Evidence that cytochrome b_5 and cytochrome b_5 Reductase can act as sole electron donors to the hepatic cytochrome P450 system

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1

Running Title: Drug metabolism and disposition in HBN, HRN and HBRN mice

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Number of text pages: 30

Number of tables: 1

Number of figures: 5

Number of references: 40

Number of words in the Abstract: 237

Number of words in the Introduction: 701

Number of words in the Discussion: 1291

Abbreviations: ADR - adverse drug reaction; AUC: area under the concentration-time curve; BFC: 7-Benzyloxy-4-trifluoromethylcoumarin; BQ: 7-benzyloxyquinoline; BR – benzyloxyresorufin; C_{max}: maximum plasma concentration; Cyb5: cytochrome b5; Cyb5R: cytochrome b5 reductase; HBN: Hepatic cytochrome b_5 Null; HRN: Hepatic Reductase Null; HBRN: Hepatic cytochrome b_5 and cytochrome P450 Reductase Null; LC-MS/MS: high performance liquid chromatography-tandem mass spectrometry; MR – methoxyresorufin; NCE: new chemical entity; P450: cytochrome P450; POR: cytochrome P450 oxidoreductase; WT: wild-type; NADPH: nicotinamide adenine dinucleotide phosphate (reduced).

MOL#84616

Abstract

We have previously described the development of genetic models to study the *in vivo* functions of the hepatic cytochrome P450 system, through the hepatic deletion of either cytochrome P450 oxidoreductase (POR; HRN line) or cytochrome b_5 (Cyb5; HBN line). However, HRN mice still exhibit low levels of mono-oxygenase activity, in spite of the absence of detectable reductase protein. To investigate whether this is because cytochrome b_5 and cytochrome b_5 reductase can act as sole electron donors to the P450 system, we have crossed HRN with HBN mice to generate a line lacking hepatic expression of both electron donors (HBRN). HBRN mice exhibited exacerbation of the phenotypic characteristics of the HRN line - liver enlargement, hepatosteatosis and increased expression of certain cytochrome P450s. Also, drug metabolising activities *in vitro* were further reduced relative to the HRN model, in some cases to undetectable levels. Pharmacokinetic studies *in vivo* demonstrated that midazolam half-life, C_{max} and area under the concentration-time curve (AUC) were increased, and clearance was decreased, to a greater extent in the HBRN line than in either the HBN or HRN model. Microsomal incubations using NADPH concentrations below the apparent K_m of cytochrome b_5 reductase, but well above that for POR, led to the virtual elimination of 7-benzyloxyquinoline turnover in HRN samples. These data provide strong evidence that cytochrome b_5/cytochrome b_5 reductase can act as a sole electron donors to the cytochrome P450 system *in vitro* and *in vivo*.

3

Introduction

The cytochrome P450 (P450) monooxygenases comprise 70-80% of Phase I xenobiotic metabolising enzymes and are critical players in protecting organisms against damage from chemical insult, as well as maintaining cellular function and homeostasis through involvement in processes including steroidogenesis, bile acid production, cholesterol biosynthesis, vitamin D pathway, prostacyclin biosynthesis, long chain fatty acid and eicosanoid signalling, inflammatory pathways and brain neurotransmitter synthesis (Ferguson and Tyndale, 2012; Miller, 2005; Nebert and Russell, 2002; Prosser and Jones, 2004; Romano et al., 1987).

More than 80% of prescribed drugs undergo P450-mediated Phase I metabolism (Eichelbaum et al., 2006). Favourable absorption, distribution, metabolism and excretion (ADME) characteristics are critical determinants in the progression of new chemical entities (NCEs) through the drug development pipeline, and pre-clinical ADME utilises both *in vitro* and *in vivo* models; however P450 metabolism is usually established solely by the *in vitro* route. Adverse drug reactions (ADRs), which are often associated with P450-dependent metabolism and are among the top five causes of drug-related deaths in the USA, are a major problem for the pharmaceutical industry. The root causes of ADRs fall into three groups: drug-drug interactions, reactive metabolite formation and genetic polymorphisms of individual P450s (Eichelbaum et al., 2006). In humans, polymorphisms in genes such as *CYP2C9*, *2C19*, and *CYP2D6* lead to significant inter-individual differences in both systemic drug exposure and pro-drug activation in the patient population, and this can have repercussions including reduced efficacy and a range of ADRs (Johansson and Ingelman-Sundberg, 2010). Characterisation of the metabolic fate of NCEs, allowing the re-engineering of efficacious

molecules to design-out metabolic liabilities, is therefore of prime importance in the early pre-clinical phase of development.

Mammalian microsomal P450s function by catalysing the insertion of one atom of molecular oxygen into a substrate molecule, while reducing the other atom to water, a reaction which requires two electrons. Two proteins can transfer these electrons to the P450: the multi-domain flavoprotein NADPH-cytochrome P450 oxidoreductase (POR) which has traditionally been attributed as providing the first (and often the second) electron (Ortiz de Montellano, 2005), and cytochrome b_5 reductase (Cyb5R) which can also supply the second electron (via Cyb5).

Studies aiming to elucidate the role of Cyb5 in P450-mediated metabolism using reconstituted systems *in vitro* have generated results which are difficult to interpret, so there is a clear need for a model which can provide information regarding interactions between P450s, POR and Cyb5 *in vivo* and their respective roles in drug disposition. To that end, we and others previously generated and characterised a conditional hepatic model of POR deletion (Hepatic Reductase Null (HRN)) (Gu et al., 2003; Henderson et al., 2003) and an inducible conditional deletion in liver and gastrointestinal tract (Finn et al., 2007), as well as two models where Cyb5 has been either conditionally deleted in the liver (Hepatic b_5 Null (HBN) (Finn et al., 2008)) or knocked out globally (Finn et al., 2011; McLaughlin et al., 2010). These models have been used to investigate the P450 metabolism and resulting toxicity of many drugs and environmental carcinogens (Arlt et al., 2006; Arlt et al., 2011; Arlt et al., 2005; Arlt et al., 2008; Finn et al., 2007; Finn et al., 2008; Levova et al., 2011; McLaughlin et al., 2010; Pass et al., 2005; Potega et al., 2011; Stiborova et al., 2005; Stiborova et al., 2008). Deletion of POR had a major effect on both *in vitro* and *in vivo* metabolism although *in vitro* P450 activity was not completely ablated (10% residual

activity) (Gu et al., 2003; Henderson et al., 2003). We also demonstrated that deletion of Cyb5 can profoundly affect P450 metabolism in a tissue- and substrate-dependent manner (Finn et al., 2008; McLaughlin et al., 2010). These data raised two questions: is the residual P450 activity observed in the HRN animals being driven by Cyb5/Cyb5R, and can the deletion of Cyb5 on a POR null background circumvent this? To address these questions we have generated a conditional hepatic POR and Cyb5 knock-out mouse (Hepatic b5 Reductase Null (HBRN). Here we describe the initial characterization of these animals with respect to *in vitro* and *in vivo* P450 activity. The data presented provide evidence that Cyb5/Cyb5R can function as sole electron donors to the cytochrome P450 system *in vivo*.

Materials and Methods

Chemicals - Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Poole, UK). Nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) was obtained from Melford Laboratories (Ipswich, UK). 7-Benzyloxy-4-trifluoromethylcoumarin (BFC), 7-hydroxy-4-trifluoromethylcoumarin, 7-benzyloxyquinoline (BQ) and hydroxy-tolbutamide, were purchased from BD Gentest, Cowley, UK. Midazolam, 1-hydroxy midazolam and 4-hydroxy midazolam were kind gifts from Roche (UK) and 1-hydroxy metoprolol and O-desmethyl-metoprolol were generous gifts from Astra Häsle (Mölndal, Sweden). Bupropion and hydroxybupropion were purchased from Toronto Research Chemicals (Toronto, Canada).

Generation of hepatic microsomal cytochrome b_5 and cytochrome P450 oxidoreductase null mice - Hepatic reductase null (HRN ($POR^{lox/lox}::Cre^{ALB}$)) and hepatic Cyb5 null (HBN ($Cyb_5^{lox/lox}::Cre^{ALB}$)) animals were generated as described previously (Finn et al., 2008; Henderson et al., 2003). HBRN ($Cytb_5^{lox/lox}::POR^{lox/lox} \pm Cre^{ALB}$) and wild-type (WT; $POR^{lox/lox}::Cyb_5^{lox/lox}$) were generated by crossing the appropriate lines and thereafter maintained by crossing of homozygous pairs within each line. All lines used in this study were C57BL/6J (N6). All mice were kept under standard animal house conditions, with free access to food and water, and a 12h light/dark cycle. All animal work was carried out on male 10-week-old mice in accordance with the Animal Scientific Procedures Act (1986) and after local ethical review.

Preparation of hepatic microsomal fractions - Microsomes were prepared from snap frozen liver tissue harvested from 8-10 week old male mice (5 per genotype) as described previously (Finn et al., 2008). Microsomes were stored at -70°C until required. Microsomal protein concentrations were determined using the Biorad Protein Assay Reagent (Bio-Rad Labs Ltd,

Herts UK). P450 oxidoreductase (POR) activity was estimated by NADPH-dependent cytochrome *c* reduction (Strobel and Dignam, 1978). The P450 content of mouse liver microsomes was determined by reduced carbon monoxide difference spectroscopy using the method of Omura and Sato (Omura and Sato, 1964). Cyb5 content was determined spectrophotometrically as described previously (McLaughlin et al., 2010).

Immunoblotting – Western immunoblot analysis was carried as described previously (Finn et al., 2008). Immunoreactive proteins were detected using polyclonal goat anti-rabbit, anti-mouse or anti-sheep horseradish peroxidase immunoglobulins as secondary antibodies (Dako, Ely, UK), and visualised using the Immobilon™ chemiluminescent substrate (Millipore, Watford, UK) on a FUJIFILM LAS-3000 mini imaging system (Fujifilm UK Ltd, UK). Densitometric analysis was performed using Multi Gauge V2.2 software (Fujifilm UK Ltd, UK).

In vitro fluorogenic assay incubations - Assays were performed essentially as described previously (Finn et al., 2008) using 20 μg hepatic microsomes and 7-benzyloxy-4-trifluoromethylcoumarin (BFC; 40 μM); BR and MR (1 μM); 7-benzyloxyquinoline (BQ; 80 μM), and NADPH as a co-factor at a final concentration of 1mM. Reactions were measured for 3 min at the recommended excitation and emission wavelengths for each probe using a Fluoroskan Ascent FL fluorimeter (Labsystems, UK). Turnover rates were calculated using authentic metabolite standards with the exception of BQ (7-hydroxy-4-trifluoromethylcoumarin for BFC; resorufin for MR and BR).

In vitro probe substrate incubations – Midazolam, tolbutamide and metoprolol assays were performed in triplicate for 5 samples per genotype as described previously (Finn et al., 2008) using midazolam (50 μM), tolbutamide (800μM) and metoprolol (240 μM). Assays were allowed to proceed for 30 min before being stopped by the addition of 1 volume of ice cold

acetonitrile and ice incubation for 10 min. Samples were centrifuged for 8 min at 16000 x *g*

to remove particulate material before analysis by high performance liquid chromatography-

tandem mass spectrometry (LC-MS/MS). Turnover was calculated based on authentic

metabolite standards.

Bupropion assays were carried out using 20 µg of hepatic microsomes and 250µM substrate.

Microsomal incubations were analysed by LC-MS/MS using a Waters 2795 HPLC and

Quattro Micro mass spectrometry system in positive electrospray ionization mode. Multiple

reaction monitoring data were acquired. The cone voltage and collision energy were

optimised for each product (Bupropion - cone voltage: 28, collision energy: 18, transition:

240.43 > 184.26; 6-hydroxy bupropion: cone voltage: 26, collision energy: 18, transition:

256.40 > 238.31). A dwell time of 0.05 s was used between multiple reaction monitoring

transitions. Bupropion and 6-hydroxy bupropion were resolved in 2 min on an ACQUITY

UPLC BEH C18 Column, 2.1 x 75 mm, 1.7 µm column (Waters, Elstree, UK). The injection

volume was 5 µl. The following elution program was used at a temperature of 30˚C and a

flow rate of 0.5 ml/min: Eluent A –formic acid; Eluent B – Acetonitrile containing 0.1%

formic acid; (1) 5% B held for 0.3 min, (2) linear gradient to 45% B over 0.35 min then held

for a further 0.35 min, (3) linear gradient to 95% B in 0.05 min, held for 0.25 min (4) re-

equilibration at 5% B for 0.25min.

Midazolam pharmacokinetics – WT, HBN, HRN and HBRN mice (n=5 for each genotype)

were dosed orally with midazolam (2.5 mg/kg). Blood samples (10µl) were taken from the

tail vein of each mouse at 10, 20, 40, 60, 120, 240, 360 and 450 min post dosing. Midazolam

analysis by LC-MS/MS and pharmacokinetic modelling was carried out as described

previously (Finn et al., 2008). The data shown represent mean ± standard error of the mean.

Results

Phenotype of HBRN (Cyb5$^{lox/lox}$::POR$^{lox/lox}$::Cre^{ALB}) mice – In order to establish the effect of simultaneous hepatic deletion of both microsomal Cyb5 and POR, we generated a mouse line with conditional knockouts of both genes. Mice lacking hepatic microsomal Cyb5 and POR were viable and exhibited no gross anatomical abnormalities. Both sexes were fertile and offspring were born at expected Mendelian ratios. *Post mortem* examination of HBRN animals revealed that all tissues except the liver were normal in appearance. The livers of HBRN animals were pale, mottled and enlarged relative to those of WT mice of the same genetic background, a phenotype indistinguishable from that observed in HRN mice (Henderson et al., 2003). When liver to body weight ratios were calculated, those of HRN and HBRN mice were significantly higher than those of WT mice, both being increased by almost 50% (**Figure 1A**). No change in the liver to body weight ratio was observed in HBN mice relative to controls. Haematoxylin and eosin stained sections of liver (**Figure 1B**) indicated a progressive increase in lipid accumulation across the genotypes. The livers of HBN mice were only mildly affected, having a mottled appearance with pale areas surrounding the central veins whereas the remainder of the liver parenchyma was normal in colour. This appearance was more marked, being associated with centrilobular vacuolation in the HRN line, while the phenotype of HBRN liver exhibited extensive vacuolation with large vacuoles which were often larger than the size of an individual hepatocyte. This micro- and macro-vesicular hepatic lipidosis is typical of steatotic liver.

Analysis of cytochrome P450-dependent monooxygenase components – As described previously (Finn et al., 2008; Henderson et al., 2003), hepatic P450 concentrations measured by Fe^{2+}-CO versus Fe^{2+} difference spectra were significantly elevated (2.6-fold) in HRN

animals compared to WT but unchanged in HBN (**Figure 1C**). The increased expression observed in HBRN animals was similar to that seen in the HRN line, approximately 2.3-fold. The concentration of hepatic microsomal Cyb5 was reduced by 85% and 88% in HBN and HBRN livers respectively but interestingly was increased 2.7-fold in HRN livers (**Figure 1C**). Hepatic POR activity, measured using cytochrome *c* as a surrogate electron acceptor, was unchanged in HBN animals compared to WT and undetectable in both HRN and HBRN livers (**Figure 1D**). These results suggest that conditional deletion of hepatic POR leads to an increase in Cyb5 concentration whereas deletion of hepatic Cyb5 has no corresponding effect on POR expression.

Western blot analysis of hepatic Cyb5, POR and P450 proteins in the various mouse lines is shown in **Figure 2** and the relative fold changes are shown in **Table 1**. Cyb5 and POR were not detectable in the HBN and HRN lines, and both proteins were absent from HBRN liver. Consistent with the data shown in **Figure 1**, Cyb5 levels were increased approximately 3-fold in HRN liver but conditional deletion of Cyb5 in the HBN liver had no corresponding effect on POR expression (**Figure 2**).

As reported previously, the expression of Cyp2b10 was increased in HRN mice, and indeed in all three conditional knockouts (2.3-fold, 8.6-fold and 12.9-fold in the HBN, HRN and HBRN, respectively; p≤0.005 in each case; **Table 1**). The greater increase in expression in the HBRN line suggests a possible additive effect when both POR and Cyb5 are deleted (p≤0.05), but the inter-individual variation in expression makes it difficult to evaluate this possibility. Cyp3a expression was also induced in the HBN, HRN and HBRN lines (1.8, 2.6- and 2.8-fold, respectively; p≤0.005); Cyp7a expression was also induced (14-15 fold; p≤0.005) in the HRN and HBRN lines. Conditional deletion of POR and/or Cyb5 had no effect on the hepatic expression of Cyp1a in any of the models (**Figure 2**). The expression of

11

Cyp2c, Cyp2d, Cyp2e and Cyp4a proteins was slightly induced by these deletions, (less than 2.5-fold).

In vitro cytochrome P450 activities – *In vitro* P450 activities in hepatic microsomes from WT, HBN, HRN and HBRN mice were determined using a panel of eight substrates, four of which are commonly designed as probes for specific P450s (**Figure 3A**) while the other four are clinically used drugs (**Figure 3B**). Deletion of hepatic Cyb5 had no effect on the O-dealkylation of BR and MR (**Figure 3A**) but caused a significant, substrate-dependent reduction in turnover for five out of the eight substrates examined (BFC, BQ, midazolam, metoprolol and tolbutamide) (**Figures 3A, B**). Interestingly, the rate of hydroxylation of bupropion by HBN liver microsomes was actually increased relative to controls, possibly due to the increased levels of Cyp2b10. As observed previously, marked reduction of all these activities was observed in HRN liver microsomes. Importantly, in the HBRN line, the activities were further reduced (except in the case of tolbutamide, but the activities in both HRN and HBRN lines were very low for this substrate).

In general, two distinct patterns of effect were observed (**Figure 3A**). Deletion of Cyb5 alone had no marked effect on microsomal activity towards MR and BR whereas deletion of POR almost abolished the corresponding activity. In contrast, deletion of either Cyb5 or POR markedly reduced activity towards BQ and BFC, and deletion of both genes had an additive effect, suggesting that both Cyb5 and POR are necessary for the metabolism of these substrates. These data suggest that the residual microsomal activity was at least in part due to cytochrome b_5 donating electrons directly into the P450 system.

The pattern of effects on the metabolism of drugs in clinical use generally reflected those observed with BQ and BFC (**Figure 3B**). Deletion of either Cyb5 or POR, in spite of the increased expression of a number of P450 isozymes, significantly reduced the rate of

metabolism of metoprolol (α-hydroxylation and O-demethylation), midazolam (1'- and 4-hydroxylation) and tolbutamide, while conditional deletion of both genes in the HBRN led to near-complete abolition of activity. These results suggest that both Cyb5 and POR are required for maximal metabolism of these substrates, although the consequences of a single gene deletion of POR were more severe than those of deletion of Cyb5 alone. Indeed, in the cases of metoprolol and tolbutamide activity was almost entirely absent in HRN microsomes, with or without additional deletion of Cyb5, suggesting electron transfer was entirely POR-dependent.

The exception to this pattern was bupropion (**Figure 3B**). Hydroxylation of this substrate was actually increased in HBN liver microsomes ($p \leq 0.005$) relative to the activity observed in WT liver, although it was significantly reduced in HRN microsomes ($p \leq 0.005$) and further compromised in HBRN microsomes ($p \leq 0.05$).

The above data is presented as reaction rates normalised against total P450 concentration. When the activities were expressed per mg microsomal protein (**Supplemental Table 1**), the effects were still observed but were less pronounced. Whether this represents the existence of homeostatic mechanisms which modulate P450 expression in an attempt to maintain total activities remains to be established.

In vivo pharmacokinetics of midazolam – To determine whether deletion of Cyb5 together with POR further altered drug metabolism *in vivo*, the pharmacokinetics of orally administered midazolam were determined in WT, HBN, HRN and HBRN mice. The elimination profiles of midazolam disappearance differed between WT and HBN mice, translating into significant alterations of pharmacokinetic parameters that were exacerbated in a genotype-dependent fashion (HBRN>HRN>HBN) (**Figure 4A and B**). Profound changes in the pharmacokinetics were observed, with the half life of midazolam extended by 1.2-, 2-

and 2.4-fold relative to WT in the three conditional knockout lines, respectively; maximal

plasma concentration (C_{max}) was increased by 3.3-, 5.5- and 6.7- fold and clearance was

decreased by 85%, 95% and 97%. Midazolam exposure, as measured by area under the curve

(AUC), showed an 8.5-, 19.6- and 29-fold increase in the HBN, HRN and HBRN models

respectively compared to WT (**Figure 4B**). Furthermore, there was a significant increase in

midazolam AUC between HRN and HBRN mice, although the observed increase in half-life

between those two genotypes failed to reach statistical significance (p=0.06).

In order to determine whether the electrons required for the residual P450-mediated activity

observed in HRN liver microsomes were supplied by the (extremely low) levels of POR

expressed in this line or by Cyb5R/Cyb5, we characterised the *in vitro* kinetics of POR in WT

liver microsomes. This analysis indicated that murine hepatic POR has an apparent K_m for

NADPH of 2.9 µM (**Figure 5A**). The literature indicates that although Cyb5R is classed as

NADH-dependent, it does also have a low affinity for NADPH (Km ~ 1mM (Roma et al.,

2006)). The *in vitro* assays illustrated in **Figure 3** were carried out in the presence of 1mM

NADPH, meaning that the necessary electrons could be coming either from the residual POR

or from Cyb5R/Cyb5. The large difference in affinity for NADPH between the two enzymes

was exploited to determine which enzyme is driving the reaction (**Figure 5B**). If the P450-

mediated activity in the HRN samples was driven by residual POR, titration of NADPH

concentration from 1 mM down to 50 µM should have little effect on its rate but if it is driven

by Cyb5R, one would predict a significant reduction in turnover. In WT liver microsomes

(containing both POR and Cyb5), reducing the concentration of NADPH down to 50 µM

caused little change in rate of BQ turnover because the lowest concentration tested was still

significantly higher than Km (2.9 µM). This is consistent with the role of POR as the electron

donor in WT liver. In HRN samples, however, the activity observed was strongly dependent

on the concentration of NADPH over the concentration range from 50 – 1,000 µM.

Reduction of the NADPH concentration to 50µM caused a 94% reduction in BQ turnover

(**Figure 5B**). When NADH was used with HRN liver microsomes, there was little change in

BQ turnover above a concentration of 100µM, but a rapid fall-off below that point consistent

with the involvement of Cyb5R (K_m for NADH estimated at ~12µM) as the source of

electrons (**Figure 5B inset**). These data provide strong evidence that, in the absence of

measurable levels of POR, electrons can be supplied to P450s from Cyb5R/Cyb5, rather than

from residual hepatic POR.

MOL#84616

Discussion

We previously used the HRN mouse line, a conditional hepatic model of POR deletion which profoundly reduces hepatic P450 activity (Henderson et al., 2003), to dissect the role of this enzyme system in the metabolism, disposition and toxicity of drugs and foreign chemicals. It is important to note that the HRN model represents a genetic deletion of POR, with >99% of hepatocytes being negative on immunohistochemistry (Pass et al., 2005). However, despite the complete absence of detectable hepatic POR in this model, measurable P450-dependent enzyme activities could still be detected in HRN liver microsomes Henderson, 2003 #1969}. In this study, we have interbred the HRN line with mice lacking hepatic Cyb5 to create the HBRN model, in order to investigate the role of Cyb5 in this residual activity.

As with the HRN (Henderson et al., 2003) and HBN (Finn et al., 2008) models, the dual knockout of POR and Cyb5 had no deleterious effects on mouse fecundity, litter size or pup development and no gross physical phenotype was observed. The steatotic liver characteristic of POR deletion (Finn et al., 2007; Gu et al., 2003; Henderson et al., 2003) was even more pronounced in the HBRN animals than in the HRN model. This appeared to be an additive effect of the double knock-out, since HBN mice also develop this phenotype after about 16 weeks of age (unpublished data). In assays using almost a milligram of protein (~ 40-fold more than normal) from HBN or HBRN hepatic microsomes, we could not detect reduction of cytochrome c (unpublished).

Consistent with our previous findings, and those of others, indicating that Cyb5 can have positive, negative or no effect on the function of the cytochrome P450 system (Finn et al., 2008; Zhang et al., 2005), the *in vitro* activities of hepatic microsomal fractions from the three models differed in a substrate-specific manner. Deletion of Cyb5 alone had no marked effect on microsomal activity towards MR and BR, as observed previously (Finn et al., 2008;

16

McLaughlin et al., 2010), whereas deletion of POR almost abolished the corresponding activity. This suggests that Cyb5 is not essential for the O-dealkylation of these substrates, which are metabolised predominantly by Cyp1a2 and Cyp2b10, respectively (Nerurkar et al., 1993). Interestingly, the metabolism of a further Cyp2b10 substrate - bupropion – was actually increased in HBN samples, probably reflecting the increased expression of Cyp2b10. In contrast, deletion of either Cyb5 or POR markedly reduced activity towards the other probe substrates and drugs used in this study, and deletion of both genes further increased this effect, suggesting that both Cyb5 and POR are necessary for the metabolism of these substrates and that in the absence of POR, Cyb5/Cyb5R can provide electrons into the mammalian P450 system.

Consideration of the redox potentials of Cyb5 (+20mV) and ferric substrate-bound cytochrome P450 (-237mV) shows that it would be thermodynamically impossible for Cyb5 to provide the first electron in the P450 catalytic cycle. However, the redox potential of Cyb5R (-265mV) is such that it could support this reaction. Given that the redox potential of oxyferrous cytochrome P450 is also approximately +20mV, it is feasible that Cyb5 can supply the second electron into the catalytic cycle. Further evidence for the role of Cyb5/Cyb5R was provided by exploiting the different affinities of POR and Cyb5R for NADPH; we were able to show that in the absence of POR, BQ metabolism became highly dependent on NADPH concentration. Furthermore, in HRN samples, titration of NADH caused BQ activity to drop sharply at co-factor concentrations below 100μM, with the K_m of the reaction consistent with that of Cyb5R for NADH. These data suggest both that residual POR is not the electron donor catalysing substrate metabolism and also provide strong evidence that Cyb5R is the electron source under such circumstances.

A number of mechanisms have been proposed for the possible interactions between Cyb5 and P450 to explain the modifier action of the former on the latter. These include direct transfer

of a rate-limiting electron; the formation of a ternary complex which allows near-simultaneous transfer of two electrons between POR and P450; improved reaction coupling; and direct effector actions (Schenkman and Jansson, 2003). It seems that, where inhibitory effects are observed, they are due to competition between Cyb5 and POR; for example, for a binding site on the proximal surface of CYP2B4 whereby formation of a Cyb5-P450 complex prevents ferric P450 from accepting and electron from POR and initiating the catalytic cycle Where stimulatory effects are observed they are due to an increase in the rapidity and efficiency of catalysis in the presence of Cyb5 compared with POR; and where no effect is observed, this represents a balance between these two opposing effects (Zhang et al., 2008).

The findings that in the absence of POR, hepatic Cyb5 mediates reactions *in vivo* was demonstrated by using the probe drug midazolam, metabolised predominantly by Cyp3a proteins in wild-type mice. As reported previously, the half-life, C_{max} and AUC of midazolam were significantly increased, and clearance substantially decreased, in the HBN and HRN models (as compared to WT). However, deletion of both genes produced a further significant increase in C_{max}, half-life and AUC and a reduction in clearance. The induction of Cyb5 in HRN and HBRN would serve to amplify its role in the disposition of midazolam under these circumstances. Furthermore, since these effects are evident following oral administration of the compound, the data suggest that the consequences of differences in hepatic metabolism are superimposed on any intestinal first pass effects on clearance. In almost all cases, the dual deletion of both Cyb5 and POR almost completely abrogates all cytochrome P450 activities, suggesting that other electron donors can at best only play a very minor role in hepatic P450 functions; the new HBRN model thus provides a more authentic hepatic-P450 null phenotype.

The cost of drug development is rising exponentially (Collier, 2009), and the failure rate for the development of new chemical entities (NCEs) is 80-90% (Cuatrecasas, 2006),

principally due to toxicity and lack of efficacy. The use of *in vitro* technologies is both time- and cost-effective, allowing the metabolic profile of a NCE to be determined early in the preclinical development process and causing the contribution from ADRs to candidate attrition to fall over the last 10 years (Plant, 2004), but ADRs are still a significant reason for failure in Phases II and III of development. One possible reason for this is that potential ADRs associated with P450 metabolism are not always detected *in vitro* during early preclinical development. The HBRN model described here, along with the HBN (Finn et al., 2008), complete Cyb5 knockout (Finn et al., 2011; McLaughlin et al., 2010), HRN (Henderson et al., 2003), inducible hepatic POR knockout (Finn et al., 2007) and gut POR knockout (Zhang et al., 2009) models, is a powerful tool in determining the impact of P450-mediated metabolism on the *in vivo* disposition and efficacy of drugs as well as on the toxicokinetics of parent compounds and their metabolites. Use of such models to screen NCEs for P450-mediated metabolism and toxicity would also address the lack of *in vivo* data in the early preclinical development of new drugs. The FDA has recommended that murine-human species differences in drug metabolism and disposition should be identified and characterised early as possible during the drug development process. The availability of mouse models which are humanised for the major drug metabolising P450s e.g. CYP3A4 and CYP2D6 (Felmlee et al., 2008; Hasegawa et al., 2011; Scheer et al., 2012; van Herwaarden et al., 2007; van Waterschoot and Schinkel, 2011; Yu et al., 2004), means that the HBRN model could be used in conjunction with such mice, allowing the identification of NCEs which are metabolised by human P450s *in vivo* prior to the initiation of clinical trials.

MOL#84616

Acknowledgements

Authorship contributions

Participated in Research design: Henderson, Wolf

Conducted experiments: McLaughlin

Contributed new reagents or analytic tools: Henderson

Performed data analysis: McLaughlin

Wrote or contributed to the writing of the manuscript: Henderson, McLaughlin, Wolf

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23

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Footnotes

Colin Henderson and Lesley McLaughlin are joint first authors

Figure legends

Figure 1. Hepatic phenotype of HBRN mice compared to WT, HBN and HRN.

A. Liver to body weight ratio of WT, HBN, HRN and HBRN mice (mean ± standard deviation; n=5 animals per genotype). **B.** Representative haematoxylin and eosin stained sections of liver from WT, HBN, HRN and HBRN mice. Original magnification: 5x (top row) and 20x (bottom row). Arrows indicate areas of lipid deposition. **C.** P450 (white bars) and cytochrome b_5 (black bars) content of hepatic microsomes from WT, HBN, HRN and HBRN mice, measured by difference spectroscopy (mean ± standard deviation; n=5 animals per genotype). Statistical significance (comparing knockout animals to WT): [*] $p \leq 0.05$; [**] $p \leq 0.01$; [***] $p \leq 0.001$. **D.** POR activity of hepatic microsomes from WT, HBN, HRN and HBRN mice as measured using cytochrome c as a surrogate electron acceptor (nmol/min/mg microsomal protein, mean ± standard deviation; 5 animals per genotype, assayed in duplicate). Statistical significance (comparing knockout animals to WT): [*] $p \leq 0.05$; [**] $p \leq 0.01$; [***] $p \leq 0.001$.

Figure 2. Cytochrome b_5, cytochrome P450 oxidoreductase and cytochrome P450 expression in livers from WT, HBN and HRN and HBRN mice.

Detection of hepatic expression of drug metabolising enzymes by Western immunoblotting of individual microsomal samples (20 µg hepatic microsomes per lane). Further experimental details are given in the Materials & Methods. On Cyp2b blot, arrow indicates Cyp2b10 band.

Figure 3. Comparison of cytochrome P450-mediated *in vitro* metabolism using hepatic microsomes from WT, HBN, HRN and HBRN mice.

In vitro assays were performed as described in Materials & Methods. Assays were performed in triplicate on liver microsomes from five mice of each genotype, with NADPH as a co-

factor at a final concentration of 1 mM. All data shown represent mean $\pm$ standard deviation for 5 mice per group assayed in triplicate. Statistical significance: [*] $p \leq 0.05$; [**] $p \leq 0.01$; [***] p ≤ 0.001. **(a)** Benzyloxyresorufin dealkylation (BR: Cyp2b), methoxyresorufin dealkylation (MR: Cyp1a), 7-Benzyloxy-4-trifluoromethylcoumarin dealkylation (BFC; Cyp3a/2b/2c), 7-benzyloxyquinolone dealkylation (BQ: Cyp3a). **(b)** Bupropion 6-hydroxylation (Cyp2b), midazolam 1'- and 4-hydroxylation (Cyp3a), metoprolol α-hydroxylation and O–demethylation (Cyp2d) and tolbutamide hydroxylation (Cyp2c).

Figure 4. *In vivo* pharmacokinetic profiles of midazolam in WT, HBN, HRN and HBRN mice

A. Midazolam was administered orally to WT, HBN, HRN and HBRN mice (n=5 per group) at a dose of 2.5 mg/kg. ○ WT; ● HBN; □ HRN; ■ HBRN. B. Midazolam disposition in WT, HBN, HRN and HBRN mice: comparison of pharmacokinetic parameters - * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, relative to WT data; (*) $p \leq 0.05$ HRN relative to HBRN data.

Figure 5. Dependence of hepatic enzyme activities on co-factor concentration in HRN mice.

A. Dependence of cytochrome *c* reduction on NADPH concentration in WT hepatic microsomes. Assays were performed in triplicate under conditions of linearity for time and protein. Standard deviations for K_m and V_{max} are from the fit of the curve as calculated using the Michaelis-Menten equation (GraFit v5; Erithacus Software, Horley, UK). **B.** Dependence of 7-benzyloxyquinoline metabolism on co-factor concentration. Assays in WT with NADPH (○), HRN with NADPH (●) and HRN with NADH (■) hepatic microsomes were performed in triplicate. Percentage of maximal activity was calculated using the following rates as 100% ACTIVITY - WT with NADPH: 6.6 +/- 0.4 pmol/min/mg, HRN with NADPH: 9.9 +/- 1.1 pmol/min/mg and HRN with NADH:21.7 +/- 1.7 pmol/min/mg.

Data points represent mean ± standard deviation. *Inset*: dependence of BQ oxidation on

NADH concentration in HRN hepatic microsomes (mean +/- standard deviation for triplicate

samples); standard deviation for K_m is from the fit of the curve as calculated using the

Michaelis-Menten equation (GraFit v5).

Table 1: Relative changes in Cyb5, POR and Cytochrome P450 protein expression in WT, HBN, HRN and HBRN livers

Protein	Fold change compared to WT			
	WT	HBN	HRN	HBRN
Cyb5	1 ± 0.2	$0.004 \pm 0.005^{***}$	$3.1 \pm 0.8^{***}$	$0.2 \pm 0.4^{***/(***)}$
POR	1 ± 0.1	$1.2 \pm 0.08^{***}$	$0.01 \pm 0.01^{***}$	$0.01 \pm 0.01^{***}$
Cyp1a	1 ± 0.1	1.1 ± 0.06	1.1 ± 0.07	1.0 ± 0.05
Cyp2b10	1 ± 0.2	$2.3 \pm 0.2^{***}$	$8.6 \pm 2.0^{***}$	$12.9 \pm 2.5^{***/(*)}$
Cyp2b	1 ± 0.2	$1.4 \pm 0.1^{**}$	$1.9 \pm 0.3^{**}$	$2.4 \pm 0.4^{***}$
Cyp2c	1 ± 0.05	$1.6 \pm 0.1^{***}$	$1.7 \pm 0.2^{***}$	$1.6 \pm 0.1^{***/(*)}$
Cyp2d	1 ± 0.003	$1.2 \pm 0.002^{**}$	$1.3 \pm 0.004^{**}$	$1.3 \pm 0.001^{**}$
Cyp2e	1 ± 0.2	$0.8 \pm 0.05^{*}$	$1.4 \pm 0.2^{*}$	$0.7 \pm 0.2^{*/(***)}$
Cyp3a	1 ± 0.3	$1.8 \pm 0.4^{**}$	$2.6 \pm 0.4^{***}$	$2.8 \pm 0.2^{***}$
Cyp4a	1 ± 0.1	1.2 ± 0.2	$1.3 \pm 0.06^{**}$	$1.4 \pm 0.08^{***}$
Cyp7a	1 ± 0.4	1.7 ± 0.9	$14.2 \pm 3.8^{***}$	$14.7 \pm 2.7^{***}$

Statistical significance - *- is shown for HBRN, HRN or HBN compared to WT, and between HRN and HBRN (*); **/(*)** = p ≤ 0.05, ****/(**)** = p ≤ 0.01, *****/(***)** = p ≤ 0.001, n=5 for each genotype.

Figure 1

Figure 2

Figure 3

A

B
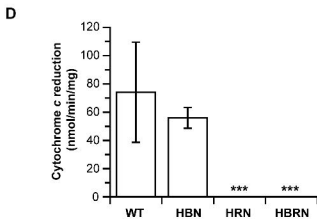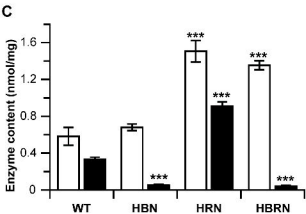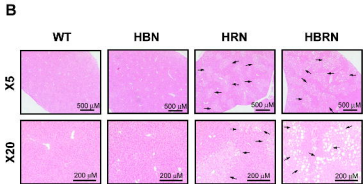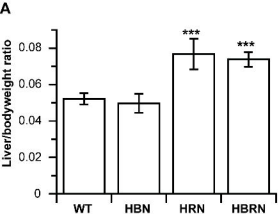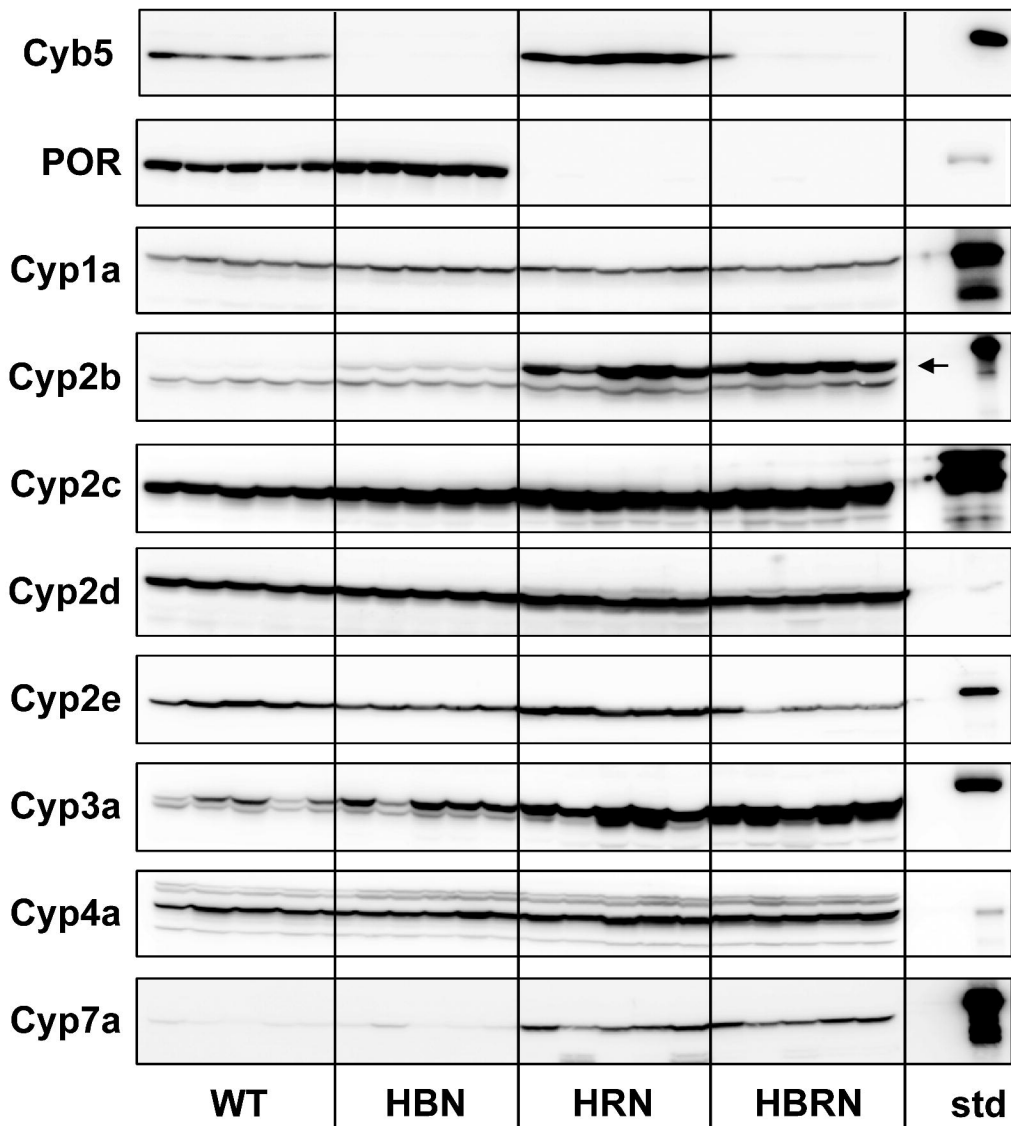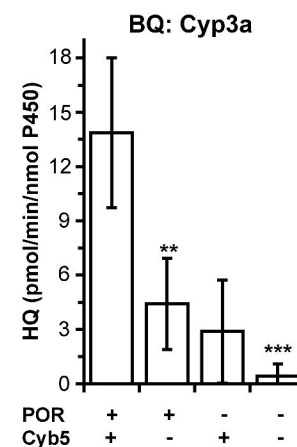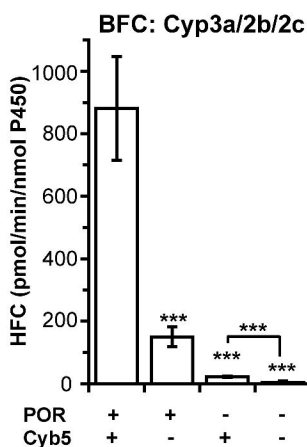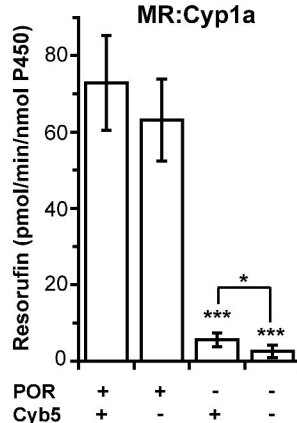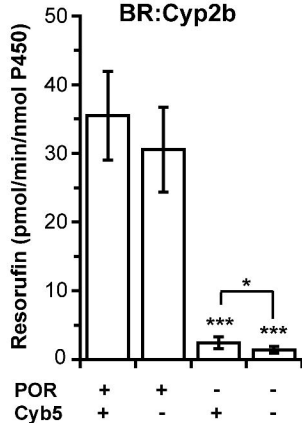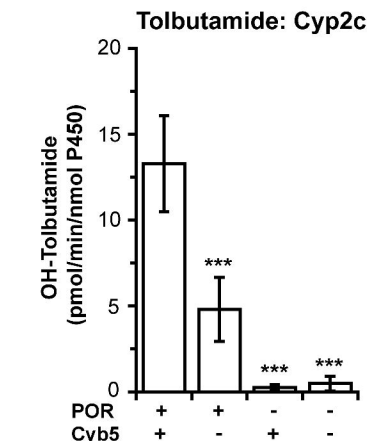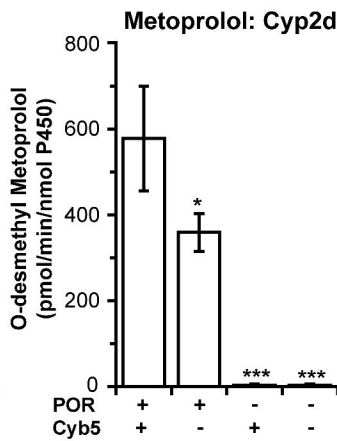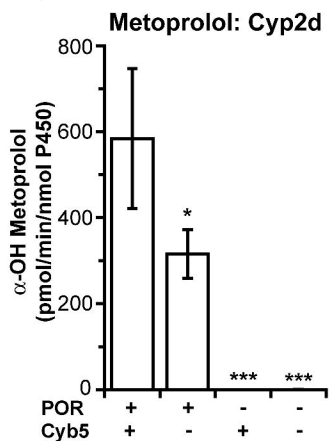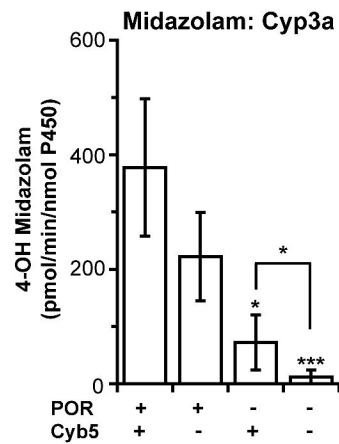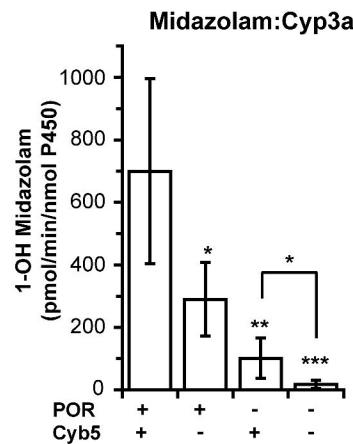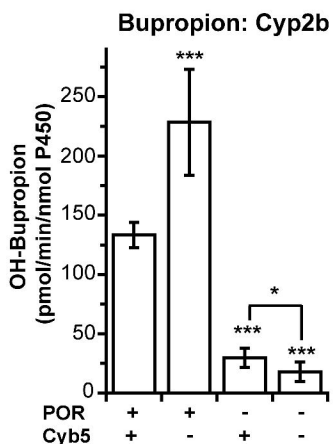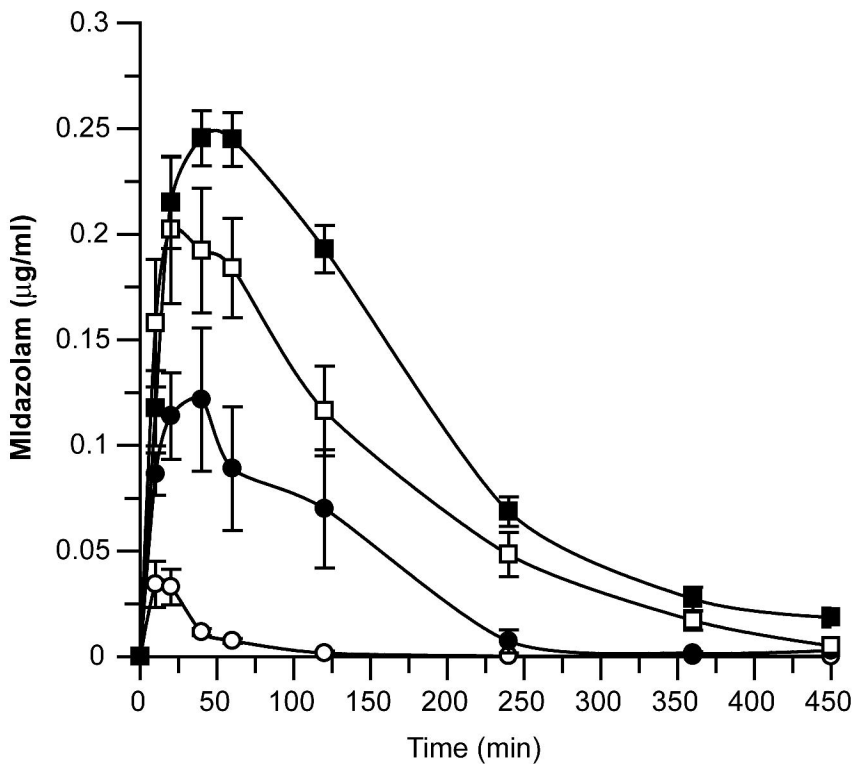
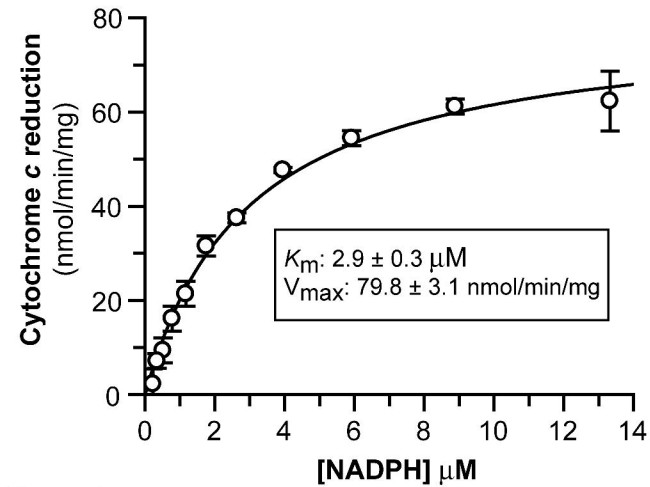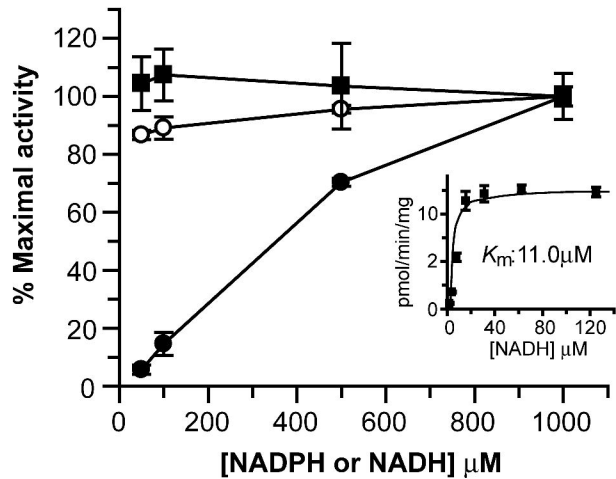|	Half-life (min)	C$_{max}$ (µg/ml)	AUC$_{0 \to 450\,min}$ (min*µg/ml)	AUC$_{\infty}$ (min*µg/ml)	Clearance (ml/min/kg)
Wild-type	37.6 ± 23.6	0.04 ± 0.02	1.73 ± 0.53	1.76 ± 0.52	1541 ± 505
HBN	43.9 ± 7.2	0.13 ± 0.07*	14.9 ± 11.6*	15.0 ± 11.7*	263 ± 126***
HRN	74.5 ± 19.6**	0.22 ± 0.07***	34.0 ± 11.4***	34.5 ± 11.4***	82.5 ± 40.4***
HBRN	89.2 ± 21.9**	0.26 ± 0.02***	48.4 ± 4.10***(*)	51.3 ± 5.66***(*)	49.5 ± 5.09***

Figure 4

A

B

Figure 5