrition has been reduced. During early development, in vitro studies in liver preparations (e.g., microsomes and hepatocytes) are widely used for preliminary assessment of drug metabolism in humans and preclinical species to select appropriate safety species and/or understand predominant metabolic pathways. However, in circumstances where in vitro studies display low substrate turnover or poor in vitro/in vivo correlation, these systems often prove inadequate or misleading (Anderson et al., 2009; Dalvie et al., 2009). Thus, a need exists for better and economical preclinical models to obtain more reliable information on the likely human metabolic profile early in the drug discovery and development process.

Recently, multiple groups have reported mouse models where the mouse hepatocytes are genetically or chemically programmed for chronic liver disease (Tateno et al., 2004; Azuma et al., 2007; Suemizu et al., 2008; Strom et al., 2010). When human hepatocytes are injected into the spleen or bloodstream of these animals, they migrate to the liver and routinely result in >70–90% humanization of the mouse liver with an apparently normal liver function (Tateno et al., 2004;
Azuma et al., 2007). Among these models, the most well-studied is the so-called "Chimeric Mouse with Humanized Liver" that expresses the urokinase plasminogen activator (uPA+) gene on a severely compromised immune-deficient (SCID) background. Studies have demonstrated that uPA/SCID chimeric mice livers have expression levels and enzymatic activity for major human P450s and phase II conjugation enzymes similar to that in donor human hepatocytes (Katoh et al., 2004, 2005b; Tateno et al., 2004; Nishimura et al., 2005) and demonstrate a human-like susceptibility to CYP450 inhibition (Katoh et al., 2004, 2007) and induction (Katoh et al., 2005a,c; Emoto et al., 2008). There have also been some attempts to evaluate whether these mice can recapitulate the in vivo human metabolic profile of various xenobiotics (Foster et al., 2014). Some examples of these studies include CYP2D6-mediated metabolism of debrisoquine to 4-hydroxy debrisoquine (Katoh et al., 2007), CYP2C9-catalyzed conversion of S-warfarin to 7-hydroxywarfarin (Inoue et al., 2008, 2009), comparison with human metabolic profile of three different GSK drug candidates (De Serres et al., 2011), metabolism of ibuprofen and naproxen (Sanoh et al., 2012), and aldehyde oxidase-catalyzed oxidation of zaleplon (Tanoue et al., 2013).

To further evaluate the chimeric mouse model for prediction of human hepatobiliary drug metabolism and excretion, we investigated the metabolic profile of a series of compounds that are known to have a qualitatively distinct metabolic profile in humans relative to rodents, including lamotrigine, diclofenac, a Merck drug candidate (named MRK-A), and propafenone. The working hypothesis of the study was that if the liver of the chimeric mice was truly functioning like human liver, a qualitative switch in metabolic pathways should be apparent in humanized mice relative to controls. Furthermore, the selected compounds underwent metabolism via oxidation as well as glucuronidation and help broaden the scope of drug metabolizing enzymes that have been evaluated for humanization in chimeric mice.

Materials and Methods

Lamotrigine, diclofenac sodium, 4’-hydroxydiclofenac, propafenone hydrochloride, and methylcellulose were obtained from Sigma Aldrich (St. Louis, MO). MRK-A was synthesized by the Medicinal Chemistry Group, Merck Research Laboratories (MRL), Rahway, NJ. [3H]lamotrigine, [14C]MRK-A, [3H]diclofenac, and [1H]propafenone with specific activity 56, 0.04, 56, and 16 mCi/mg, respectively, were prepared by the Labeled Compound Synthesis Group, Merck Research Laboratories (MRL). All radioactive tracers were >95% pure based on high-performance liquid chromatography (HPLC) analysis. Diclofenac acyl glucuronide was also synthesized by the Labeled Compound Synthesis Group, Merck Research Laboratories (MRL). All radioactive tracers were >95% pure based on high-performance liquid chromatography (HPLC) analysis.

Production of Chimeric Mouse with Humanized Liver. The uPA+/+SCID transgenic mice and the chimeric mice with humanized liver (PBX-mice) were generated and characterized as described before (Tateno et al., 2004). Briefly, the uPA+/+SCID mice were injected with 0.8 × 10⁸ to 1.0 × 10⁸ human hepatocytes into the inferior splenic pole of 2- to 3-week-old male uPA+/+SCID mice. Mice were used for studies between 11 and 14 weeks of age and a body weight of 15–23 g. The replacement index or the extent of repopulation of human hepatocytes was >80%, as determined by the measurement of human albumin levels in plasma (Tateno et al., 2004). The native SCID mice were used as controls.

Metabolism in Hepatocytes. Cryopreserved human and CD1 mouse hepatocytes were prepared for studies according to the procedures provided by the vendor. Briefly, cryopreserved hepatocytes were thawed at 37°C for 90 seconds and added to prewarmed InVitroGRO thawing media, inverted gently, and centrifuged at 50 g for 5 minutes at room temperature. The cells were resuspended in InVitroGRO incubation media, and cell count and viability were determined using the trypan blue exclusion method. Additional incubation media was added to bring the final cell concentration to two million cells per milliliter.

Compounds (10 µM) were incubated with hepatocytes (2 million cells/ml) at 37°C for 2 hours in capped vials in a shaking water bath (final acetonitrile concentration in incubations was 1%). The vials were purged with a mixture of 5% CO₂ + 95% O₂ gas every 30 minutes. The incubation was terminated by adding an equal volume of ice-cold acetonitrile, vortexing, sonicating briefly, and centrifuging at 10,000 g for 10 minutes. Samples were counted for radioactivity before and after addition of acetonitrile to determine the extraction efficiency.

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Table 1

<table>
<thead>
<tr>
<th>Radioactivity Excretion</th>
<th>Chimeric Mice</th>
<th>Control Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>[% of administered dose]</td>
<td></td>
<td>--------------</td>
</tr>
<tr>
<td>[3H]lamotrigine</td>
<td>Bile 3.3 (3.0, 3.6)</td>
<td>8.0 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>Urine 43 (42, 44)</td>
<td>26 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>Feces 2.0 (1.8, 2.1)</td>
<td>1.8 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Total 48 (48, 49)</td>
<td>36 ± 7.5</td>
</tr>
<tr>
<td>[3H]MRK-A</td>
<td>Bile 58 ± 30</td>
<td>49 ± 5.3</td>
</tr>
<tr>
<td></td>
<td>Urine 5.1 ± 2.8</td>
<td>14 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>Feces 4.1 (3.1, 5.2)</td>
<td>2.4 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Total 65 ± 34</td>
<td>65 ± 11</td>
</tr>
<tr>
<td>[3H]diclofenac</td>
<td>Bile 25 ± 6.6</td>
<td>21 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>Urine 41 ± 12</td>
<td>35 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>Feces 1.7 ± 0.9</td>
<td>1.5 (0.6, 2.4)</td>
</tr>
<tr>
<td></td>
<td>Total 68 ± 9.1</td>
<td>57 ± 12</td>
</tr>
<tr>
<td>[3H]propafenone</td>
<td>Bile 14 (14, 15)</td>
<td>6.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Urine 44 (38, 50)</td>
<td>83 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Feces 3.0 (2.4, 3.7)</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>Total 61 (55, 68)</td>
<td>89 ± 3.0</td>
</tr>
</tbody>
</table>

N.D., not determined.
recovery of the radioactive material; the recovery was greater than 90% for all analyses. The supernatants were analyzed by LC/MS (see below).

Metabolism in Bile Duct-Cannulated Chimeric and Control Mice. [3H]lamotrigine, [14C]MRK-A, [3H]diclofenac, and [3H]propafenone were individually formulated as suspensions in 0.25% methocel and dosed orally at 10 mg/kg (10 ml/kg, 50 µCi/ml for lamotrigine, diclofenac, and propafenone, 39 µCi/ml for MRK-A) to bile duct-cannulated chimeric and control mice (n = 3/group). Bile, urine, and feces were collected continuously at room temperature up to 24 hours postdose and stored at −80°C until analysis.

Treatment of Bile, Urine, and Feces. Radioactivity in bile and urine was determined by liquid scintillation counting using a Tri-Carb 3100 TR scintillation counter (Perkin Elmer, Waltham, MA). Prior to LC/MS analysis, bile and urine samples were treated with an equal volume of acetonitrile, vortex mixed, and centrifuged at 10,000 g for 10 minutes. The radioactivity recovery in the supernatant was determined and was >90% in all cases. Hydrolysis of glucuronic acid conjugates of diclofenac and its oxidative metabolites was performed by adjusting the pH to 10 by the addition of 5 N sodium hydroxide and shaking in a water bath at 37°C for 1 hour. The fecal samples were homogenized after mixing with water (in a 1:3 w/v ratio). The total radioactivity in fecal homogenates was determined as follows. Aliquots of fecal homogenate totaling ~250 mg were accurately weighed in triplicate, treated with 500 µl Solvable (Perkin Elmer) in a shaking water bath at 60°C for 30 minutes, followed by treatment with 500 µl sodium hypochlorite (4–6%) for an additional 30 minutes to dissolve and bleach color. Samples were allowed to cool, and 15 ml Hionic-Fluor (Perkin Elmer) scintillation fluid was added. Radioactivity was counted using a Tri-carb 3100TR liquid scintillation counter (Perkin Elmer).

Measurement of Intrinsic Clearance of Propafenone in Hepatocytes from Human and uPA+/+SCID Mice. Cryopreserved hepatocytes were thawed as described earlier and cell count and viability were determined using trypan blue exclusion. Cells were resuspended in Krebs-Henseleit buffer to a final cell concentration of 200,000 cells/ml. Propafenone (1 µM final concentration) was added to triplicate incubations from pooled human and uPA+/+SCID mouse hepatocytes that had been prewarmed to 37°C, and incubations were continued for 1 hour. Samples (75 µl aliquots) were drawn at 0, 5, 15, 30, and 60 minutes and added to 150 µl ice-cold acetonitrile containing 100 nM labetalol (internal standard). The supernatants were analyzed by LC-MS/MS. Intrinsic clearance (CL\text{int}) estimates were obtained using the first-order rate of propafenone disappearance, as described earlier (Obach et al., 1997).

Instrumentation. Samples for metabolite profiling were analyzed using an Accela UHPLC system coupled to an LTQ-Orbitrap mass spectrometer.

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**Fig. 2.** HPLC-radioactivity metabolite profiles in urine after oral dosing of [3H]lamotrigine to chimeric humanized mice (A) and control mice (B).
(Thermo Fisher Scientific, Waltham, MA) and equipped with a parallel Packard online radio-flow detector (Perkin Elmer). Mass spectrometer spray voltage was set at 5 kV; capillary temperature at 250°C; and a capillary voltage of 9, 10, 12, and 49 V for lamotrigine, MRK-A, diclofenac, and propafenone, respectively. Full-scan spectra were collected in positive ion mode from m/z 150-800 using fourier-transform mass spectrometry at a resolution of 60,000. Product ion spectra were acquired using both collision-induced dissociation (CID) with a normalized collision energy of 30 in the LTQ, as well as higher energy collisional dissociation with a normalized collision energy of 100 in the C-trap. Fourier-transform mass spectrometry resolution for product-ion spectra was set at 30,000. The chromatographic separation was performed on a Halo C8 column (4.6 × 150 mm, 2.7 µm) (MAC-MOD Analytical, Inc., Chadds Ford, PA). The mobile phase A consisted of 100% water and B of 100% acetonitrile, each containing 0.1% formic acid. A linear gradient was applied from 10 to 40% B over 22 minutes, 20 to 50% B over 40 minutes, 5 to 80% B over 50 minutes, or 2 to 40% B over 35 minutes for lamotrigine, MRK-A, diclofenac, and propafenone, respectively, followed by a 5-minute column wash with 90% B in each case. The UHPLC flow rate was 750 µl/min. The eluate was split postcolumn with 20% proceeding to the MS and 80% to the radio-flow detector.

Samples for intrinsic clearance determination of propafenone were analyzed using LC-MS/MS on a Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) interfaced with a Shimadzu HPLC system equipped with two series LC-10ADVP pumps (Shimadzu Scientific Instruments, Columbia, MD) and a Leap HTS PAL auto sampler (Leap Technologies, Carrboro, NC). Compounds were eluted on a 20 × 2.1 mm Betasil C18 Dash column (Thermo Fisher Scientific). Mobile phase A consisted of water with 0.1% formic acid and mobile phase B consisted of acetonitrile with 0.1% formic acid. The HPLC flow rate was 1.5 ml/min, and the elution gradient was 10 to 40% B over 1 minute. The API 4000 was operated in positive ion mode with a spray voltage of 5.5 kV and a capillary temperature of 450°C. Propafenone and the internal standard (labetalol) were monitored by multiple reaction monitoring using precursor to product ion transitions of m/z 342 → 116 and m/z 329 → 162, respectively.

Results

Figure 1 shows the chemical structures of the compounds evaluated in this investigation.

When [3H]lamotrigine was incubated with human and mouse cryopreserved hepatocytes, negligible metabolic turnover of the drug occurred after incubation for 2 hours, and only parent drug was detected in the HPLC radioactivity profile. The lack of metabolic turnover over the course of 2-hour duration of incubations is likely related to the fact that lamotrigine is a low-clearance and long half-life compound in both mice and humans (Biton, 2006; Argikar et al., 2009).

After oral administration of [3H]lamotrigine, the majority of the radioactivity that was excreted over the 24-hour sample collection period was recovered in urine (~43% and 26% of the administered dose in chimeric and control mice, respectively; Table 1). Figure 2, A and B, depicts the radiochromatograms of urine from chimeric and

![Fig. 3. High-resolution MS and MS/MS data of lamotrigine metabolites detected in chimeric mouse urine at retention times of 11 minutes (A) and 14.9 minutes (B). The proposed structure of lamotrigine N-2 glucuronide is derived from comparison of these MS data with those published earlier.](image-url)
control mice, respectively. The metabolite profile in the urine of chimeric mice was different from that observed in the urine of control mice. The mass spectrum corresponding to the major radioactive peak at a retention time of 11 minutes in chimeric mice urine is shown in Fig. 3A. The molecular ion \([M + H]^+\) at \(m/z\) 432.0472 is consistent with the elemental composition of the protonated lamotrigine glucuronide \((C_{15}H_{16}Cl_2N_5O_6)^+, \Delta M 0.0 \text{ ppm})\). The CID of the ion at \(m/z\) 432 yielded a major fragment ion at \(m/z\) 256.0150 corresponding to lamotrigine \((\Delta M -0.4 \text{ ppm})\). A second radioactive peak of lesser intensity at retention time 14.9 minutes (Fig. 2A) also exhibited a molecular ion at \(m/z\) 432.0474 and a major daughter ion after CID at \(m/z\) 256.0151, indicating a second lamotrigine glucuronide (Fig. 3B). However, this second glucuronic acid conjugate had a distinct fragmentation pattern compared with that eluting at 11 minutes as shown in Fig. 3. On the basis of mass spectral fragmentation and reverse-phase HPLC elution profile of these conjugates in comparison with those reported earlier (Doig and Clare, 1991), the radioactive peaks at 11 and 14.9 minutes in mouse urine were designated as isomeric glucuronic acid conjugates of lamotrigine at positions N-2 and N-5 of the triazine ring, respectively. The major drug-related components detected in control mouse urine were the parent lamotrigine and lamotrigine N-oxide, as reported earlier in rats (Fig. 2B; Parsons and Miles, 1984; Maggs et al., 2000). The small amounts of radioactivity excreted in the bile of both chimeric and control mice were comprised largely of unchanged lamotrigine (data not shown).

The metabolism profiles of \(^{14}\text{C}]\text{MRK-A}\) after incubation with human and CD-1 mouse hepatocytes are shown in Fig. 4, A and B, respectively. LCMS analysis indicated that the dominant radioactive peak at retention time of 7 minutes formed in human hepatocytes corresponded to \([M+H]^+\) ion with \(m/z\) 711.1896, which agrees well with the elemental composition of the parent molecule conjugated to glucuronic acid \((C_{29}H_{30}F_7N_4O_9)^+, \Delta M 0.0 \text{ ppm})\). The CID of the ion at \(m/z\) 711 yielded a dominant fragment ion at \(m/z\) 535.1571, corresponding to the \([M+H]\) ion of MRK-A \((\Delta M 0.7 \text{ ppm})\), indicating the loss of glucuronic acid (176 Da, Fig. 5). This conjugate was previously identified as an \(O\)-glucuronide of MRK-A at the hydroxymethyl moiety (unpublished data). In contrast, parent glucuronide was only a minor metabolite formed upon incubation of MRK-A with

![Fig. 4. HPLC-radioactivity profiles of \(^{14}\text{C}]\text{MRK-A}\) after incubation with human (A) and mouse (B) hepatocytes.](image-url)
mouse hepatocytes (Fig. 4B). After oral dosing of [14C]MRK-A to chimeric and control mice, the majority of administered radioactivity was excreted in the bile in both groups (58% in bile versus 5% in urine for chimeric mice and 49% in bile versus 14% in urine for control mice). The HPLC-radiochromatographic analysis of the bile from chimeric and control mice showed distinct metabolite profiles in the two groups of mice, as shown in Fig. 6, A and B. The LC-MS analysis indicated that the major metabolite in the bile of chimeric mice (Fig. 6A, retention time 16.5 minutes) was the ether glucuronide of the parent compound at the hydroxymethyl moiety, whereas this was only a minor metabolite in the bile of the control mice (Fig. 6B).

Furthermore, this parent glucuronide was also the major radioactive species observed in the urine of chimeric mice, but was not detected in the urine of control mice (data not shown). In contrast to chimeric mice, the major metabolite observed in the bile of control mice (retention time 25 minutes) corresponded to [M+H]+ ion at m/z 551, which involves oxidation of MRK-A at one of the triazole nitrogens (exact position of oxidation is unknown; Fig. 6B). A number of additional oxidative metabolites of MRK-A also were detected in bile from control mice (Fig. 6B). These data collectively indicate that MRK-A was cleared largely via glucuronidation at the hydroxymethyl moiety in chimeric humanized mice, whereas oxidation was the primary clearance route in control animals.

After oral dosing with [3H]diclofenac to both chimeric and control mice, radioactivity was excreted relatively evenly in both bile and urine (25 and 21% in bile, and 44 and 35% in urine of chimeric and control mice, respectively) (Table 1). Representative HPLC radiochromatograms of urine from chimeric and control mice after administration of [3H]diclofenac are shown in Fig. 7, A and C, respectively. Urine samples from chimeric mice were also profiled after base treatment with sodium hydroxide to hydrolyze the glucuronic acid conjugates (Fig. 7, B and D, respectively). LC-MS analysis of the dominant metabolite in the urine of chimeric mice (Fig. 7A, retention time 20.5 minutes) revealed the presence of [M+H]+ ion at m/z 488.0514, suggesting the addition of 192 Da to the parent compound, which is consistent with hydroxylation and glucuronidation of diclofenac (C20H20Cl2NO9+). To determine the regiochemistry of hydroxylation, the urine extract was subjected to base treatment with sodium hydroxide. After base treatment, the peak at 20.5 minutes corresponding to the glucuronic acid conjugate was decreased and the peak at 26.8 minutes was increased (Fig. 7B). The retention time and the CID mass spectrum of the peak at 26.8 minutes matched well with 4′-hydroxydiclofenac authentic standard, indicating that the major metabolite peak at 20.5 minutes in the original urine sample in Fig. 7A corresponded to the glucuronic acid conjugate of 4′-hydroxydiclofenac. In contrast to chimeric mice, LC-MS analysis of the dominant metabolite in the urine of control mice (Fig. 7C, retention time 24.5 minutes) revealed that the [M+H]+ ion of this metabolite was at m/z 472.0569, suggesting the addition of glucuronic acid (176 Da) to the parent compound (C20H20Cl2NO8+). The retention time of this conjugate was consistent with that of diclofenac acyl glucuronide authentic standard, and it readily converted to diclofenac upon base treatment (Fig. 7D). Multiple isomeric peaks corresponding to this m/z ratio likely represent rearranged isomers resulting from the same acyl glucuronide conjugate of the parent compound. Bile samples from chimeric and control mice also were subjected to the same analysis as urine samples, and the corresponding HPLC radiochromatograms are shown in Fig. 8. LC/MS analysis indicated that the two major components of radioactivity excreted in the bile of the chimeric mice corresponded to two 4′OH-diclofenac glucuronides (Fig. 8A, retention times 16 and 20.5 minutes), whereas radioactivity in the bile of control mice consisted primarily of the acyl glucuronide of the parent molecule (Fig. 8C, retention time 25 minutes). Similar to urine samples, these
biliary glucuronic acid conjugates also were converted to their respective aglycones upon base treatment (Fig. 8, B and D). These data on the composition of radioactivity excreted in urine and bile clearly suggest that 4'-hydroxylation followed by glucuronidation was the predominant metabolism route for diclofenac in chimeric mice, whereas parent glucuronidation was the major pathway in control mice.

After oral dosing with [3H]propafenone to bile duct-cannulated mice, 44 and 83% of the administered radioactivity was excreted in the urine, whereas 14 and 6% was excreted in the bile of chimeric and control mice, respectively (Table 1). The major metabolite observed in the urine of both chimeric and control animals was the glucuronic acid conjugate of the 4'-hydroxy derivative of propafenone at the ω-phenyl ring (Fig. 9). This metabolite also represented the major radioactive species in the bile of both chimeric and control groups (data not shown). To follow up on this unexpected result, kinetic studies using control SCID mouse hepatocytes and human hepatocytes were performed. In this analysis it was determined that the CL_{int} (or rate of disappearance) was ~30-fold faster in SCID mouse hepatocytes relative to that in pooled human hepatocytes (2.1 versus 0.07 ml/min/10^6 cells).

**Discussion**

The goal of the studies described here was to test the hypothesis that the PXB chimeric mice with humanized liver can reproduce the human metabolic profile of a range of drugs that are known to be cleared via both phase I and phase II metabolism pathways and demonstrate qualitatively different metabolic pathways in man relative to rodents. The drugs selected for this evaluation were the antiepileptic lamotrigine; the anti-inflammatory agent diclofenac; a proprietary NK₁-antagonist, MRK-A; and the antiarrhythmic propafenone.

Previous studies indicated that urinary excretion represents the major route of elimination for lamotrigine and its metabolites in humans and animals. The metabolite profile in human urine is distinct from that in rodents and is comprised of a major N-2 quaternary...
glucuronide (80–90% of urinary radioactivity versus 5–10% in rodents), with lower levels of the N-5 glucuronide (10% versus 5–10% in rodents), unchanged lamotrigine (7–30% versus 50–60% in rodents), and lamotrigine N-oxide (0–5% versus 10–25% in rodents) (Parsons and Miles, 1984; Cohen et al., 1987; Doig and Clare, 1991; Remmel and Sinz, 1991). Similar to many other tertiary amine drugs that form quaternary ammonium glucuronides, formation of the lamotrigine N-2 glucuronide appears to be restricted to monkeys and humans and is not observed to a significant extent in rodents (Green et al., 1995; Luo et al., 1995; Hawes, 1998). In rats, lamotrigine is eliminated largely via urinary excretion of the intact parent drug and, to a lesser extent, via N-2-oxidation (to form an N-oxide) and sequential epoxidation and glutathione conjugation of the O-dichlorophenyl ring (Parsons and Miles, 1984; Maggs et al., 2000). This species difference in the formation of quaternary ammonium glucuronides appears related to the fact that this metabolic pathway is catalyzed by the UGT1A4 enzyme, which is a pseudogene in rodents (Green et al., 1995; Green and Tephly, 1998; Ogura et al., 2006; Argikar et al., 2009). Therefore, the extent of formation of this N-2 glucuronide in chimeric mice could serve as an indication of the humanization of the mouse liver in terms of UGT1A4 expression and activity. Similar to humans, the majority (~90%) of the excreted radioactivity after oral dosing of [3H]lamotrigine to chimeric mice was recovered in urine, and a large fraction of this radioactivity was comprised of the N-2 glucuronide, with only small amounts of the parent drug and oxidative metabolites (Fig. 2A). In contrast, intact lamotrigine and its N-2-oxide were the major drug-related excretory products in the urine of the native SCID mice and much smaller amounts of the glucuronide conjugates were excreted (Fig. 2B).

It is noteworthy that in vitro metabolism studies did not provide any useful clues on the important clearance pathways for lamotrigine, because no metabolic turnover was observed in incubations with cryopreserved human or mouse hepatocytes. This is a relatively common occurrence during lead optimization because an important goal of many drug discovery programs is to slow metabolic clearance to improve pharmacokinetic properties of compounds, and the commonly used tissue preparations such as liver microsomes or hepatocytes remain metabolically viable for relatively short periods in incubation. Thus, in cases such as lamotrigine, chimeric mice with humanized liver could serve as a useful model to prospectively predict the human-specific metabolism of drug candidates.

MRK-A was an investigational NK1-antagonist for chemotherapy-induced nausea and vomiting. This compound was metabolized primarily via glucuronidation at the hydroxymethyl moiety in human (and dog) hepatocytes, whereas oxidation was the predominant pathway in rodents (both rats and mice) (Fig. 4). Furthermore, MRK-A exhibited acceptable pharmacokinetic properties in both rats and dogs (systemic plasma clearance of ~20–30 and ~3–5 ml/min/kg and elimination half-lives of 2–3 and 6–7 hours, respectively). Interestingly, the systemic clearance of MRK-A was reasonably well-predicted in rats (within twofold of actual) by scaling from in vitro metabolic clearance in hepatocytes using a well-stirred model of hepatic elimination (Obach et al., 1997) but under-predicted by ~3- to 4-fold in dogs (unpublished data). Because of the discordant pathways of
metabolism in hepatocytes from preclinical species and humans and the lack of a good in vitro-in vivo correlation in the dog, MRK-A was progressed to the clinic to determine its pharmacokinetic properties in humans. Unfortunately, however, it exhibited relatively poor pharmacokinetics in humans (elimination half-life of 2–3 hours), and its development had to be halted. One possible explanation for this outcome is that the rapid glucuronidation of MRK-A in humans was responsible for its short half-life, and, similar to dogs, human hepatocytes underpredicted the actual rates of glucuronidation in vivo. Chimeric mice afforded an interesting opportunity to experimentally test this hypothesis in that MRK-A would be anticipated to show distinct metabolic profiles in humanized versus control mice. The results presented in Fig. 6 show that MRK-A does in fact undergo extensive glucuronidation in chimeric mice, whereas this pathway represented only a small fraction of the overall metabolism in control mice. It remains to be determined if the chimeric mice would also have quantitatively predicted the rates of glucuronidation and the resulting rapid clearance/short half-life of MRK-A in humans.

In rats and mice, the major pathway of diclofenac metabolism is via glucuronidation of the carboxylic acid moiety (~60% of dose); in contrast, at least 50% of an oral dose of diclofenac is excreted as the 4'-hydroxydiclofenac metabolite (and its conjugates) in humans, with acyl glucuronidation of the parent representing <20% of the overall elimination (Riess et al., 1978; Stierlin and Faigle, 1979; Stierlin et al., 1979; Seitz et al., 1998; Lagas et al., 2010). The formation of 4'-hydroxydiclofenac is widely known to be catalyzed by CYP2C9 (Zhou et al., 2009), whereas acyl glucuronidation of diclofenac is mediated by UGT2B7 (King et al., 2001). We demonstrated previously that a unique human-specific CYP2C8-mediated oxidation of diclofenac acyl glucuronide to 4'-hydroxydiclofenac acyl glucuronide is also likely responsible for the apparent larger fraction of the diclofenac dose eliminated as the 4'-hydroxydiclofenac metabolite and its conjugates in humans relative to rodents (Kumar et al., 2002). Thus, the relative amounts of diclofenac acyl glucuronide and 4'-hydroxydiclofenac (unconjugated + conjugated) recovered in excreta after an oral dose of diclofenac are likely dependent on the relative activities of UGT2B7, CYP2C9, and CYP2C8 enzymes. The metabolite profile of diclofenac in bile and urine from chimeric mice was consistent with humans in that 4'-hydroxydiclofenac (largely in conjugated form) was the major metabolic product, with smaller amounts of the parent acyl glucuronide. It appears reasonable to speculate that the “humanization” of the metabolic profile of diclofenac in chimeric mice relative to control SCID mice is indicative of the human-like expression and activity of UGT2B7, CYP2C9, and likely CYP2C8 enzymes in the chimeric mouse liver.

Metabolism of the antiarrhythmic agent, propafenone, in humans involves the CYP2D6-mediated hydroxylation to 5-hydroxypropafenone, direct glucuronidation of the parent, as well as CYP3A4- and CYP1A2-catalyzed N-dealkylation to N-despropylpropafenone (Hege et al., 1984; Botsch et al., 1993; Dilger et al., 1999). The available clinical data indicate that CYP2D6-catalyzed formation of 5-hydroxypropafenone (and its subsequent glucuronidation) and direct glucuronidation of the parent represent major clearance routes for propafenone in humans (Hege et al., 1984). Although N-despropyl-propafenone is an important pharmacologically active metabolite that circulates in human plasma at comparable levels to those of

**Fig. 8.** HPLC-radioactivity metabolite profiles in bile after oral dosing of [3H]diclofenac to chimeric humanized (A) and control mice (C). Panels B and D show metabolite profiles in bile from chimeric and control mice, respectively, after treatment of samples with sodium hydroxide to hydrolyze glucuronic acid conjugates.
5-hydroxypropafenone, this pathway is of minor importance in the overall clearance of propafenone (Hege et al., 1984). This is consistent with our data on the metabolism of [³H]propafenone in human hepatocytes where 5-hydroxypropafenone (and its glucuronide conjugate), propafenone glucuronide, and \( N \)-despropylpropafenone accounted for \( \sim 60 \), 30, and 10\%, respectively, of the total metabolic turnover of propafenone (data not shown). In contrast to humans, the major metabolic pathway of propafenone in rodents involves hydroxylation at the 4'-position of the \( \omega \)-phenyl ring (Tan et al., 1998). Therefore, our hypothesis was that the relative extent of formation of 4'- and 5-hydroxypropafenone in chimeric mice could serve as an index of the human-like expression and functional activity of the CYP2D6 enzyme. However, similar metabolite profiles were observed in the bile and urine of both chimeric and control mice after oral dosing with [³H]propafenone, and the metabolism appeared to proceed primarily via the rodent-specific 4'-hydroxylation pathway; neither the human-specific metabolite 5-hydroxypropafenone nor other minor metabolites such as \( N \)-despropylpropafenone were observed in urine and bile of both sets of mice. This result was somewhat unexpected given that previous studies in the same chimeric mouse model did demonstrate the expression and functional viability of CYP2D6 activity in the livers of these mice (Katoh et al., 2004, 2007).

One potential reason for the lack of human-like propafenone metabolism in chimeric mice is that the \( \text{CL}_{\text{int}} \) of propafenone in mouse hepatocytes is much greater than that in human hepatocytes such that in chimeric mice, whose livers are \( \sim 80\% \) humanized, the residual mouse propafenone 4'-hydroxylase activity remains dominant relative to the human CYP2D6 activity and results in apparent mouse-like phenotype of propafenone biotransformation. Our studies did in fact reveal that the \( \text{CL}_{\text{int}} \) of propafenone in SCID mouse hepatocytes was \( \sim 30\text{-fold} \) higher relative to pooled human hepatocytes. Given such large difference in mouse versus human \( \text{CL}_{\text{int}} \), \( \sim 80\% \) humanization of the mouse liver does not appear sufficient to change the phenotype of propafenone metabolism from mouse to human. This dataset with propafenone suggests that before evaluating a particular drug or drug candidate in chimeric mice, it would be valuable to assess relative intrinsic clearances in mouse versus human hepatocytes to ascertain that the residual mouse metabolic activity will not mask the human metabolism phenotype.

In summary, our data indicate that chimeric mice with humanized liver were able to successfully reproduce the human-specific metabolic profile covering a range of phase I and phase II metabolic pathways for three out of the four drugs that were evaluated; the fourth drug, propafenone, with a much greater mouse hepatic \( \text{CL}_{\text{int}} \), does not appear suitable for study in such a model that retains a residual complement of mouse metabolic activities. On the basis of this and previously published data, it appears that chimeric mice offer promise for gaining understanding of the anticipated human metabolism profile of drug candidates during discovery and development; this, in turn, can help de-risk a variety of issues related to drug metabolites at an early stage or help devise appropriate and timely mitigation strategies (e.g., metabolite exposure in preclinical safety assessment species.

![HPLC-radioactivity metabolite profiles in urine after oral dosing of [³H]propafenone to chimeric humanized mice (A) and control mice (B).](image-url)
drug-drug interactions). Furthermore, our own and others’ investigations in these mice thus far have largely focused on studying qualitative aspects of human metabolism phenotype, but as further characterization data emerge, these mice could also find applications in the areas of quantitative translational ADME such as prediction of human pharmacokinetics and drug-drug interactions mediated via phase I and phase II enzymes as well as transporters. These aspects warrant continued future investigation.

**Authorship Contributions**

**Participated in research design:** Bateman, Reddy, and Kumar.

**Conducted experiments:** Bateman and Kakuni.

**Contributed new reagents or analytic tools:** Kakuni and Morikawa.

**Performed data analysis:** Bateman, Reddy, and Kumar.

**Wrote or contributed to the writing of the manuscript:** Bateman, Reddy, Kakuni, Morikawa, and Kumar.

**References**


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