Cytochrome b{sub}5 is a major determinant of human cytochrome
P450 CYP2D6 & CYP3A4 activity in vivo

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Abbreviations: Cyb5: cytochrome b₅; AUC: area under the curve; Cₘₐₓ: peak plasma concentration; CLₘᵢₙ: intrinsic clearance; POR – P450 Oxidoreductase; HBN: hepatic cytochrome b₅ null; DDI: PCN: pregnenolone-16α-carbonitrile; drug-drug interaction; POR: cytochrome P450 oxidoreductase; UPLC-MS/MS: ultra performance liquid chromatography-tandem mass spectrometry.
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. P450 activity is regulated transcriptionally and by the rate of electron transfer from P450 reductase. *In vitro* studies have demonstrated that cytochrome *b*\(_5\) (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 humanised 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 to 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 *E. coli* membranes containing recombinant Cyb5. *In vivo*, the \(C_{\text{max}}\) and \(AUC_{0-8h}\) 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, \(AUC_{0-8h}\) 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 potential impact on the metabolism, efficacy and side-effects of numerous therapeutic drugs.
Introduction

The cytochrome P450-dependent monooxygenase 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, in order 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 (Akhtar et al., 2005; Lamb et al., 2001; Yamaori et al., 2003; Yamazaki et al., 1996; Yamazaki et al., 2002). In order 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., 2014a; Henderson et al., 2013; McLaughlin et al., 2010).

This raises the question “Does human P450 system exhibits the same level of Cyb5 dependency?” We have addressed this question using models created as part of a project to humanise 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; Scheer et al., 2012b). These models have been shown to exhibit in vivo functionality reflecting that predicted in man.

In the present study we crossed the hepatic Cyb5 null (HBN) mouse (Finn et al., 2008) with CYP3A4 and CYP2D6 humanised mice (Hasegawa et al., 2011; Scheer et al., 2012a; Scheer et al.,
2012b) in order to understand whether Cyb5 modulates human P450 activity \textit{in vivo} and demonstrate profound effects on drug pharmacokinetics. These data demonstrate that Cyb5 is a key determinant of human P450 activity \textit{in vivo}. 
Materials & Methods

Chemicals

Reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. NADPH came from Melford Laboratories (Ipswich, UK) and nifedipine oxide from BD Gentest, Cowley, UK. 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 (Cyb5<sup>lox/lox</sup>::Cre<sup>ALB</sup> (HBN)), huCYP3A4/3A7 (Cyp3a<sup>-/-</sup>::CYP3A4/7::Cyb5<sup>lox/lox</sup> (CYP3A4)) and huCYP2D6.1 (Cyp2d<sup>-/-</sup>::CYP2D6::Cyb5<sup>lox/lox</sup> (CYP2D6)) mice was described previously (Finn et al., 2008; Hasegawa et al., 2011; Scheer et al., 2012b). HBN animals were crossed with CYP3A4 or CYP2D6 mice to generate CYP3A4-HBN (Cyp3a<sup>-/-</sup>::CYP3A4/7::Cyb5<sup>lox/lox</sup>::Cre<sup>ALB</sup>) and CYP2D6-HBN (Cyp2d<sup>-/-</sup>::CYP2D6::Cyb5<sup>lox/lox</sup>::Cre<sup>ALB</sup>) lines, which were maintained homozygous for floxed Cyb5, heterozygous for Cre<sup>ALB</sup> and humanised for either CYP3A4 or CYP2D6. All mice were kept under standard animal house conditions, with free access to food and water and a 12h light/12h dark cycle. Animal work was carried out in accordance with the Animals (Scientific Procedures) Act 1986 and local ethical review. To induce expression of cytochrome P450 CYP3A4, which is constitutively expressed at very low levels in humanised mice, CYP3A4 and CYP3A4-HBN mice were always pre-dosed with either corn oil or PCN (10 mg/kg q.d. x3) 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 pregnenolone-16α-carbonitrile (PCN)-treated CYP3A4 and CYP3A4-HBN mice and untreated CYP2D6 and CYP2D6-HBN mice (n=3 per group). Lipid content was analysed 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 Labs Ltd, Herts, UK).

**Characterisation of hepatic microsomes**

Microsomal P450 content was determined by reduced carbon monoxide difference spectroscopy (Omura and Sato, 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, UK) were used as standards. Immunoreactive proteins were detected using polyclonal goat anti-rabbit or anti-mouse horseradish peroxidase immunoglobulins as secondary antibodies (Dako, Ely, UK), and visualised and protein expression semi-quantified using Immobilon™ chemiluminescent substrate (Millipore, Watford, UK) and a FUJIFILM LAS-3000 mini imaging system (Fujifilm UK Ltd, UK). Densitometry was performed using Multi Gauge V2.2 software (Fujifilm UK Ltd, UK).

**In vitro microsomal incubations**

*In vitro* triazolam, nifedipine, metoprolol, bufuralol and debrisoquine incubations were performed as described in Supplemental Information. In some experiments, *E. coli* membranes containing recombinant human Cyb5 (0-32.9 pmol/incubation) were added to hepatic microsomes (final volume 6 µl) and incubated for 20 min at room temperature before the
addition of buffer and substrate. Triplicate incubations were initiated, terminated and processed as described in Supplemental Methods.

**Stimulation of recombinant CYP2D6 activity**

*E. coli* membranes co-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 min at room temperature before the addition of buffer and substrate. Incubations were initiated, terminated and processed as described in Supplemental Methods.

**UPLC-MS/MS analysis**

Analysis of metoprolol and metabolites was carried out as described previously (Finn et al., 2008). Other microsomal incubations and pharmacokinetic samples were analysed using a Waters Acquity UPLC and Micromass Quattro Premier mass spectrometer (Micromass, Manchester, UK) as described in Supplemental 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 Supplemental Information. Pharmacokinetic parameters were calculated using WinNonLin software, v3.1 (Pharsight, St Louis, MO, USA). A non-compartmental model was used to calculate $\text{AUC}_{0-\text{inf}}$, $\text{AUC}_{\text{inf}}$, terminal half-life, $C_{\text{max}}$, and clearance. The 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 which 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 Cre<sub>ALB</sub>. The mice were viable, exhibited no gross anatomical abnormalities and had histologically normal livers (Supplemental Figure 1). Both sexes were fertile and offspring were born at expected Mendelian ratios. Administration of pregnenolone-16α-carbonitrile (PCN) to CYP3A4 and CYP3A4-HBN mice resulted in a significant increase in liver:body weight ratio (by ~1.2 fold). No other changes in 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 to 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 vs 3071 ± 228 μg/g liver), mean ± SD, p<0.001. In CYP2D6-HBN mice, hepatic γ-linolenic, adrenic and docosapentaenoic acid (18:3n-6, 22:4n-6 and 22:5n-3) levels were slightly reduced in comparison with those in CYP2D6 mice (0.217 ± 0.015 vs 0.283 ± 0.032 % of total fatty acids, 96.2 ± 16.5 vs 126.4 ± 7.9 μg/g liver and 79.7 ± 7.8 vs 108.8 ± 15.5 μg/g liver, respectively), mean ± SD, all p<0.05.

**Analysis of cytochrome P450 mono-oxygenase components**

The effects of Cyb5 deletion and/or P450 humanisation on mono-oxygenase components were analysed in hepatic microsomal fractions (Table 1, Figure 1). Spectroscopically-detectable Cyb5 levels were ~85% lower in CYP2D6-HBN and corn oil (CO)-treated CYP3A4-HBN mice compared to CYP2D6 and corn oil (CO)-treated CYP3A4, respectively, and by ~91% in PCN-treated CYP3A4-HBN compared to 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 to corn oil-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 corn oil-treated controls.

Western blot analysis of hepatic Cyb5, POR and P450 isozyme expression in CYP3A4 and CYP3A4-HBN mouse liver microsomes is illustrated in Figure 1 and Supplemental Figure 2. No Cyb5 was detected in CYP3A4-HBN samples with or without PCN treatment, but such 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 Cyp2b, 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 PXR.

Analysis of male CYP2D6-HBN liver microsomes revealed increased expression of hepatic Cyp2b10 and Cyp3 proteins. Unexpectedly, a 50% reduction in CYP2D6 expression was observed in male CYP2D6-HBN mice compared to CYP2D6 mice with intact Cyb5 (Figure 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 (Figure 2 and Supplemental Figure 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 humanised, 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 humanised mouse liver: in Cyp3a null mice (the background strain for the CYP3A4 humanised 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 to 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 metabolise this compound. A 23% reduction in nifedipine metabolism was observed in PCN-treated CYP3A4-HBN samples compared to 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 carried out using samples from female mice in order 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 α-hydroxylation activities of Cyp2d null samples were <2% of those of CYP2D6 liver microsomes indicating that the activity of the humanised 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 0-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 0-demethylation in CYP2D6-HBN samples was reduced by 36% and 41% compared to CYP2D6, demonstrating an interaction between Cyb5 and CYP2D6 in these reactions.

The kinetics of bufuralol 1'-hydroxylation by CYP2D6 and CYP2D6-HBN microsomes were characterised and analysed according to the Michaelis-Menten model. $V_{\text{max}}$ was markedly reduced in CYP2D6-HBN microsomes (495.4 ± 34.3 vs 793.6 ± 64.2 pmol/min/mg); however no change in $K_m$ was observed (11.8 ± 1.5 vs 12.9 ± 1.9 μM).

**Reconstitution of P450 activity in CYP3A4- and CYP2D6- HBN hepatic microsomes**

In order 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, addition of Cyb5 increased triazolam turnover up to 5-fold; the maximum resulting activity was 1.45 times that in PCN-treated CYP3A4 microsomes (Figure 3A). Addition of Cyb5 to CYP3A4 microsomes caused a comparatively small increase in activity (up to 1.18 fold), while 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 (Figure 3B). This activity was marginally higher than that of CYP2D6 samples. The activity of CYP2D6 samples was unaffected at low concentrations of exogenous Cyb5 but, for unknown reasons, the highest concentration of Cyb5 caused a 92% reduction in CYP2D6 turnover. This effect (also seen with bufuralol, below) was reproducible and observed in two independent experiments. Addition of Cyb5 did not affect debrisoquine turnover in the
Cyp2d knockout samples. When bufuralol was used as a CYP2D substrate, the addition of Cyb5 increased turnover in CYP2D6-HBN samples from 59% of that in CYP2D6 samples to an equivalent rate (Figure 3C). Again, the activity was reduced at the highest concentration of Cyb5, although not as markedly as with debrisoquine. 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 co-expressing 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 Figure 3).

**Effect of Cyb5 deletion on in vivo pharmacokinetics**

The pharmacokinetic profile of triazolam was significantly altered in CYP3A4-HBN animals compared to CYP3A4 mice (Figure 4A). Peak plasma concentration ($C_{\text{max}}$) and area under the curve (AUC$_{0-8h}$) 4- and 5.7 fold greater, respectively, with a concomitant 82% reduction in clearance (Supplemental Table 2). Counterintuitively, the AUC$_{0-8h}$ of 4-hydroxytriazolam was also greater in CYP3A4-HBN than CYP3A4 mice ($36.9 \pm 3.3$ vs $16.2 \pm 4.5$ min*µg/ml, Figure 4B).

Nifedipine disposition was similarly altered in CYP3A4-HBN animals; AUC$_{0-8h}$ was $431.6 \pm 84.2$ compared with $181.2 \pm 52.5$ min*µg/ml and clearance was correspondingly reduced ($64.1 \pm 12.5$ vs $162.6 \pm 45.0$ ml/min/kg) (Figure 4C, Supplemental Table 2). Again, increased plasma levels of oxidised nifedipine were observed in CYP3A4-HBN animals compared to CYP3A4 mice (AUC$_{0-8h}$ $35.6 \pm 4.5$ vs. $13.2 \pm 2.2$ min* µg/ml, Figure 4D). The reasons for the increases in plasma metabolite levels are likely to be complex; they may include enhanced extra-hepatic metabolism (since 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\(_{0-8h}\) of bufuralol increased (1.4 fold) and clearance decreased (by 60%) in the absence of hepatic Cyb5 (Figure 4E, Supplemental Table 2) but plasma 1'-hydroxybufuralol concentrations remained unchanged (Figure 4F). The pharmacokinetic profile of debrisoquine, but not 4-hydroxydebrisoquine, was also altered in hepatic Cyb5 null mice: increased C\(_{\text{max}}\) half-life and AUC\(_{0-8h}\) (1.3, 1.2 and 1.5 fold respectively) were associated with a 40% reduction in clearance in CYP2D6-HBN compared to CYP2D6 mice (Figure 4G and H, Supplemental Table 2).
Discussion

Cytochrome P450 CYP3A4 and CYP2D6 are responsible for the metabolism and disposition of the majority of drugs in man. It is widely accepted that CYP3A4 activity can be modulated \textit{in vitro} by Cyb5, and we have previously shown that the \textit{in vitro} and \textit{in vivo} activity of murine Cyp3a is reduced in the absence of Cyb5 (Finn et al., 2008; McLaughlin et al., 2010). Evidence concerning the \textit{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 characterise the \textit{in vivo} consequences of hepatic Cyb5 deficiency on CYP3A4- and CYP2D6-dependent drug disposition. Such studies cannot be conducted in humans, therefore the availability of P450-humanised mouse lines crossed with the hepatic Cyb5 deletion model HBN was critical in allowing them to be undertaken. Accordingly, we investigated the role of Cyb5 in human CYP3A4- and CYP2D6-mediated drug metabolism \textit{in vitro} and \textit{in vivo} using mouse models generated by crossing mice humanised for the key drug metabolising 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 humanised HBN models (for those murine genes which had not been deleted) and CYP3A4 expression in humanised mouse liver was also increased by Cyb5 deletion. Conversely, however, deletion of hepatic Cyb5 in the CYP2D6 line reduced basal CYP2D6 expression in male, but not female, mice.

The \textit{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 \textit{in vitro}
reconstitution experiments (Kumar et al., 2005; Lee and Goldstein, 2012; Voice et al., 1999; Yamaori et al., 2003; Yamazaki et al., 1999) or its inclusion in baculovirus expression systems (Jushchysyn et al., 2005). Similar results were obtained using nifedipine, although the interpretation of these data was complicated by the ability of non-Cyp3a P450s to metabolise 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, in order 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 in a similar system (Muller-Enoch, 1999).

The interaction between CYP2D6 and Cyb5 is less well-characterised 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 towards 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 humanised 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_max and AUC. This reduction in triazolam turnover is almost certainly an underestimate since 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 counter-intuitive 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 extra-hepatic 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_{max} and AUC, although with these compounds there were no marked changes in exposure to oxidative metabolites.

The evidence from our studies in humanised mice shows that lack of hepatic Cyb5 activity compromises CYP3A4 and CYP2D6-mediated drug metabolism both in vitro and in vivo. This suggests that inter-individual 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 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 metabolisers who also have compromised Cyb5 activity.

In summary, the results of this study demonstrate the importance of Cyb5, speculatively, is a potential determinant of rates of drug metabolism in man. Our results show that Cyb5 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 Cyb5 in P450 activity can include suppression (Bakken et al., 2009; Muller-Enoch, 1999), no effect (Dehal and Kupfer, 1997; Yamaori et al., 2003; Yamazaki et al., 1997; Yamazaki et al., 2002) or enhancement (Jushchyshyn et al., 2005; Kumar et al., 2005; Lee and Goldstein, 2012; Voice et al., 1999; Yamaori et al., 2003; Yamazaki et al., 1999) of CYP3A4 activity and can redirect metabolism from detoxification towards metabolic activation of carcinogens (Stiborova et al., 2012a; Stiborova et al., 2012b), our observations illustrate the importance of undertaking case-by-case investigations of the role of Cyb5 in determining patterns of drug disposition in vivo. In addition, we speculate that variation in hepatic Cyb5 expression may contribute to the heterogeneity in plasma levels of many commonly prescribed drugs, potentially affecting both efficacy and toxicity, and may contribute to inter-individual variability in drug response. In order to demonstrate whether this is the case, a mouse model could be generated where the
hepatic levels of Cyb5 are regulated conditionally, for example using the Cyp1a1 promoter system we have described previously (Finn et al., 2007; Henderson et al., 2014b). Clinical studies will be difficult because there are no specific Cyb5 inhibitors and assessing Cyb5 levels by other means e.g. from liver biopsies could be problematic.
Acknowledgements: We thank Julia Carr for technical assistance with animal work, Dr Yury Kapelyukh for his interpretation of pharmacokinetic data. CRW & CJH acknowledge a research agreement with Dr Nico Scheer & TaconicArtemis GmbH, from whom the humanised mice were obtained.
Author contributions:

Participated in research design (CJH, LAM, CRW)

Conducted experiments (CJH, LAM)

Contributed new reagents or analytic tools (NS, CRW)

Performed data analysis (CJH, LAM, CRW)

Wrote or contributed to the writing of the manuscript (CJH, LAM, LAS, CRW)
References


Peng HM and Auchus RJ (2014) Two surfaces of cytochrome b5 with major and minor contributions to CYP3A4-catalyzed steroid and nifedipine oxygenation chemistries. *Arch Biochem Biophys* **541**: 53-60.


Yamazaki H, Gillam EM, Dong MS, Johnson WW, Guengerich FP and Shimada T (1997) Reconstitution of recombinant cytochrome P450 2C10(2C9) and comparison with


Footnotes:

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Colin J. Henderson & Lesley A. McLaughlin are joint first authors.
Figure Legends:

Figure 1: Cytochrome b$_5$, cytochrome P450 oxidoreductase and cytochrome P450 expression in livers from CYP3A4 and CYP3A4-HBN mice. Detection of hepatic drug metabolising enzymes by Western immunoblotting of individual microsomal samples (15µg protein per lane except Cyb5, 30 µg).

Figure 2: Cytochrome b$_5$, cytochrome P450 oxidoreductase and cytochrome P450 expression in livers from CYP2D6 and CYP2D6-HBN mice. Detection of drug metabolising enzymes by Western immunoblotting of individual microsomal samples (15µg protein per lane except Cyb5, 30 µg).

Figure 3: The effect of exogenous cytochrome b$_5$ addition on microsomal cytochrome P450 activities. Activities of CYP3A4, CYP3A4-HBN and Cyp3a null liver microsomes towards triazolam (A), CYP2D6, CYP2D6-HBN and Cyp2d null liver microsomes towards debrisoquine (B) or bufuralol (C) in the absence and presence of increasing concentrations of recombinant Cyb5. Samples were treated as described. Values (mean ± standard deviation) are presented as a percentage of the CYP3A4 or CYP2D6 (as appropriate) sample activity from triplicate incubations.

Figure 4: In vivo pharmacokinetic profiles of triazolam, nifedipine, debrisoquine, bufuralol and their respective metabolites in CYP3A4, CYP3A4-HBN or CYP2D6, CYP2D6-HBN mice. A-D: Pharmacokinetic plots of triazolam (A) and 4-hydroxytriazolam (B), nifedipine (C) and oxidised nifedipine (D) after administration to CYP3A4 and CYP3A4-HBN mice. Open circles: CYP3A4; closed circles: CYP3A4-HBN. E-H: Pharmacokinetic plots of bufuralol (E), 1'-hydroxybufuralol (F), debrisoquine (G) and 1-hydroxydebrisoquine (H) after administration to CYP2D6 and CYP2D6-HBN mice. Open circles: CYP2D6; closed circles: CYP2D6-HBN. CYP3A4 and CYP3A4-HBN mice were pre-treated with PCN to induce CYP3A4 levels and probe drugs were dosed as described in Materials and Methods. Data represent drug or metabolite blood concentrations (mean ± standard error of the mean) at the individual time points. n= 4 per group for triazolam and nifedipine, 6 or 10 for bufuralol and 8 for debrisoquine.
Table 1: Levels of cytochrome $b_5$, cytochrome P450 and cytochrome P450 oxidoreductase activities in CYP3A4, CYP3A4-HBN CYP2D6 and CYP2D6-HBN mice

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cytochrome $b_5$</th>
<th>Cytochrome P450</th>
<th>POR activity</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 ± 6.2</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>

CYP3A4 and CYP3A4-HBN animals were (+) or were not (-) pre-treated with PCN (controls were treated with corn oil) and assays were performed as described. n = 3 for cytochrome $b_5$ measurement, 4 for cytochrome P450 measurement (both expressed in nmol/mg) and 3 (in duplicate) for POR activity (expressed in nmol cytochrome reduced/min/mg). ** = p ≤ 0.01 for CYP3A4 (PCN) vs. CYP3A4-HBN (PCN); * = p ≤ 0.01 for CYP3A4 (CO) vs. CYP3A4-HBN (CO); ¥ = p ≤ 0.05, ¥¥ = p ≤ 0.01 for CYP3A (CO) vs. CYP3A (PCN) and Ψ = p ≤ 0.05 for CYP2D6 vs. CYP2D6-HBN.
Table 2: *In vitro* activities of hepatic microsomes from CYP3A4, CYP3A4-HBN, CYP2D6 and CYP2D6-HBN mice

<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>
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<tbody>
<tr>
<td>Triazolam (4-OH)</td>
<td>1180 ± 458.0</td>
<td>552.9 ± 70.9</td>
<td>18.4 ± 1.0</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
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<tr>
<td>Nifedipine</td>
<td>2358 ± 253.4</td>
<td>1818 ± 60.2</td>
<td>708.5 ± 61.6</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Debrisoquine (4-OH)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>136.1 ± 11.5</td>
<td>72.0 ± 7.3 ***</td>
<td>2.2 ± 1.05</td>
</tr>
<tr>
<td>Bufuralol (1'-OH)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>205.2 ± 37.2</td>
<td>131.9 ± 17.6 ***</td>
<td>38.1 ± 5.0</td>
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<tr>
<td>Metoprolol (α-OH)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>91.4 ± 25.6</td>
<td>51.5 ± 7.4 *</td>
<td>1.0 ± 0.5</td>
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<tr>
<td>Metoprolol (O-desmethyl)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>554.0 ± 120.0</td>
<td>324.4 ± 33.8 *</td>
<td>28.2 ± 8.6</td>
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</table>

CYP3A4 and CYP3A4-HBN animals were pre-treated with PCN, as described. CYP3A4 activities were determined in male and CYP2D6 in female mice. Metabolite production is presented as mean (pmol/min/mg) ± standard deviation for each genotype (n = 4 or 5 performed in triplicate per mouse; null data were determined in triplicate from a pool of 3 livers). * = p<0.05; *** p<0.001. n.a. - not applicable.
<table>
<thead>
<tr>
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<th>HLM</th>
<th>CYP3A4</th>
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<td>Cyp3a/CYP3A4</td>
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<td>Cyb5</td>
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<td>Cyp1a</td>
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<td>Cyp2b10</td>
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</table>

**Figure 1**

*Graphical representation of protein expression levels for various Cytochrome P450 enzymes under different conditions.*
Figure 2
Figure 3
Figure 4