Cytochrome b₅ Is a Major Determinant of Human Cytochrome P450 CYP2D6 and CYP3A4 Activity In Vivo

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ABSTRACT

The cytochrome P450-dependent mono-oxygenase system is responsible for the metabolism and disposition of chemopreventive agents, chemical toxins and carcinogens, and >80% of therapeutic drugs. Cytochrome P450 (P450) activity is regulated transcriptionally and by the rate of electron transfer from P450 reductase. In vitro studies have demonstrated that cytochrome b₅ (Cyb5) also modulates P450 function. We recently showed that hepatic deletion of Cyb5 in the mouse (HBN) markedly alters in vivo drug pharmacokinetics; a key outstanding question is whether Cyb5 modulates the activity of the major human P450s in drug disposition in vivo. To address this, we crossed mice humanized for CYP2D6 or CYP3A4 with mice carrying a hepatic Cyb5 deletion. In vitro triazolam 4-hydroxylation (probe reaction for CYP3A4) was reduced by >50% in hepatic microsomes from CYP3A4-HBN mice compared with controls. Similar reductions in debrisoquine 4-hydroxylation and metoprolol α-hydroxylation were observed using CYP2D6-HBN microsomes, indicating a significant role for Cyb5 in the activity of both enzymes. This effect was confirmed by the concentration-dependent restoration of CYP3A4-mediated triazolam turnover and CYP2D6-mediated bufuralol and debrisoquine turnover on addition of Escherichia coli membranes containing recombinant Cyb5. In vivo, the peak plasma concentration and area under the concentration time curve from 0 to 8 hours (AUC₀–₈h) of triazolam were increased 4- and 5.7-fold, respectively, in CYP3A4-HBN mice. Similarly, the pharmacokinetics of bufuralol and debrisoquine were significantly altered in CYP2D6–HBN mice, the AUC₀–₈h being increased ~1.5-fold and clearance decreased by 40–60%. These data demonstrate that Cyb5 can be a major determinant of CYP3A4 and CYP2D6 activity in vivo, with a potential impact on the metabolism, efficacy, and side effects of numerous therapeutic drugs.

Introduction

The cytochrome P450-dependent mono-oxygenase system is responsible for the metabolism and disposition of over 80% of all therapeutic drugs and is a key determinant of drug efficacy and side effects. The functions of this system are complex, but a common feature is that, to carry out the mono-oxygenation reaction, all the microsomal cytochrome P450 enzymes (P450s) involved in the drug metabolism receive electrons from NADPH cytochrome P450 oxidoreductase (POR) (Pandey and Fluck, 2013). Furthermore, it was proposed over 40 years ago that a second electron donor, cytochrome b₅ (Cyb5), could also modulate the activity of this system (Baron et al., 1973). Since that time, numerous in vitro studies have confirmed this important function (Yamazaki et al., 1996, 2002; Lamb et al., 2001; Yamaori et al., 2003; Akhtar et al., 2005). To establish the in vivo relevance of these findings, we recently undertook conditional deletion of Cyb5 in the mouse, demonstrating marked changes in the pharmacokinetics of a number of murine P450 substrates (Finn et al., 2008; Henderson et al., 2013, 2014a; McLaughlin et al., 2010).

This raises the question, does the human P450 system exhibit the same level of Cyb5 dependency? We have addressed this question using models created as part of a project to humanize mice for pathways of drug disposition. A particular focus of our studies has been the creation of mouse models in which murine P450 gene clusters were exchanged for their human counterparts. These include models in which the seven closely linked mouse Cyp3a genes on mouse chromosome 5 were replaced with human CYP3A4 and CYP3A7 (Hasegawa et al., 2011) and the Cyp2c and Cyp2d gene clusters with human CYP2C9 and CYP2D6, respectively (Scheer et al., 2012a,b). These models have been shown to exhibit in vivo functionality reflecting that predicted in humans.

In the present study, we crossed the hepatic Cyb5 null (HBN) mouse (Finn et al., 2008) with CYP3A4 and CYP2D6 humanized mice (Hasegawa et al., 2011; Scheer et al., 2012a,b) to understand whether Cyb5 modulates human P450 activity in vivo and demonstrates profound effects on drug pharmacokinetics. These

ABBREVIATIONS: AUC, area under the curve; CO, corn oil; Cyb5, cytochrome b₅; HBN, hepatic cytochrome b₅ null; PCN, pregnenolone-16α-carbonitrile; P450, cytochrome P450; POR, cytochrome P450 oxidoreductase.
data demonstrate that Cyb5 is a key determinant of human P450 activity in vivo.

Materials and Methods

Chemicals. Reagents were purchased from Sigma-Aldrich (Poole, United Kingdom) unless otherwise stated. NADPH came from Melford Laboratories (Ipswich, United Kingdom) and nifedipine oxide from BD Gentest (Cowley, United Kingdom). 4-Hydroxytriazolam, 1-hydroxybufuralol, and bufuralol were from Toronto Research Chemicals (Toronto, Canada).

Generation of CYP3A4-HBN and CYP2D6-HBN Mice. Generation of hepatic Cyb5 null [Cyb5lox/lox::CreALB (HBN)], huCYP3A4/2A7 (Cybp 5 VII::CYP3A4/7::Crelox/lox (CYP3A4)), and huCYP2D6.1 (Cyp2d14::CYP2D6::Cyb5lox/lox (CYP2D6)) mice was described previously (Finn et al., 2008; Hasegawa et al., 2011; Scheer et al., 2012b). HBN animals were born at expected Mendelian ratios. Administration of PCN to CYP3A4 and CYP3A4-HBN mice were always predosed with either corn oil (CO) or pregnenolone-16α-carbonitrile (PCN) (10 mg/kg daily × 3) to induce CYP3A4 expression. CYP2D6 and CYP2D6-HBN mice were left untreated.

Hepatic Lipid Profiling. Hepatic lipid profiles were measured on snap-frozen livers from PCN-treated CYP3A4 and CYP3A4-HBN mice and untreated CYP2D6 and CYP2D6-HBN mice (n = 3 per group). The lipid content was analyzed by Nutrition Analytical Services, Department of Aquaculture, University of Stirling.

Preparation of Hepatic Microsomal Fractions. Microsomes were prepared from snap-frozen liver samples as described previously (Meehan et al., 1988) and stored at −80°C until required. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hertfordshire, United Kingdom).

Characterization of Hepatic Microsomes. Microsomal P450 content was determined by reduced carbon monoxide difference spectroscopy (Oumura and Sató, 1964), POR activity was estimated as NADPH-dependent cytochrome c reduction (Strobel and Dignam, 1978), and Cyb5 content was determined spectrophotometrically as described previously (McLaughlin et al., 2010).

Immunoblotting. Immunoblot analysis of microsomes (15 µg protein) was performed as described previously (Finn et al., 2008). Human liver microsomes (15 µg; BD Biosciences, Oxford, United Kingdom) were used as standards. Immunoreactive proteins were detected using polyclonal goat anti-rabbit or anti-mouse horseradish peroxidase immunoglobulin as secondary antibodies (Dako, Ely, United Kingdom) and were visualized and protein expression semiquantitated using Immobilon chemiluminescent substrate (Millipore, Watford, United Kingdom) and a Fujifilm LAS-3000 mini-imaging system (Fujifilm UK, Bedford, United Kingdom). Densitometry was performed using Multi Gauge V2.2 software (Fujifilm UK).

In Vitro Microsomal Incubations. In vitro triazolam, nifedipine, meperidine, metoprolol, bufuralol, and debrisoquine incubations were performed as described in the Supplemental Materials and Methods.

In some experiments, Escherichia coli membranes containing recombinant human Cyb5 (0–32.9 pmol/incubation) were added to hepatic microsomes (final volume 6 µl) and incubated for 20 minutes at room temperature before the addition of buffer and substrate. Triplicate incubations were initiated, terminated, and processed as described in Supplemental Materials and Methods.

Stimulation of Recombinant CYP2D6 Activity. E. coli membranes expressing recombinant CYP2D6 and POR (Pritchard et al., 1998) were mixed with E. coli membranes expressing recombinant human Cyb5 in molar ratios of 1:0, 1:5, 1:10, 1:15, and 1:20 (5 pmol of CYP2D6 per incubation; final volume 6 µl) and incubated for 20 minutes at room temperature before the addition of buffer and substrate. Incubations were initiated, terminated, and processed as described in the Supplemental Materials and Methods.

Ultraprecision Liquid Chromatography with Tandem Mass Spectrometry Analysis. Analysis of metoprolol and metabolites was performed as described previously (Finn et al., 2008). Other microsomal incubations and pharmacokinetic samples were analyzed using a Waters Acquity ultraperformance liquid chromatography (Waters Corporation, Dublin, Ireland) and Micromass Quattro Premier mass spectrometer (Micromass, Manchester, United Kingdom) as described in the Supplemental Materials and Methods.

In Vivo Pharmacokinetics. CYP3A4 and CYP3A4-HBN mice were dosed orally with triazolam or nifedipine; CYP2D6 and CYP2D6-HBN mice were dosed with bufuralol or debrisoquine. Dosing and sampling procedures are described in the Supplemental Materials and Methods. Pharmacokinetic parameters were calculated using WinNonLin software, v3.1 (Pharsight, St. Louis, MO). A noncompartmental model was used to calculate the area under the concentration time curve from 1 to 8 hours (AUC0-8h, area under the plasma concentration time curve from time 0 extrapolated to infinity (AUCinf), terminal half-life, peak plasma concentration (Cmax), and clearance. An unpaired t test was used to calculate P values.

Results

Phenotype of CYP3A4-HBN and CYP2D6-HBN Mice. We generated CYP3A4-HBN and CYP2D6-HBN mice that are humanized for either CYP3A4 or CYP2D6 (and deleted for the respective Cyp3a or Cyp2d gene clusters) and also contain a floxed Cyb5 gene, hepatic deletion of which is driven by CreALB. The mice were viable, exhibited no gross anatomic abnormalities, and had histologically normal livers (Supplemental Fig. 1). Both sexes were fertile, and offspring were born at expected Mendelian ratios. Administration of PCN to CYP3A4 and CYP3A4-HBN mice resulted in a significant increase in the liver/body weight ratio (by ∼1.2-fold). No other changes in the liver, kidney, or lung to body weight ratios were observed in any of the lines (Supplemental Table 1).

Hepatic lipid profiling indicated only slight changes in HBN mice compared with their counterparts with intact Cyb5. The only alteration in PCN-treated CYP3A4-HBN animals compared with similarly treated CYP3A4 mice was a slight reduction in arachidonic acid (20:4n-6) levels (3620 ± 68 versus 3071 ± 228 µg/g liver, mean ± S.D.; P < 0.001). In CYP2D6-HBN mice, hepatic γ-hydroxilinic, adrenic, and docosapentaenoic acid (18:3n-6, 22:4n-6, and 22:5n-3, respectively) levels were slightly reduced in comparison with those in CYP2D6 mice (0.217 ± 0.015% versus 0.283 ± 0.032% of total fatty acids, 96.2 ± 16.5 versus 126.4 ± 7.9 µg/g liver, and 79.7 ± 7.8 versus 108.8 ± 15.5 µg/g liver, respectively, mean ± S.D.; all P < 0.05).

Analysis of Cytochrome P450 Mono-Oxygenase Components. The effect of Cyb5 deletion and/or P450 humanization on mono-oxygenase components was analyzed in hepatic microsomal fractions (Fig. 1; Table 1). Spectroscopically detectable Cyb5 levels were ∼85% lower in CYP2D6-HBN and CO-treated CYP3A4-HBN mice compared with CYP2D6 and CO-treated CYP3A4, respectively, and by ∼91% in PCN-treated CYP3A4-HBN compared with similarly treated CYP3A4 animals. Total hepatic P450 levels were elevated in PCN-treated CYP3A4 (1.45-fold) and CYP3A4-HBN (2.88-fold) animals compared with CO-treated controls but were similar in untreated
CYP2D6 and CYP2D6-HBN mice. Hepatic POR activity in PCN-treated CYP3A4-HBN mice was 1.64-fold higher than in CO-treated controls.

Western blot analysis of hepatic Cyb5, POR, and P450 isozyme expression in CYP3A4 and CYP3A4-HBN mouse liver microsomes is illustrated in Fig. 1 and Supplemental Fig. 2. No Cyb5 was detected in CYP3A4-HBN samples with or without PCN treatment, but this treatment increased Cyb5 expression in CYP3A4 mice (2.3-fold). Basal levels of CYP3A4 in both lines were extremely low but increased 17- and 29-fold, respectively, in response to PCN. These values were obtained by optical scanning from the luminescent output from the bands observed by Western blotting.

Microsomes from PCN-treated CYP3A4-HBN mice had CYP3A4 levels 70% higher than those from PCN-treated CYP3A4 mice. Deletion of Cyb5 increased Cyp2b10 expression 4-fold in CYP3A4-HBN mice, and it was further upregulated (to 8.9-fold) by PCN treatment; neither Cyb5 deletion nor PCN treatment affected the expression of other P450s. This protein was identified as Cyp2b10 based on electrophoretic mobility and the high level of induction by compounds such as phenobarbital. When samples from PCN-treated CYP3A4-HBN and CYP3A4 mice were compared, slight elevations of Cyp2b2, Cyp2b10, Cyp2d, and POR (1.5-, 2.3-, 2.0-, and 1.4-fold, respectively) were apparent. This is most likely due to direct ligand activation of the pregnane X receptor.

Analysis of male CYP2D6-HBN liver microsomes revealed an increased expression of hepatic Cyp2b10 and Cyp3 proteins. Unexpectedly, a 50% reduction in CYP2D6 expression was observed in male CYP2D6-HBN mice compared with CYP2D6 mice with intact Cyb5 (Fig. 2). This difference could complicate interpretation of the effects of Cyb5 deletion on CYP2D6 activity. In females, however, CYP2D6 expression was similar in CYP2D6 and CYP2D6-HBN lines (Fig. 2; Supplemental Fig. 2). As in males and the CYP3A4-HBN line, deletion of Cyb5 led to increased expression of Cyp2b10, but no other marked effects on P450 expression were observed in female CYP2D6 and CYP2D6-HBN mice.

### In Vitro Cytochrome P450 Activities

Studies with the human CYP3A4 substrates triazolam and nifedipine and the CYP2D6 substrates debrisoquine, bufuralol, and metoprolol were performed using liver microsomes from humanized, Cyp3a null and Cyp2d null mice (Table 2). The results indicated that triazolam 4-hydroxylation was almost exclusively mediated by the product of the human CYP3A4 transgene in humanized mouse liver: in Cyp3a null mice (the background strain for the CYP3A4 humanized line) this activity was only 1.5% of that in PCN-treated CYP3A4 mice. Despite the presence of higher levels of CYP3A4 protein, triazolam turnover was reduced by 53% in liver microsomes from PCN-treated CYP3A4-HBN animals compared with those from similarly treated CYP3A4 mice. Nifedipine metabolism was still measurable in Cyp3a null microsomes, representing 30% of the activity in PCN-treated CYP3A4 microsomes, suggesting that non-Cyp3a murine P450s can metabolize this compound. A 23% reduction in nifedipine metabolism was observed in PCN-treated CYP3A4-HBN samples compared with PCN-treated CYP3A4 samples. Although effects on residual murine P450-dependent metabolism cannot be excluded, these data suggest that the effects of Cyb5 on nifedipine oxidation, like those on triazolam 4-hydroxylation, are mediated via reduced CYP3A4 activity.

In vitro studies on CYP2D6 activity were performed using samples from female mice to circumvent potential difficulties due to the difference in CYP2D6 expression between male CYP2D6-HBN and CYP2D6 mice (Table 2). Debrisoquine 4-hydroxylation and metoprolol a-hydroxylation activities of Cyp2d null samples were <2% of those of CYP2D6 liver microsomes, indicating that the activity of the humanized samples was indeed mediated by the product of the human transgene. These activities were reduced 47 and 44%, respectively, in CYP2D6-HBN samples compared with those from CYP2D6 mice. Analysis of bufuralol 1’-hydroxylation and metoprolol O-demethylation activity in Cyp2d null liver microsomes suggested that a small proportion (19 and 5%, respectively) of these activities in CYP2D6 samples was mediated by non-Cyp2d murine P450s. Bufuralol 1’-hydroxylation and metoprolol O-demethylation in CYP2D6-HBN samples was reduced by 36 and 41% compared with CYP2D6, demonstrating an interaction between Cyb5 and CYP2D6 in these reactions.

The kinetics of bufuralol 1’-hydroxylation by CYP2D6 and CYP2D6-HBN microsomes were characterized and analyzed according to the Michaelis-Menten model. Vmax was markedly

### Table 1

Levels of cytochrome b5, cytochrome P450, and cytochrome P450 oxidoreductase activities in CYP3A4, CYP3A4-HBN, CYP2D6, and CYP2D6-HBN mice

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cytochrome b5</th>
<th>Cytochrome P450</th>
<th>Cytochrome P450 oxidoreductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4 (-)</td>
<td>0.24 ± 0.09</td>
<td>0.38 ± 0.07</td>
<td>149.8 ± 45.0</td>
</tr>
<tr>
<td>CYP3A4 (+)</td>
<td>0.32 ± 0.02</td>
<td>0.55 ± 0.05*</td>
<td>158.8 ± 35.1</td>
</tr>
<tr>
<td>CYP3A4-HBN (-)</td>
<td>0.04 ± 0.02*</td>
<td>0.25 ± 0.04</td>
<td>131.6 ± 62.1</td>
</tr>
<tr>
<td>CYP3A4-HBN (+)</td>
<td>0.03 ± 0.01†</td>
<td>0.72 ± 0.19†</td>
<td>216.2 ± 31.1†</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>0.18 ± 0.04</td>
<td>0.36 ± 0.04</td>
<td>111.9 ± 1.6</td>
</tr>
<tr>
<td>CYP2D6-HBN</td>
<td>0.03 ± 0.02†</td>
<td>0.42 ± 0.16</td>
<td>127.5 ± 44.9</td>
</tr>
</tbody>
</table>

*P = 0.05 for CYP3A4 (CO) versus CYP3A4 (PCN).
†P = 0.05 for CYP3A4 (PCN) versus CYP3A4-HBN (PCN).
‡P = 0.05 for CYP3A4 (CO) versus CYP3A4 (PCN).
§P < 0.05 for CYP2D6 versus CYP2D6-HBN.
Reduced in CYP2D-HBN microsomes (495.4 ± 34.3 versus 793.6 ± 64.2 pmol/min per milligram), but no change in $K_m$ was observed (11.8 ± 1.5 versus 12.9 ± 1.9 μM).

Reconstitution of P450 Activity in CYP3A4- and CYP2D6-HBN Hepatic Microsomes. To clarify whether the reduction in drug metabolism observed in CYP3A4-HBN and CYP2D6-HBN mice was attributable to the absence of Cyb5, E. coli membranes containing recombinant human Cyb5 were added to in vitro incubations. In PCN-treated CYP3A4-HBN microsomes, the addition of Cyb5 increased triazolam turnover up to 5-fold; the maximum resulting activity was 1.45 times that in PCN-treated CYP3A4 microsomes (Fig. 3A). The addition of Cyb5 to CYP3A4 microsomes caused a comparatively small increase in activity (up to 1.18-fold) whereas addition of Cyb5 to Cyp3a null microsomes had no effect on triazolam turnover.

Addition of Cyb5 to incubations with CYP2D6-HBN microsomes likewise increased CYP2D6-mediated debrisoquine turnover, the maximal activity observed being 3.2 times higher than in the absence of Cyb5 (Fig. 3B). This activity was marginally higher than that of CYP2D6 samples. The activity of CYP2D6 samples was unaffected at low concentrations of exogenous Cyb5; however, for unknown reasons, the highest concentration of Cyb5 caused a 92% reduction in CYP2D6 turnover. This effect (also seen with bufuralol) was reproducible and observed in two independent experiments.

The addition of Cyb5 did not affect debrisoquine turnover in the Cyp2d knockout samples. When bufuralol was used as a CYP2D6 substrate, the addition of Cyb5 increased turnover in CYP2D6-HBN samples from 59% of that in CYP2D6 samples to an equivalent rate (Fig. 3C). Again, the activity was reduced at the highest concentration of Cyb5, although not as markedly as with debrisoquin. This effect was also observed in the CYP2D6-HBN samples, but Cyb5 addition had no effect on bufuralol activity in Cyp2d null microsomes.

The effect of Cyb5 addition to a reconstituted system comprising E. coli membranes coexpressing recombinant CYP2D6 and POR was also investigated. Bufuralol turnover was increased ~2-fold at all Cyb5 concentrations compared with that in the absence of Cyb5 (Supplemental Fig. 3).

Effect of Cyb5 Deletion on In Vivo Pharmacokinetics. The pharmacokinetic profile of triazolam was significantly altered in CYP3A4-HBN animals compared with CYP3A4 mice (Fig. 4A). The peak plasma concentration ($C_{\text{max}}$) and area under the curve (AUC$_0$–8) were 4- and 5.7-fold greater, respectively, with a concomitant 82% reduction in clearance (Supplemental Table 2). Counterintuitively, the AUC$_0$–8 of 4-hydroxytriazolam was also greater in CYP3A4-HBN than CYP2D6 mice (36.9 ± 3.3 versus 16.2 ± 4.5 min·μg/ml) (Fig. 4B).

The nifedipine disposition was similarly altered in CYP3A4-HBN animals. The AUC$_0$–8 was 431.6 ± 84.2 compared with 181.2 ± 52.5 min·μg/ml, and clearance was correspondingly reduced (64.1 ± 12.5 versus 162.6 ± 45.0 ml/min per kilogram) (Fig. 4C, Supplemental Table 2). Again, increased plasma levels of oxidized nifedipine were observed in CYP3A4-HBN animals compared with CYP3A4 mice (AUC$_0$–8 was 35.6 ± 4.5 versus 13.2 ± 2.2 min·μg/ml) (Fig. 4D). The reasons for the increases in plasma metabolite levels are likely to be complex; they may include enhanced extrahepatic metabolism (because the Cyb5 deletion in the HBN lines is liver specific) and changes in secondary metabolism and excretion pathways.

Bufuralol exposure was significantly increased in male CYP2D6-HBN mice relative to controls (data not shown). However, the lower expression level of CYP2D6 in the CYP2D6-HBN mice made it difficult to ascribe this effect to the absence of hepatic Cyb5. In female mice, the AUC$_0$–8 of bufuralol increased

### TABLE 2

<table>
<thead>
<tr>
<th>Substrate (Reaction)</th>
<th>CYP3A4 (PCN)</th>
<th>CYP3A4-HBN (PCN)</th>
<th>Cyp3a Null</th>
<th>CYP2D6</th>
<th>CYP2D6 HBN</th>
<th>Cyp2d Null</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triazolam (4-OH)</td>
<td>1180 ± 458.0</td>
<td>552.9 ± 70.9$^a$</td>
<td>18.4 ± 1.0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>2358 ± 253.4</td>
<td>1818 ± 60.2$^a$</td>
<td>708.5 ± 61.6</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Debrisoquine (4-OH)</td>
<td>NA</td>
<td>NA</td>
<td>136.1 ± 11.5</td>
<td>72.0 ± 7.3$^b$</td>
<td>2.2 ± 1.05</td>
<td></td>
</tr>
<tr>
<td>Bufuralol (1’-OH)</td>
<td>NA</td>
<td>NA</td>
<td>205.2 ± 37.2</td>
<td>131.9 ± 17.6$^b$</td>
<td>38.1 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>Metoprolol (α-OH)</td>
<td>NA</td>
<td>NA</td>
<td>91.4 ± 25.6</td>
<td>51.5 ± 7.4$^b$</td>
<td>1.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Metoprolol (O-desmethyl)</td>
<td>NA</td>
<td>NA</td>
<td>554.0 ± 120.0</td>
<td>324.4 ± 33.8$^b$</td>
<td>28.2 ± 8.6</td>
<td></td>
</tr>
</tbody>
</table>

NA, not applicable.

$P < 0.05.$

$P < 0.001.$
null mice: increased $C_{\text{max}}$ half-life and AUC$_{0-8}$ (1.3-, 1.2-, and 1.5-fold, respectively) were associated with a 40% reduction in clearance in CYP2D6-HBN compared with CYP2D6 mice (Fig. 4, G and H; Supplemental Table 2).

**Discussion**

Cytochrome P450 CYP3A4 and CYP2D6 are responsible for the metabolism and disposition of the majority of drugs in humans. It is widely accepted that CYP3A4 activity can be modulated in vitro by Cyb5, and we have previously shown that the in vitro and in vivo activity of murine Cyp3a is reduced in the absence of Cyb5 (Finn et al., 2008; McLaughlin et al., 2010). Evidence concerning the in vivo consequences of Cyb5 deficiency in terms of human P450-dependent drug metabolism has, however, not yet emerged.

The primary aim of this study was to characterize the in vivo consequences of hepatic Cyb5 deficiency on CYP3A4- and CYP2D6-dependent drug disposition. Such studies cannot be conducted in humans, so the availability of P450-humanized mouse lines crossed with the hepatic Cyb5 deletion model HBN was critical. Accordingly, we investigated the role of Cyb5 in human CYP3A4- and CYP2D6-mediated drug metabolism in vitro and in vivo using mouse models generated by crossing mice humanized for the key drug metabolizing P450s CYP3A4 and CYP2D6 (Hasegawa et al., 2011; Scheer et al., 2012b) with our hepatic Cyb5-deficient HBN line (Finn et al., 2008).

The CYP3A4-HBN and CYP2D6-HBN mouse lines exhibited no deleterious effects with respect to fecundity, litter size, pup development, or gross physical phenotype. Deletion of Cyb5 in the HBN line upregulated hepatic Cyp2b10 and Cyp3a expression (Finn et al., 2008; Henderson et al., 2013); this trait was also apparent in the humanized HBN models (for those murine genes which had not been deleted) and CYP3A4 expression in humanized mouse liver was also increased by Cyb5 deletion. Conversely, deletion of hepatic Cyb5 in the CYP2D6 line reduced basal CYP2D6 expression in male, but not female, mice.

The in vitro metabolism of triazolam was attenuated by at least 50% in CYP3A4-HBN liver microsomes but could be restored by addition of exogenous Cyb5. This was consistent with the increase in certain CYP3A4-dependent activities observed upon the addition of Cyb5 to in vitro reconstitution experiments (Voice et al., 1999; Yamazaki et al., 1999; Yamaori et al., 2003; Kumar et al., 2005; Lee and Goldstein, 2012) or its inclusion in baculovirus expression systems (Jushchyshyn et al., 2005). Similar results were obtained using nifedipine, although the interpretation of these data were complicated by the ability of non-Cyp3a P450s to metabolize this substrate, as indicated by the results obtained using hepatic microsomes from Cyp3a null mice. It is, however, likely that this effect was mediated mainly via the heterologously expressed human CYP3A4 enzyme in the CYP3A4-HBN mouse; otherwise, to account for the magnitude of the effects observed, the deletion of Cyb5 would have had to obliterate endogenous murine P450 activity almost completely.

Variable results have been obtained in published studies using reconstituted systems with human CYP3A4, POR, and Cyb5 and nifedipine in vitro; an approximate doubling of nifedipine oxidation activity was observed in an osmotically shocked E. coli membrane system on inclusion of Cyb5 (Voice et al., 1999), but in another study a slight reduction in nifedipine oxidation was observed on incorporation of Cyb5 a similar system (Muller-Enoch, 1999).
The interaction between CYP2D6 and Cyb5 is less well characterized than that between CYP3A4 and Cyb5. Studies in *E. coli* expression systems in vitro do not indicate stimulation of CYP2D6 activity by Cyb5 (Dehal and Kupfer, 1997; Yamazaki et al., 1997; Yamazaki et al., 2002), but we observed reduced hydroxylation of both debrisoquine and bufuralol by CYP2D6-HBN microsomes. Activity was restored by the addition of exogenous Cyb5 to microsomal incubations, and furthermore the activity toward bufuralol of *E. coli* membranes containing recombinant CYP2D6 and POR could be stimulated by adding Cyb5. This, together with the finding that the lipid composition of hepatocytes was similar in the humanized and HBN lines, indicates that the observed changes in metabolism can be ascribed to a direct interaction between Cyb5 and cytochrome P450 proteins rather than effects mediated via alterations in membrane lipids. Direct modulation by Cyb5 could be due to altered electron transfer rates or allosteric effects; the evidence for the latter is becoming increasingly compelling (Estrada et al., 2013; Johnson et al., 2014; Peng and Auchus, 2014).

Pharmacokinetic studies with triazolam demonstrated that CYP3A4 clearance is significantly impaired in response to in vivo deletion of hepatic Cyb5, leading to significantly increased exposure to the parent drug and corresponding increases in *C*<sub>max</sub> and AUC. This reduction in triazolam turnover is almost certainly an underestimate because CYP3A4-HBN mice treated with PCN express almost twice as much CYP3A4 as similarly treated CYP3A4 animals. Exposure to 4-hydroxytriazolam was also greater in CYP3A4-HBN than CYP3A4 mice, and similar results were obtained using nifedipine; however, the effects observed were less marked confirming the in vivo relevance of in vitro evidence that the role of Cyb5 in drug disposition is substrate specific.

The counterintuitive observation that suppression of hepatic clearance can be associated with elevated plasma levels of oxidative metabolites may be a consequence of the fact that the Cyb5 deletion in HBN mice is liver specific, leading to higher plasma concentrations of parent compounds due to reduced hepatic metabolism and possibly driving an increase P450-mediated oxidation in extrahepatic tissues. The determinants of plasma metabolite concentrations are multifactorial, being dependent on the conversion of parent drug to metabolite, metabolic clearance of parent compound by other pathways, and elimination clearance of the metabolite itself. Differential relative changes in these processes can, in theory, result in increased, decreased, or unchanged metabolite concentrations (Levy et al., 1983). Our pharmacokinetic data do not allow firm conclusions to be drawn regarding the origins of the observed increases in metabolite concentrations; additional experiments would be required to explain these observations.

As with the CYP3A4 substrates in CYP3A4 and CYP3A4-HBN mice, in vivo pharmacokinetic studies with debrisoquine and bufuralol in CYP2D6 and CYP2D6-HBN mice revealed significant decreases in parent drug clearance, again associated with increases in *C*<sub>max</sub> and AUC, although with these compounds there were no marked changes in exposure to oxidative metabolites.

The evidence from our studies in humanized mice shows that lack of hepatic Cyb5 activity compromises CYP3A4- and CYP2D6-mediated drug metabolism both in vitro and in vivo. This suggests that interindividual variation in Cyb5 activity is likely to be an important determinant of P450-mediated drug metabolism in humans. Both CYP3A4 and CYP2D6 are expressed at highly variable levels in the human liver; the finding that the absence of Cyb5 can alter the pharmacokinetics of CYP2D6 substrates in vivo adds further complexity to the prediction of therapeutic responses to drugs of this class, especially in poor metabolizers who also have compromised Cyb5 activity.

In summary, the results of this study demonstrate the importance of Cyb5, speculatively, as a potential determinant of rates of drug metabolism in humans. Our results show that
Cytochrome b5 can modulate the in vivo pharmacokinetics of CYP3A4 and CYP2D6 substrates in a substrate-dependent manner. Taken together with in vitro evidence that the substrate-dependent effects of Cytochrome b5 in P450 activity can include suppression (Muller-Ehmsen, 1999; Bakken et al., 2009), no effect (Dehal and Kupfer, 1997; Yamazaki et al., 1997, 2002; Yamaori et al., 2003), or enhancement (Voice et al., 1999, Yamaori et al., 1999; Yamaori et al., 2003; Jushchyshyn et al., 2005; Kumar et al., 2006; Lee and Goldstein, 2012) of CYP3A4 activity and can redirect metabolism from detoxification toward metabolic activation of carcinogens (Stiborova et al., 2012a,b). Our observations illustrate the importance of undertaking case-by-case investigations of the role of Cytochrome b5 in determining patterns of drug disposition in vivo. In addition, we speculate that variation in hepatic Cytochrome b5 expression may contribute to the heterogeneity in plasma levels of many commonly prescribed drugs, potentially affecting both efficacy and toxicity, and may contribute to interindividual variability in drug response. To demonstrate whether this is the case, a mouse model could be generated where the hepatic levels of Cytochrome b5 are regulated conditionally, for example, using the Cyp1a1 promoter system we have described previously elsewhere (Finn et al., 2007; Henderson et al., 2015). Clinical studies will be difficult because there are no specific Cytochrome b5 inhibitors, and assessing Cytochrome b5 levels by other means such as from liver biopsies could be problematic.

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