

**Cytochrome b_5 is a major determinant of human cytochrome
P450 CYP2D6 & CYP3A4 activity *in vivo***

Colin J. Henderson, Lesley A. McLaughlin, Nico Scheer, Lesley A. Stanley
and C. Roland Wolf

Medical Research Institute, University of Dundee, Ninewells Hospital & Medical School,
University of Dundee, Dundee DD1 9SY, UK (CJH, LAM, CRW)

TaconicArtemis, Neurather Ring 1, 51063 Köln, Germany (NS)

Consultant in Investigative Toxicology, Linlithgow, EH49 6AX, UK (LAS)

Running title: Cytochrome *b*₅ alters human P450 activity *in vivo*

Corresponding author: Professor C. Roland Wolf, Medical Research Institute, University of Dundee, Level 9, Jacqui Wood Cancer Centre, Ninewells Hospital & Medical School, Dundee, DD1 9SY, UK; Tel: 01382-383134; Fax: 01382-386419; email: c.r.wolf@dundee.ac.uk.

Number of text pages: 30

Number of Tables: 2

Number of Figures: 4

Number of references: 35

Number of words in the Abstract: 250

Number of words in the Introduction: 376

Number of words in the Discussion: 1354

Abbreviations: Cyb5: cytochrome *b*₅; AUC: area under the curve; C_{max}: peak plasma concentration; CL_{int}: intrinsic clearance; POR – P450 Oxidoreductase; HBN: hepatic cytochrome *b*₅ null; DDI: PCN: pregnenolone-16 α -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_{\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 b_5 (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 *in vivo* and demonstrate profound effects on drug pharmacokinetics. These data demonstrate that Cyb5 is a key determinant of human P450 activity *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^{lox/lox}::Cre^{ALB}* (HBN)), huCYP3A4/3A7 (*Cyp3a^{-/-}::CYP3A4/7::Cytb^{5lox/lox}* (CYP3A4)) and huCYP2D6.1 (*Cyp2d^{-/-}::CYP2D6::Cytb^{5lox/lox}* (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^{-/-}::CYP3A4/7::Cytb^{5lox/lox}::Cre^{ALB}*) and CYP2D6-HBN (*Cyp2d^{-/-}::CYP2D6::Cytb^{5lox/lox}::Cre^{ALB}*) lines, which were maintained homozygous for floxed *Cyb5*, heterozygous for *Cre^{ALB}* 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-16 α -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 μ l) 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 AUC_{1-8h} , AUC_{inf} , terminal half-life, C_{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^{ALB}. 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-16 α -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 $\mu\text{g/g}$ liver), mean \pm 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 $\mu\text{g/g}$ liver and 79.7 ± 7.8 vs 108.8 ± 15.5 $\mu\text{g/g}$ liver, respectively), mean \pm 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 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 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_{\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 μ M).

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 CYP2D6 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_{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 ± 3.3 vs 16.2 ± 4.5 min* μ g/ml, Figure 4B).

Nifedipine disposition was similarly altered in CYP3A4-HBN animals; AUC_{0-8h} was 431.6 ± 84.2 compared with 181.2 ± 52.5 min* μ g/ml and clearance was correspondingly reduced (64.1 ± 12.5 vs 162.6 ± 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 ± 4.5 vs. 13.2 ± 2.2 min* μ g/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_{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 *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 characterise the *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 *in vitro* and *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 *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 (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 (Jushchyshyn 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 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 promotor 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

- Akhtar MK, Kelly SL and Kaderbhai MA (2005) Cytochrome b(5) modulation of 17 α hydroxylase and 17-20 lyase (CYP17) activities in steroidogenesis. *J Endocrinol* **187**(2): 267-274.
- Bakken GV, Rudberg I, Christensen H, Molden E, Refsum H and Hermann M (2009) Metabolism of quetiapine by CYP3A4 and CYP3A5 in presence or absence of cytochrome B5. *Drug Metab Dispos* **37**(2): 254-258.
- Baron J, Hildebrandt AG, Peterson JA and Estabrook RW (1973) The role of oxygenated cytochrome P-450 and of cytochrome b5 in hepatic microsomal drug oxidations. *Drug Metab Dispos* **1**(1): 129-138.
- Dehal SS and Kupfer D (1997) CYP2D6 catalyzes tamoxifen 4-hydroxylation in human liver. *Cancer Res* **57**(16): 3402-3406.
- Estrada DF, Laurence JS and Scott EE (2013) Substrate-modulated cytochrome P450 17A1 and cytochrome b5 interactions revealed by NMR. *J Biol Chem* **288**(23): 17008-17018.
- Finn RD, McLaren AW, Carrie D, Henderson CJ and Wolf CR (2007) Conditional deletion of cytochrome P450 oxidoreductase in the liver and gastrointestinal tract: a new model for studying the functions of the P450 system. *J Pharmacol Exp Ther* **322**(1): 40-47.
- Finn RD, McLaughlin LA, Ronseaux S, Rosewell I, Houston JB, Henderson CJ and Wolf CR (2008) Defining the in Vivo Role for Cytochrome b5 in Cytochrome P450 Function through the Conditional Hepatic Deletion of Microsomal Cytochrome b5. *J Biol Chem* **283**(46): 31385-31393.
- Hasegawa M, Kapelyukh Y, Tahara H, Seibler J, Rode A, Krueger S, Lee DN, Wolf CR and Scheer N (2011) Quantitative prediction of human pregnane X receptor and cytochrome P450 3A4

mediated drug-drug interaction in a novel multiple humanized mouse line. *Mol Pharmacol* **80**(3): 518-528.

Henderson CJ, McLaughlin LA, Finn RD, Ronseaux S, Kapelyukh Y and Wolf CR (2014a) A role for cytochrome b5 in the In vivo disposition of anticancer and cytochrome P450 probe drugs in mice. *Drug Metab Dispos* **42**(1): 70-77.

Henderson CJ, McLaughlin LA, Osuna-Cabello M, Taylor M, Gilbert I, McLaren AW and Wolf CR (2014b) Application of a novel regulatable Cre recombinase system to define the role of liver and gut metabolism in drug oral bioavailability. *Biochem J*.

Henderson CJ, McLaughlin LA and Wolf CR (2013) Evidence that cytochrome b5 and cytochrome b5 reductase can act as sole electron donors to the hepatic cytochrome P450 system. *Mol Pharmacol* **83**(6): 1209-1217.

Johnson EF, Connick JP, Reed JR, Backes WL, Desai MC, Xu L, Estrada DF, Laurence JS and Scott EE (2014) Correlating structure and function of drug-metabolizing enzymes: progress and ongoing challenges. *Drug Metab Dispos* **42**(1): 9-22.

Jushchyshyn MI, Hutzler JM, Schrag ML and Wienkers LC (2005) Catalytic turnover of pyrene by CYP3A4: evidence that cytochrome b5 directly induces positive cooperativity. *Arch Biochem Biophys* **438**(1): 21-28.

Kumar S, Davydov DR and Halpert JR (2005) Role of cytochrome B5 in modulating peroxide-supported cyp3a4 activity: evidence for a conformational transition and cytochrome P450 heterogeneity. *Drug Metab Dispos* **33**(8): 1131-1136.

Lamb DC, Kaderbhai NN, Venkateswarlu K, Kelly DE, Kelly SL and Kaderbhai MA (2001) Human sterol 14alpha-demethylase activity is enhanced by the membrane-bound state of cytochrome b(5). *Arch Biochem Biophys* **395**(1): 78-84.

- Lee SJ and Goldstein JA (2012) Comparison of CYP3A4 and CYP3A5: the effects of cytochrome b5 and NADPH-cytochrome P450 reductase on testosterone hydroxylation activities. *Drug metabolism and pharmacokinetics* **27**(6): 663-667.
- Levy RH, Lane EA, Guyot M, Brachet-Liermain A, Cenraud B and Loiseau P (1983) Analysis of parent drug-metabolite relationship in the presence of an inducer. Application to the carbamazepine-clobazam interaction in normal man. *Drug Metab Dispos* **11**(4): 286-292.
- McLaughlin LA, Ronseaux S, Finn RD, Henderson CJ and Roland Wolf C (2010) Deletion of microsomal cytochrome b5 profoundly affects hepatic and extrahepatic drug metabolism. *Mol Pharmacol* **78**(2): 269-278.
- Meehan RR, Forrester LM, Stevenson K, Hastie ND, Buchmann A, Kunz HW and Wolf CR (1988) Regulation of phenobarbital-inducible cytochrome P-450s in rat and mouse liver following dexamethasone administration and hypophysectomy. *Biochem J* **254**(3): 789-797.
- Muller-Enoch D (1999) Investigations on the role of cytochrome b5 and divalent cations in the maximal nifedipine oxidase activity of human liver. *Arzneimittel-Forschung* **49**(5): 470-475.
- Omura T and Sato R (1964) The Carbon Monoxide-Binding Pigment Of Liver Microsomes. I. Evidence For Its Hemoprotein Nature. *J Biol Chem* **239**: 2370-2378.
- Pandey AV and Fluck CE (2013) NADPH P450 oxidoreductase: structure, function, and pathology of diseases. *Pharmacology & therapeutics* **138**(2): 229-254.
- 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.
- Pritchard MP, Glancey MJ, Blake JA, Gilham DE, Burchell B, Wolf CR and Friedberg T (1998) Functional co-expression of CYP2D6 and human NADPH-cytochrome P450 reductase in *Escherichia coli*. *Pharmacogenetics* **8**(1): 33-42.

- Scheer N, Kapelyukh Y, Chatham L, Rode A, Buechel S and Wolf CR (2012a) Generation and characterization of novel cytochrome P450 Cyp2c gene cluster knockout and CYP2C9 humanized mouse lines. *Mol Pharmacol* **82**(6): 1022-1029.
- Scheer N, Kapelyukh Y, McEwan J, Beuger V, Stanley LA, Rode A and Wolf CR (2012b) Modeling human cytochrome P450 2D6 metabolism and drug-drug interaction by a novel panel of knockout and humanized mouse lines. *Mol Pharmacol* **81**(1): 63-72.
- Stiborova M, Indra R, Moserova M, Cerna V, Rupertova M, Martinek V, Eckschlager T, Kizek R and Frei E (2012a) Cytochrome b5 increases cytochrome P450 3A4-mediated activation of anticancer drug ellipticine to 13-hydroxyellipticine whose covalent binding to DNA is elevated by sulfotransferases and N,O-acetyltransferases. *Chem Res Toxicol* **25**(5): 1075-1085.
- Stiborova M, Poljakova J, Martinkova E, Ulrichova J, Simanek V, Dvorak Z and Frei E (2012b) Ellipticine oxidation and DNA adduct formation in human hepatocytes is catalyzed by human cytochromes P450 and enhanced by cytochrome b5. *Toxicology* **302**(2-3): 233-241.
- Strobel HW and Dignam JD (1978) Purification and properties of NADPH-cytochrome P-450 reductase. *Methods Enzymol* **52**: 89-96.
- Voice MW, Zhang Y, Wolf CR, Burchell B and Friedberg T (1999) Effects of human cytochrome b5 on CYP3A4 activity and stability in vivo. *Arch Biochem Biophys* **366**(1): 116-124.
- Yamaori S, Yamazaki H, Suzuki A, Yamada A, Tani H, Kamidate T, Fujita K and Kamataki T (2003) Effects of cytochrome b(5) on drug oxidation activities of human cytochrome P450 (CYP) 3As: similarity of CYP3A5 with CYP3A4 but not CYP3A7. *Biochem Pharmacol* **66**(12): 2333-2340.
- Yamazaki H, Gillam EM, Dong MS, Johnson WW, Guengerich FP and Shimada T (1997) Reconstitution of recombinant cytochrome P450 2C10(2C9) and comparison with

cytochrome P450 3A4 and other forms: effects of cytochrome P450-P450 and cytochrome P450-b5 interactions. *Arch Biochem Biophys* **342**(2): 329-337.

Yamazaki H, Johnson WW, Ueng YF, Shimada T and Guengerich FP (1996) Lack of electron transfer from cytochrome b5 in stimulation of catalytic activities of cytochrome P450 3A4. Characterization of a reconstituted cytochrome P450 3A4/NADPH-cytochrome P450 reductase system and studies with apo-cytochrome b5. *J Biol Chem* **271**(44): 27438-27444.

Yamazaki H, Nakajima M, Nakamura M, Asahi S, Shimada N, Gillam EM, Guengerich FP, Shimada T and Yokoi T (1999) Enhancement of cytochrome P-450 3A4 catalytic activities by cytochrome b(5) in bacterial membranes. *Drug Metab Dispos* **27**(9): 999-1004.

Yamazaki H, Nakamura M, Komatsu T, Ohyama K, Hatanaka N, Asahi S, Shimada N, Guengerich FP, Shimada T, Nakajima M and Yokoi T (2002) Roles of NADPH-P450 reductase and apo- and holo-cytochrome b5 on xenobiotic oxidations catalyzed by 12 recombinant human cytochrome P450s expressed in membranes of Escherichia coli. *Protein Expr Purif* **24**(3): 329-337.

Footnotes:

This work was funded by a Cancer Research UK Programme grant awarded to Roland Wolf [C4639/A12330].

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

Sample	Cytochrome b_5	Cytochrome P450	POR activity
CYP3A4 (-)	0.24 ± 0.09	0.38 ± 0.07	149.8 ± 45.0
CYP3A4 (+)	0.32 ± 0.02	0.55 ± 0.05 ^{YY}	158.8 ± 35.1
CYP3A4-HBN (-)	0.04 ± 0.02 ^{ΦΦ}	0.25 ± 0.04	131.6 ± 6.2
CYP3A4-HBN (+)	0.03 ± 0.01 ^{**}	0.72 ± 0.19 ^{YY}	216.2 ± 31.1 [¥]
CYP2D6	0.18 ± 0.04	0.36 ± 0.04	111.9 ± 1.6
CYP2D6-HBN	0.03 ± 0.02 ^Ψ	0.42 ± 0.16	127.5 ± 44.9

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); ^{ΦΦ} = p ≤ 0.01 for CYP3A4 (CO) vs. CYP3A4-HBN (CO); [¥] = p ≤ 0.05, ^{YY} = 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

Substrate (reaction)	CYP3A4 (PCN)	CYP3A4-HBN (PCN)	Cyp3a null	CYP2D6	CYP2D6 HBN	Cyp2d null
Triazolam (4-OH)	1180 ± 458.0	552.9 ± 70.9*	18.4 ± 1.0	n.a.	n.a.	n.a.
Nifedipine	2358 ± 253.4	1818 ± 60.2*	708.5 ± 61.6	n.a.	n.a.	n.a.
Debrisoquine (4-OH)	n.a.	n.a.	n.a.	136.1 ± 11.5	72.0 ± 7.3***	2.2 ± 1.05
Bufuralol (1'-OH)	n.a.	n.a.	n.a.	205.2 ± 37.2	131.9 ± 17.6***	38.1 ± 5.0
Metoprolol (α -OH)	n.a.	n.a.	n.a.	91.4 ± 25.6	51.5 ± 7.4*	1.0 ± 0.5
Metoprolol (O-desmethyl)	n.a.	n.a.	n.a.	554.0 ± 120.0	324.4 ± 33.8*	28.2 ± 8.6

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.

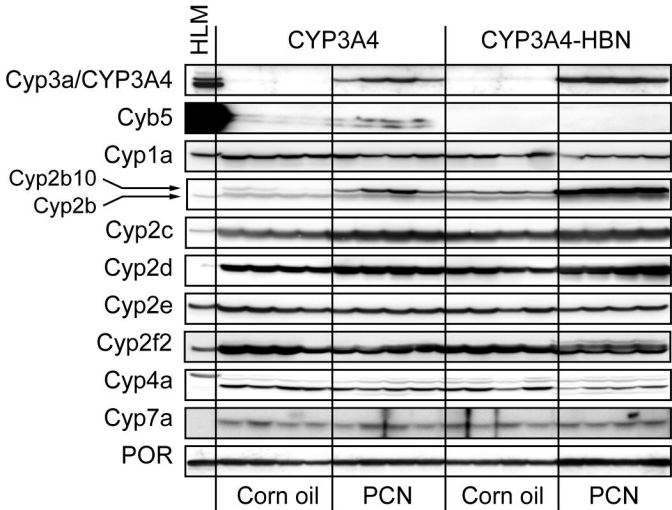


Figure 1

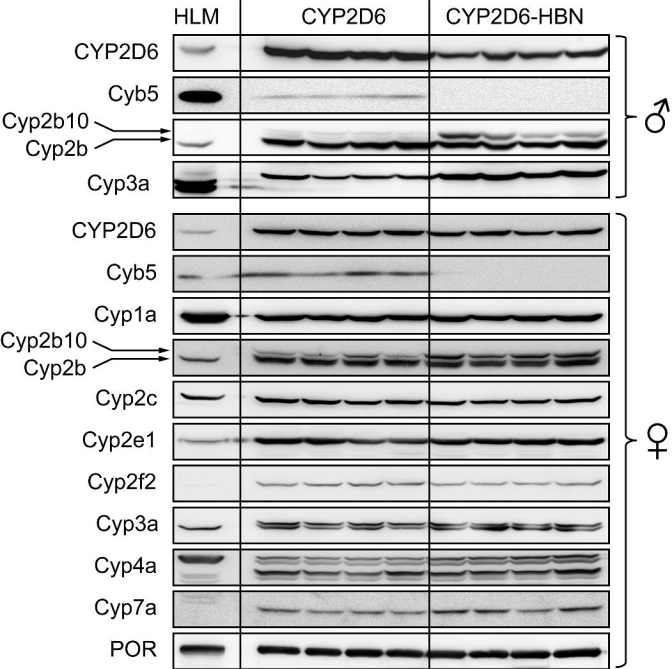


Figure 2

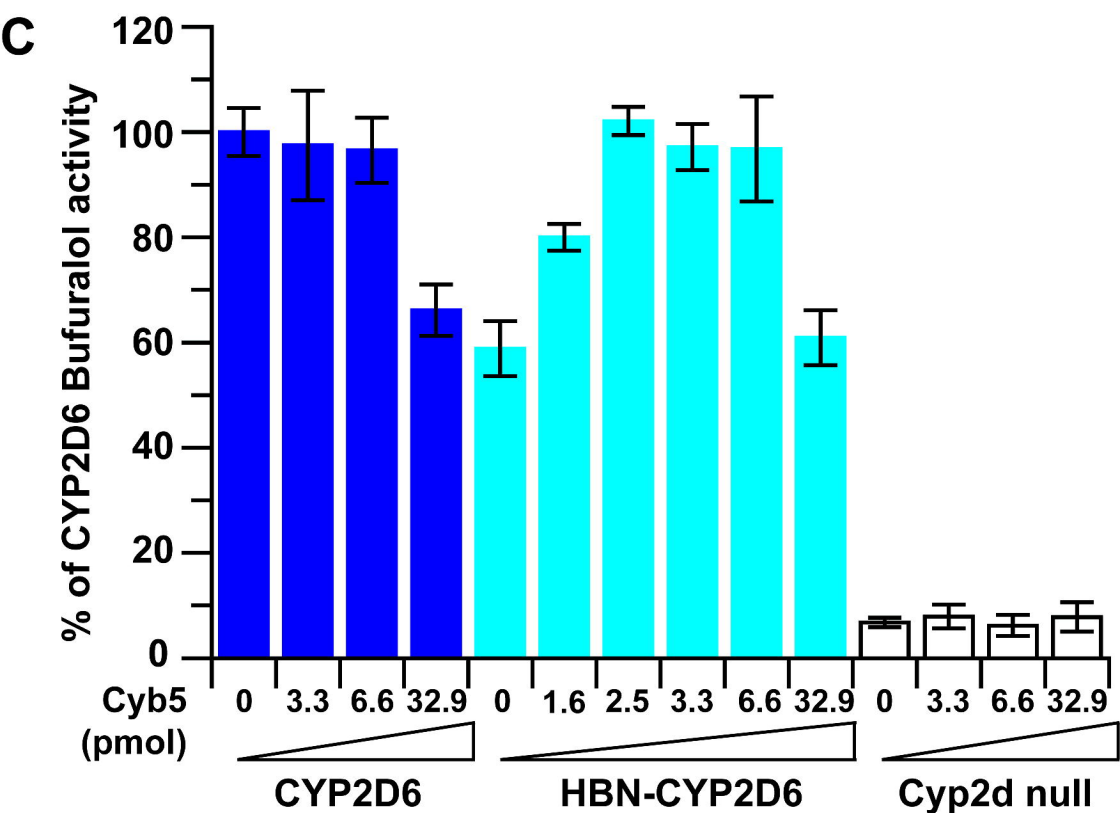
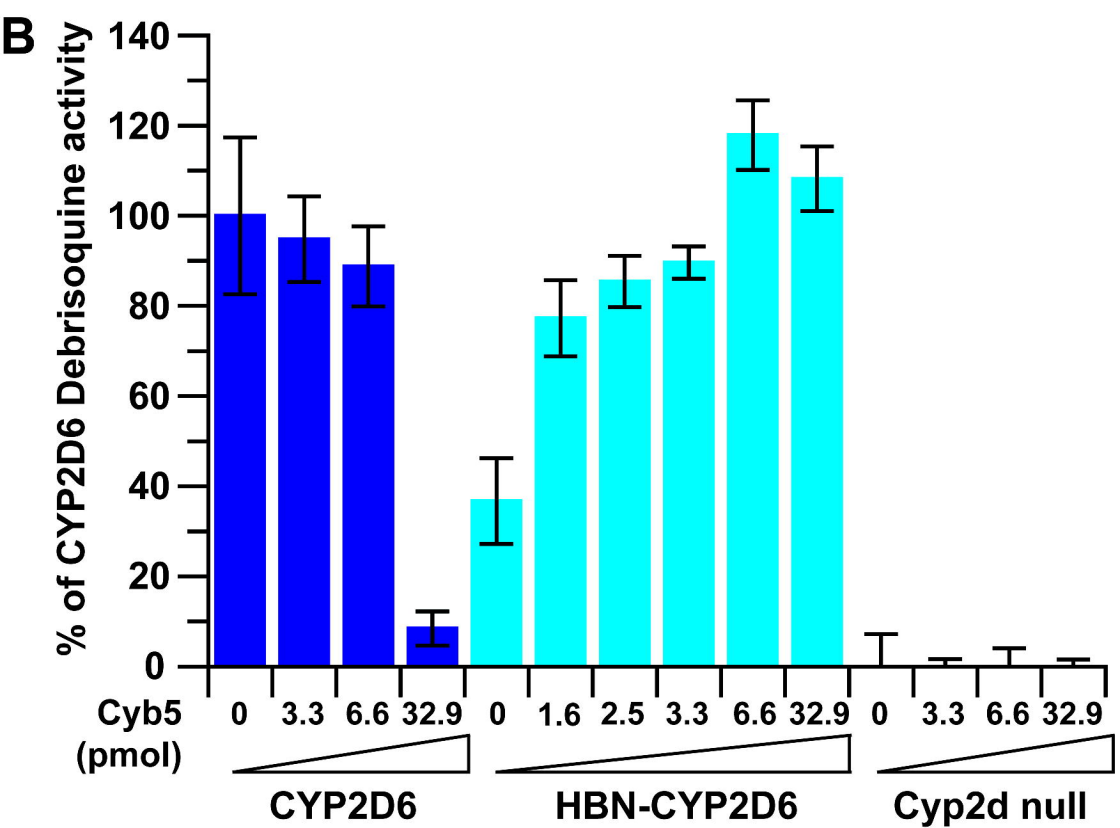
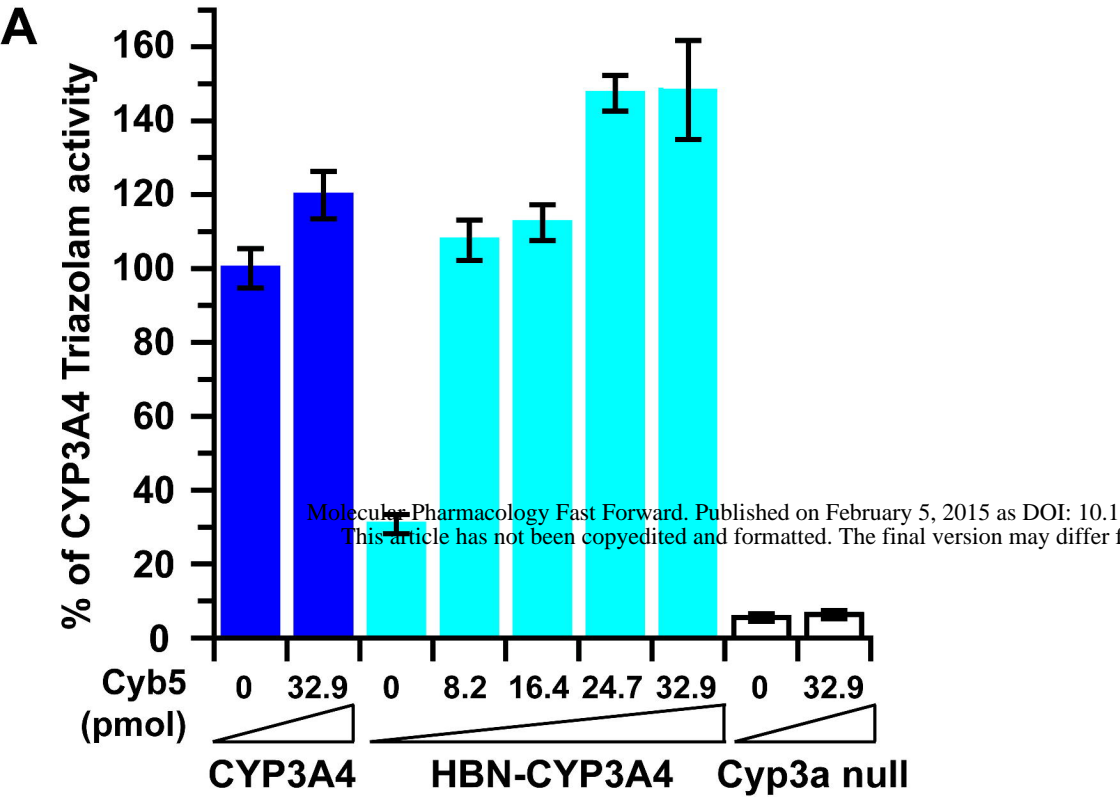


Figure 3

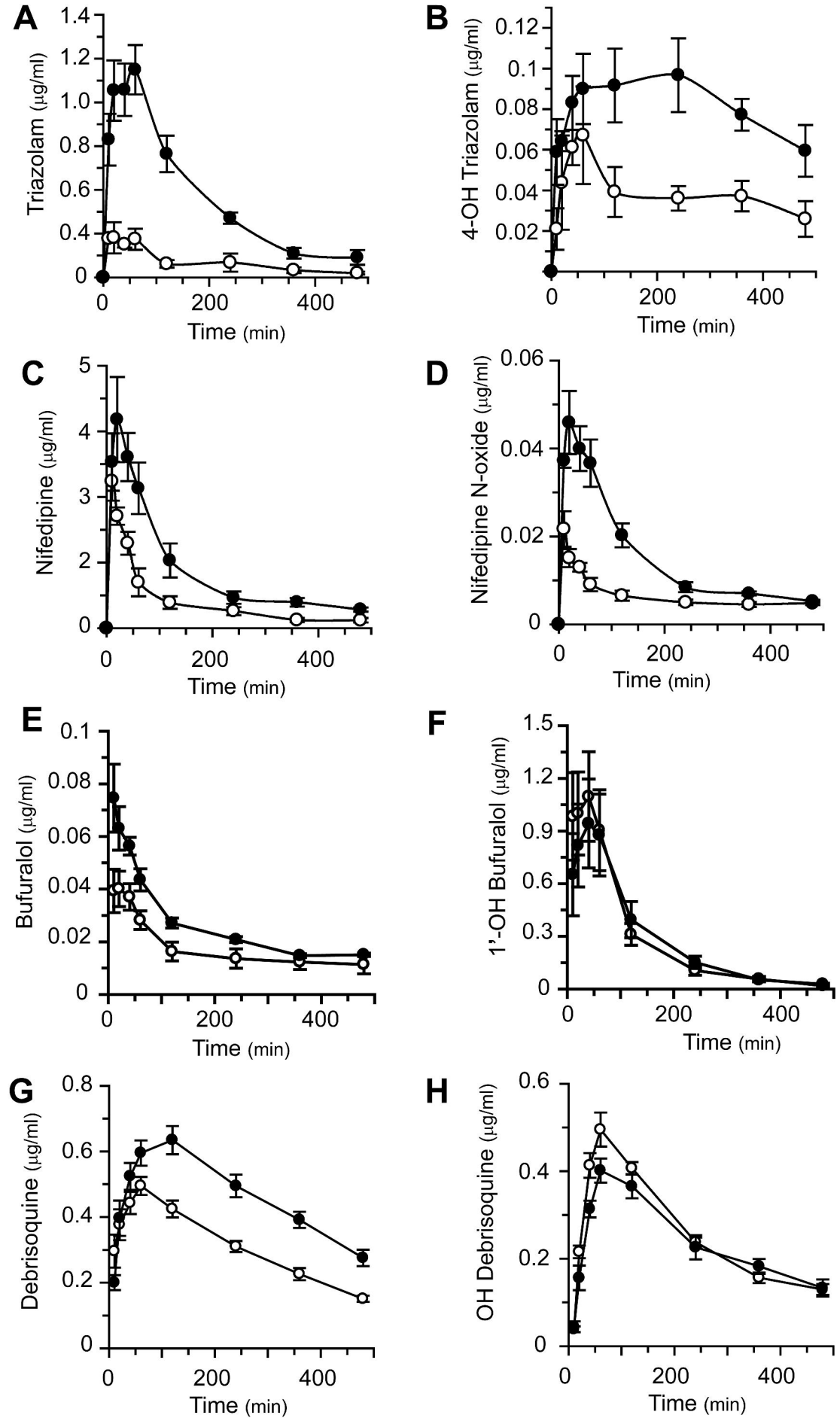


Figure 4

**Cytochrome b_5 is a major determinant of human cytochrome
P450 CYP2D6 & CYP3A4 activity *in vivo***

Colin J. Henderson, Lesley A. McLaughlin, Nico Scheer, Lesley A. Stanley
and C. Roland Wolf

Supplemental Information

Materials and Methods

***In vitro* drug incubations**

Triazolam, nifedipine, metoprolol, bufuralol and debrisoquine assays were performed in triplicate (n=3-4 per genotype) using 20, 10, 20, 3.25 or 10 µg of hepatic microsomes and 250, 25, 100, 5 and 250 µM substrate in a final volume of 50 µl. Samples were pre-incubated for 3 min at 37 °C before addition of NADPH (final concentration 1 mM). The reaction was allowed to proceed for 9 (triazolam), 6 (nifedipine), 12 (metoprolol), 3 (bufuralol) or 20 (debrisoquine) min. All reactions except those with nifedipine were stopped by the addition of 1 volume of ice cold acetonitrile containing 0.25 µg/ml midazolam (internal standard). Nifedipine samples were terminated using methanol instead of acetonitrile. Samples were incubated on ice for 10 min and centrifuged for 10 min at 16000 x *g* to remove particulate material and analysed by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

Assays to determine the apparent kinetics of bufuralol 1'-hydroxylation were performed with CYP2D6 and CYP2D6-HBN liver microsomes in triplicate under conditions of linearity for time and protein. Incubations contained 5µg of microsomal protein and 0-20 µM bufuralol (final concentrations) in a final volume of 50 µl and lasted for 4 min. Reaction mixtures were terminated, processed and analysed as described above.

UPLC-MS/MS analysis

For all compounds, UPLC was performed using an Acquity UPLC BEH C18 (1.7 µ, 50 x 2.1mm) column (Waters, Dublin, Ireland), with an injection volume of 5 µl. Mobile phases of 0.1% formic acid (A) and acetonitrile & 0.1% formic acid (B) were used for all drugs except that methanol replaced acetonitrile for nifedipine. The chromatographic gradients applied were:

- Bufuralol, 1'-hydroxy bufuralol, triazolam, 1'- and 4-hydroxytriazolam - flow rate 0.5 ml/min; 0-0.25 min: 95% - 67.5% A; 0.25-1.0 min: 67.5-63% A; hold for 0.5 min; return to initial conditions for 0.5 min.
- Nifedipine and oxidised nifedipine - flow rate 0.5 ml/min; 0-0.8 min: 80% - 35% A; hold for 0.2 min; return to initial conditions for 0.5 min.
- Debrisoquine and 4-hydroxy debrisoquine - flow rate 0.5 ml/min; 0-0.45 min: 95% - 35% A; hold for 1.05 min; return to initial conditions for 0.5 min.

Multiple reaction monitoring data were acquired in positive electrospray ionization mode for all compounds except debrisoquine and 4-hydroxydebrisoquine, for which single reaction monitoring was employed. The cone voltages and collision energies used for MS/MS analysis were optimized for each compound (**Table S3**). Concentrations of parent drugs and metabolites were calculated using authentic standards.

***In vivo* pharmacokinetics**

CYP3A4 and CYP3A4-HBN mice (n=4 per genotype) were dosed with triazolam (3 mg/kg p.o.) or nifedipine (30 mg/kg p.o.); CYP2D6 and CYP2D6-HBN mice were dosed with bufuralol (12 mg/kg p.o.; n=4 per genotype) or debrisoquine (10 mg/kg p.o.; n= 8 per genotype). Whole blood (10 µl) was taken from the tail vein at intervals after drug administration (10, 20, 40, 60, 120, 240, 360 and 480 min), transferred into a tube containing heparin (10µl, 15 IU/ml) and stored at -20 °C until processed. Acetonitrile or, for nifedipine samples, methanol (70 µl containing 0.25 mg/ml midazolam) was added to thawed samples which were shaken for 15 min, centrifuged for 10 min at 16,000 x g and analysed using the method as described for *in vitro* incubations.

Table S1: Organ:body weight ratios in CYP3A4, CYP3A4-HBN, CYP2D6 and CYP2D6-HBN mice

Genotype	Liver: body weight	Kidney: body weight	Lung: body weight
CYP3A4 (CO)	0.048 ± 0.003	0.013 ± 0.001	0.006 ± 0.0008
CYP3A4 (PCN)	0.057 ± 0.002**	0.012 ± 0.0005	0.006 ± 0.001
CYP3A4-HBN (CO)	0.049 ± 0.009	0.011 ± 0.0005	0.007 ± 0.001
CYP3A4-HBN (PCN)	0.059 ± 0.003**	0.012 ± 0.001	0.006 ± 0.001
CYP2D6	0.049 ± 0.004	0.013 ± 0.0005	0.007 ± 0.0002
CYP2D6-HBN	0.053 ± 0.004	0.013 ± 0.0006	0.006 ± 0.001

Prior to microsome preparation CYP3A4 and CYP3A4-HBN animals were pre-treated with either corn oil (CO) or PCN as described. n= 4 or 5 mice for each genotype; ** p = ≤ 0.01 for CO vs. PCN treatment (same genotype).

Table S2: Pharmacokinetic parameters of drugs examined in CYP3A4, CYP3A4-HBN, CYP2D6 and CYP2D6-HBN mice

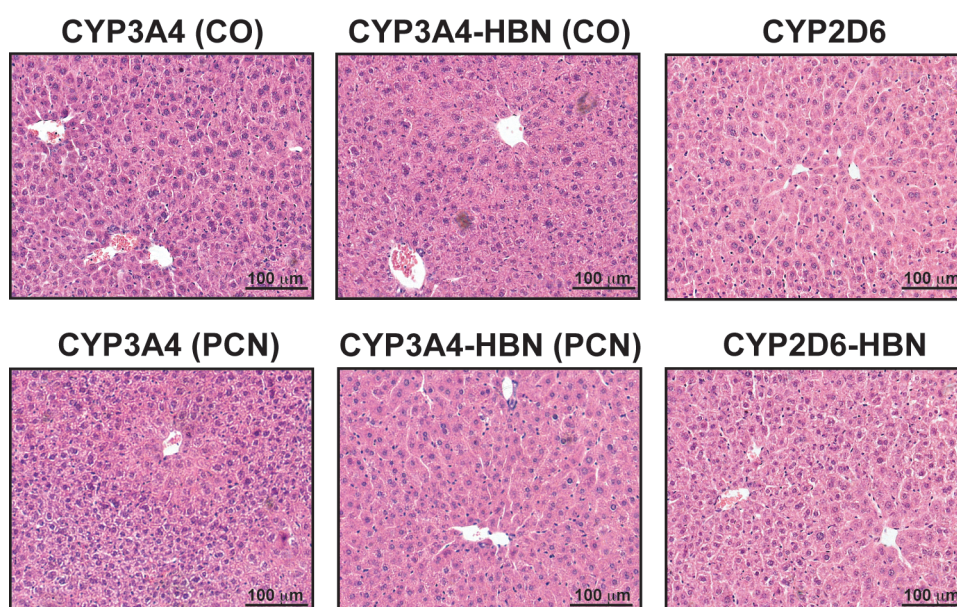
Drug	Parameter	CYP3A4	CYP3A4-HBN	Drug	Parameter	CYP2D6	CYP2D6-HBN
	C _{max}	0.26 ± 0.11	1.03 ± 0.20 ^{***}		C _{max}	0.05 ± 0.02	0.08 ± 0.03
	Half-life	112.9 ± 54.6	98.8 ± 34.6		Half-life	174.7 ± 71.5	205.5 ± 73.6
Triazolam	AUC _{0-8h}	29.4 ± 10.5	167.5 ± 29.1 ^{**}	Bufuralol	AUC _{0-8h}	8.5 ± 3.6	12.1 ± 1.9 ^{**}
	AUC _{0-inf}	32.3 ± 14.0	181.8 ± 24.3 ^{***}		AUC _{0-inf}	11.8 ± 5.8	16.6 ± 1.0 ^{**}
	Clearance	104.6 ± 36.5	18.5 ± 2.2 ^{**}		Clearance	1840 ± 2147	725.1 ± 43.5 [*]
	C _{max}	2.32 ± 0.58	3.4 ± 1.07		C _{max}	0.53 ± 0.06	0.67 ± 0.10 ^{**}
	Half-life	106.9 ± 36.9	119.8 ± 18.0		Half-life	265.0 ± 43.9	325.4 ± 59.2 [*]
Nifedipine	AUC _{0-8h}	181.2 ± 52.4	431.6 ± 84.2 ^{***}	Debrisoquine	AUC _{0-8h}	149.7 ± 17.3	223.8 ± 35.9 ^{***}
	AUC _{0-inf}	196.8 ± 56.7	480.8 ± 88.5 ^{***}		AUC _{0-inf}	207.8 ± 27.5	357.3 ± 89.7 ^{***}
	Clearance	162.6 ± 45.0	64.1 ± 12.5 ^{**}		Clearance	48.8 ± 6.0	29.5 ± 7.4 ^{***}

CYP3A4 and CYP3A4-HBN animals were pre-treated with PCN, as described, before being dosed with either triazolam or nifedipine. Units are: C_{max} (µg/ml), half-life (min), AUC_{0-8h} (min*µg/ml) AUC_{0-inf} (min*µg/ml) and Clearance (ml/min/kg). * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001; n= 4 per group for triazolam and nifedipine, 6 or 10 for bufuralol and 8 for debrisoquine.

Table S3: Mass spectrometer settings and transitions for drug analysis

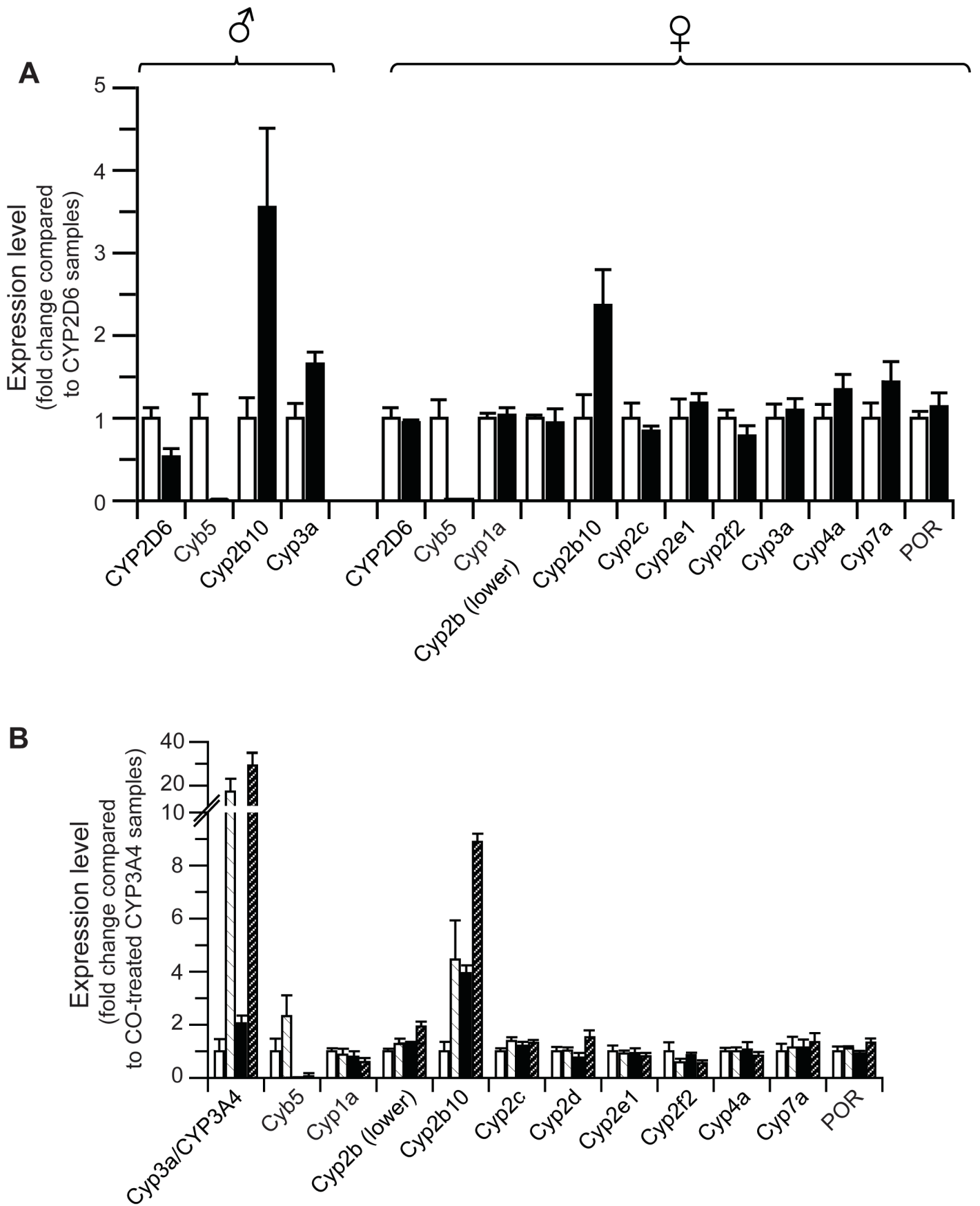
Compound	MRM Transition	Cone (V)	Collision energy
Bufuralol	262.37 > 188.33	22	16
1'-Hydroxy bufuralol	278.29 > 186.22	23	22
Debrisoquine	176.15 (SRM)	35	n.a.
4- Hydroxy debrisoquine	192.12 (SRM)	28	n.a.
Nifedipine	345.12 > 291.35	41	29
Oxidised nifedipine	347.21 > 315.32	18	9
Midazolam	326.25 > 291.35	46	26
Triazolam	343.13 > 308.10	55	37
1'-Hydroxy triazolam	359.12 > 176.21	23	30
4-Hydroxy triazolam	359.16 > 110.9	46	60

Supplemental Figure 1



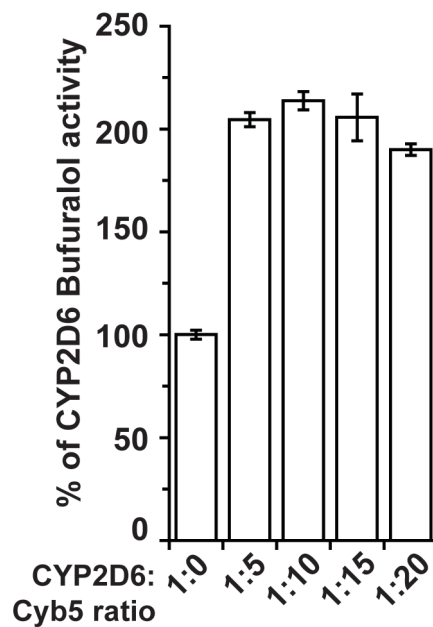
Representative Haematoxylin and Eosin stained sections of liver from CYP3A4, CYP3A4-HBN, CYP2D6 and CYP2D6 HBN mice. Original magnification 20x; CYP3A4 and CYP3A4-HBN mice were treated with corn oil (CO) or PCN (10 mg/kg q.d. x3) as described.

Supplemental Figure 2



Quantification of cytochrome b5, cytochrome P450 oxidoreductase and cytochrome P450 expression in livers from CYP3A4, CYP3A4-HBN, CYP2D6 and CYP2D6-HBN mice. Fold-change in protein expression in A, CYP2D6 (white bars) and CYP2D6-HBN (black bars) and B, corn-oil (CO) and PCN-treated CYP3A4 and CYP3A4-HBN mice (white bars, CO-treated CYP3A4; light hatched bars, PCN-treated CYP3A4; black bars, CO-treated CYP3A4-HBN; dark hatched bars, PCN-treated CYP3A4-HBN) by densitometric quantification of Western immunoblotting of liver microsomal samples (Figure 1). Fold change in P450 protein expression levels compared to CYP2D6 samples (A) and to CO-treated CYP3A4 samples (B). POR: P450 oxidoreductase.

Supplemental Figure 3



Stimulation of recombinant CYP2D6 activity towards bufuralol by increasing molar ratios of Cyb5. E.coli membranes co-expressing recombinant CYP2D6 and POR were mixed with E.coli membranes expressing recombinant human Cyb5 in molar ratios as shown, and as described. Values (mean \pm SD) are presented as a percentage of the CYP2D6 sample activity from triplicate incubations.