Deletion of Microsomal Cytochrome b\(_5\) Profoundly Affects Hepatic and Extra-hepatic Drug Metabolism

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Nonstandard abbreviations: BCN – Cytochrome $b_5$ complete knockout mice ($Cytb_5^{-/-}$), BFC - 7-Benzylxy-4-trifluoromethylcoumarin; BR – Benzyloxyresorufin; EFC - 7-Ethoxy-4-trifluoromethylcoumarin; ER – Ethoxyresorufin; HPLC – High performance liquid chromatography; LC/MS-MS – Liquid chromatography-tandem mass spectrometry; MFC - 7-Methoxy-4-trifluoromethylcoumarin; MR – Methoxyresorufin; P450 – Cytochrome P450; POR – Cytochrome P450 oxidoreductase; PR – Pentoxyresorufin; WT – Wild-type ($Cytb_5^{+/+}$).
ABSTRACT

We recently demonstrated that cytochrome b5 plays an important *in vivo* role in hepatic cytochrome P450 (P450) function (Finn *et al.*, J. Biol. Chem. (2008)). We have now generated a model where cytochrome b5 has been deleted in all tissues (BCN; cytochrome b5 complete null), which surprisingly results in a viable mouse in spite of the putative *in vivo* roles of this protein in lipid and steroid hormone metabolism, and the reduction of methemoglobin. In contrast to the liver-specific deletion, complete deletion of cytochrome b5 leads to a neonatal increase in the expression of many hepatic P450s, at both the protein and mRNA level. In extra-hepatic tissues, some changes in P450 expression were also observed, which were isoform-dependent. *In vitro* cytochrome P450 activities in liver, kidney, lung and small intestine of BCN mice were determined for a range of model substrates and probe drugs; a profound reduction in the metabolism of some substrates, particularly in lung, kidney and small intestine was observed. *In vivo*, the metabolism of metoprolol was significantly altered in BCN mice, in contrast to the previous finding in the liver-specific cytochrome b5 deletion, suggesting that extra-hepatic cytochrome b5 plays a significant role in its disposition. Testicular Cyp-17 hydroxylase and lyase activities were also significantly reduced by cytochrome b5 deletion, leading to significantly lower levels of testicular testosterone. The BCN mouse provides an additional model system with which to further investigate the functions of cytochrome b5, particularly in extra-hepatic tissues.
Microsomal cytochrome \( b_5 \) is a ubiquitous, 15.2 kDa hemoprotein associated primarily with the endoplasmic reticulum of eukaryotic cells. In conjunction with its primary electron donor cytochrome \( b_5 \) reductase, this protein has been associated with many important cellular processes. Probably the most characterized is in the cytochrome P450 (P450) – mediated metabolism of xenobiotics and drugs (Porter, 2002; Schenkman and Jansson, 2003; Yamaori et al., 2003; Yamazaki et al., 2002; Zhang et al., 2005); and in cholesterol and steroid hormone homeostasis (Akhtar et al., 2005; Lamb et al., 2001). These effects on cytochrome P450 activity are very much substrate- and P450-specific, with evidence of both stimulation and inhibition of P450 turnover (Porter, 2002; Schenkman and Jansson, 2003; Waskell et al., 1986; Zhang et al., 2008; Zhang et al., 2007). After almost 40 years of research however, the mechanism of this interaction is still being described as “enigmatic” (Schenkman and Jansson, 2003) and “controversial” (Hildebrandt and Estabrook, 1971; Porter, 2002); and has been the focus of many, often contradictory, literature reports (Guryev et al., 2001; Yamazaki et al., 2002).

The other (non-P450 related) functions of cytochrome \( b_5 \) include the reduction of heavy metals, carcinogenic arylhydroxylamines (Borthiry et al., 2007; Kurian et al., 2007), fatty acid desaturation and methemoglobin /haemoglobin cycling (Jeffcoat et al., 1977; Umbreit, 2007). Furthermore, cytochrome \( b_5 \) is also thought to play a role in the biosynthesis of plasmalogens which are glycerol-based phospholipids, found in heart and neuronal tissues (Lee et al., 1991; Paltuaf et al., 1974; Woelk and Jahrreiss, 1978).

In order to establish the \textit{in vivo} functions of this protein, we have recently generated a mouse where microsomal cytochrome \( b_5 \) was conditionally deleted in the liver (HBN mice) (Finn et al., 2008). HBN mice had significantly reduced \textit{in vitro} rates of hepatic NADPH- and NADH-dependent metabolism of a range of model P450 substrates and probe drugs;
furthermore, significant changes in drug pharmacokinetics occurred on administration of a probe drug cocktail, demonstrating that hepatic cytochrome \( b_5 \) can play a significant role in \textit{in vivo} drug disposition.

We have now generated a mouse model where the cytochrome \( b_5 \) has been deleted in all mouse tissues (\( b_5 \) complete null (BCN)). BCN mice were viable and fertile and in this report we describe the effects of cytochrome \( b_5 \) deletion on the expression of drug metabolising enzymes and on hepatic and extra-hepatic foreign compound metabolism.
Materials and Methods

Chemicals - All reagents unless stated were purchased from Sigma-Aldrich (Poole, UK). NADPH was obtained from Melford Laboratories (Ipswich, UK). 7-Benzyl oxy-4-trifluoromethylcoumarin (BFC), 7-methoxy-4-trifluoromethylcoumarin (MFC) and 7-hydroxy-4-trifluoromethylcoumarin (HFC) 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 Hälsle (Mölndal, Sweden). PCR primers were obtained from Eurofins MWG Operon (Ebersberg, Germany)

Generation of Cytochrome b5 Complete Null (BCN) Mice - Cytb5lox/lox mice were generated as previously described (Finn et al., 2008) and maintained by random breeding on a 129P2 X C57BL/6 genetic background. Cytb5lox/lox mice were crossed with a transgenic mouse line expressing Cre recombinase under the control of a phosphoglycerate kinase promoter (CrePGK) (Lallemand et al., 1998) on a C57BL/6 background, and Cytb5+/−:CrePGK offspring were backcrossed with Cytb5+/+ mice to generate heterozygous cytochrome b5 complete knockout mice minus CrePGK (Cytb5+/−), which were subsequently crossed to generate homozygous cytochrome b5 complete knockout mice (BCN, Cytb5−/−) and control (wild-type, Cytb5+/+) mice. The BCN line was thereafter maintained using a combination of crosses - Cytb5−/− females with Cytb5−/− males, Cytb5−/− females with Cytb5+/− males and Cytb5−/− females with Cytb5−/− males. Mice carrying the null cytochrome b5 allele were identified by multiplex PCR using the following primer set: (1) wild type forward primer 5′-TCCCCCCTGAGAACGTAATTG-3′ (2) null forward primer 5′-GGTCTCTCCTTGGTCCACAC-3′ and (3) common reverse primer 5′-GAGTCTTTCGTCAGTGCGTGA-3′ (Figure S1). The presence of the CrePGK transgene was
determined as previously described (Henderson et al., 2003). All mice were maintained on a standard chow (RM1 – Special Diet Services, Essex, UK) under standard animal house conditions, with free access to food and water, and 12h light/12h dark cycle. All animal work was carried out on 10 week old male mice, except where indicated, in accordance with the Animal Scientific Procedures Act (1986) and after local ethical review.

Preparation of microsomes - Microsomes were prepared from wild-type and BCN mouse tissues, using 0.3 – 0.5g of tissue, by the method of Meehan et. al. (Meehan et al., 1988) for the small intestine and by a modified method of this method for all other tissues, using sonication instead of mechanical homogenisation (Pritchard et al., 1998). 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). Microsomes were stored at -70˚C until required.

Quantitation of microsomal cytochrome b5 - The specific amount (pmol cytochrome b5/mg of microsomal protein) of cytochrome b5 expressed in various tissues was determined by NADH-reduced spectrophotometry as follows: a known volume of microsomes were resuspended in 2ml of 100 mM potassium phosphate buffer pH 7.4 and the suspension split between two matched quartz cuvettes. An absorbance baseline was plotted between 400 – 500 nm, following which NADH was added to the sample cuvette to a concentration of 200 μM and a reduced spectrum determined. The amount of cytochrome b5 in the sample was calculated by determining the absorbance difference between 426 nm and 409 nm using an extinction coefficient of 185,000 L/mol. Data is the mean of three animals (per tissue) ± s.d.

Immunoblotting - Immunoblot analysis was carried as previously described (Finn et al., 2008). Cyp17 antibody was a kind gift from Professor Mike R. Waterman, Vanderbilt University, Tennessee. Immunoreactive proteins were detected using polyclonal goat anti-
rabbit, anti-mouse or anti-sheep horseradish peroxidase (HRP) immunoglobulins as secondary antibodies (Dako, Ely, UK), and visualised using Immobilon™ chemiluminescent HRP substrate (Millipore, Watford, UK) and 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).

**RNA isolation and Real-Time Quantitative PCR analysis** - Total RNA was isolated from snap-frozen liver samples using Trizol (Invitrogen, Paisley, UK). Genomic DNA was removed using RQ1 DNase (Promega, Southampton, UK), and RNA (600 ng) reverse transcribed using random hexamers and Superscript II RNase H reverse transcriptase polymerase (Invitrogen, Paisley UK). Real-Time Quantitative PCR analysis was carried out with optimised gene-specific primer sets (Applied Biosystems, Warrington, UK) except in the case of cytochrome b₅ where the following primer-probe set was used:

Forward primer: 5’-GGGTGATGCTACCGAGAATTTT-3’;
Reverse primer: 5’-AGTTCCCCGATGATGTATGTTTTG-3’;
Probe: [6-FAM] CGTCGGGCACTCTACGGATGCAC [TAMRA-6-FAM].

Reactions were carried out in triplicate and monitored by measuring fluorescence at 518 nm with excitation at 494 nm. mRNA levels were quantified using the Prism 7700 associated software and normalised to the levels 18s mRNA (Applied Biosystems, Warrington, UK).

**P450 spectral analysis** - P450 content of mouse liver microsomes was determined by reduced CO difference spectroscopy for three samples of each genotype, using the method described by Omura and Sato (Omura and Sato, 1964).

**Generic P450 microsomal incubations** - Microsomal incubations were carried out in triplicate in 50 mM Hepes pH 7.4, 30 mM MgCl₂ containing mouse tissue microsomes and substrate pre-warmed to 37°C before initiation of reaction by addition of either NADPH or NADH to a final concentration of 0.67 mM.
**Fluorogenic assay incubations** - Assays were performed essentially as previously described (Finn et al., 2008) using the following substrate and microsome concentrations: BFC - 40 µM substrate, 20 µg mouse tissue microsomes; EFC - 40 µM substrate, 20 µg mouse liver microsomes; MFC - 140 µM substrate, 20 µg mouse liver microsomes; ER, BR, MR and PR – 1 µM substrate, 20 µg mouse liver microsomes. Reactions were measured in real time for 3 min at the recommended excitation and emission wavelengths for each probe using a Fluroskan Ascent FL plate reading fluorimeter (Labsystems, UK). Turnover rates were calculated using authentic metabolite standards (HFC for BFC, EFC and MFC assays and resorufin for BR, ER, MR and PR assays).

**Recapitulation of P450 activity** - BCN liver microsomes (20 μg) were mixed with increasing amounts of *E. coli* membranes containing recombinant murine cytochrome *b*5 (3.3, 6.6, 13.2 and 26.4 pmol of cytochrome *b*5). Tubes were capped and incubated at room temperature for 20 min. Potassium phosphate buffer (190 µl of 100 mM, pH 7.4) containing 30 µM BFC or 0.8 µM BR was then added and tubes mixed before adding into the wells of a 96 well plate. Reactions were initiated with 10 µl of 10 mM NADPH and run for 3-5 min and metabolites measured and quantified as previously described. Cytochrome *b*5, wild-type and wild-type plus cytochrome *b*5 controls were also included. Experiments using lung microsomes were carried out in the same manner except that a single concentration of cytochrome *b*5 was used (26.4 pmol).

**BFC and MR kinetics** - Assays to determine the apparent kinetic parameters were performed in triplicate with wild-type and HBN liver microsomes under conditions of linearity for time and protein (data not shown) using the same buffer/NADPH conditions as described above, with the following concentrations of substrates: MR 0.002-1 µM and BFC 0.01-100 µM, using 10 and 11 concentration points respectively.
**LC MS-MS assay incubations** - Incubations were performed under the following conditions:

- Chlorzoxazone – 1 mM substrate, 20 µg microsomes in a final volume of 150µl for 30 min;
- Midazolam – 50 µM substrate, 20 µg microsomes in a final volume of 100µl for 9 min;
- Metoprolol – 800 µM substrate, 30 µg microsomes in a final volume of 100µl for 60 min;
- Phenacetin - 50 µM substrate, 20 µg microsomes in a final volume of 100µl for 9 min;
- Tolbutamide – 800 µM substrate, 30 µg microsomes in a final volume of 10µl for 60 min.

Assays were stopped by the addition of either 0.5 volumes (chlorzoxazone) or 1 volume (tolbutamide, midazolam and phenacetin) of ice cold methanol and incubated on ice for 10 min. Samples were centrifuged for 8 min at 16000 x g to remove particulate material before HPLC (chlorzoxazone) or LC MS-MS (tolbutamide, midazolam and phenacetin) analysis was carried out as previously described (Finn et al., 2008).

**Testicular testosterone content** - Testosterone was extracted from the testes of 10 week old mice using the method of Zhang et al (Zhang et al., 2003) and analyzed by reversed phase HPLC (Paine et al., 2003).

**Progesterone and 17α hydroxy-progesterone incubations** - Incubations were performed in triplicate in a final volume of 150 µl of 50 mM potassium phosphate buffer; pH 7.4 containing 25 µg of testicular microsomes and either 8 µM progesterone or 15 µM 17α hydroxy-progesterone. Reactions were incubated at 37°C and were initiated by the addition of NADPH. After 20 min, incubations were stopped by the addition of 75 µl of ice-cold methanol. Samples were incubated on ice for 10, before being centrifuged for 8 min at 16000 x g to remove particulate material before HPLC analysis.

**Progesterone and 17α hydroxy progesterone assay HPLC conditions** - Substrates and metabolites (progesterone, 17α hydroxy-progesterone, androstenedione and testosterone) were separated by reversed phase HPLC using a Hypersil ODS column (5 µm; 4 x 250mm (Agilent, Stockport, UK)) maintained at 35 ºC with the following mobile phase conditions: 1-
3 min: 2.5% acetonitrile/41% water/56.5% methanol; 3-9 min a linear gradient was applied to reach 5% acetonitrile/25% water/72.5% methanol. This was maintained for a further 2 min before applying a linear gradient for 50 sec to reach the original solvent concentrations, which were maintained for a further 3 min. Metabolites were detected at 240 nm and quantified using authentic standards.

**Statistical methods** - Average rates of metabolism were calculated for each triplicate incubation of mouse liver microsomes from each genotype \((n = 3)\) and these data were then used to calculate \(p\) values using an Unpaired \(t\)-test.

**In vitro pharmacokinetics of metoprolol** – Five, ten week old male mice of each genotype were orally gavaged with metoprolol (2mg/kg). Blood samples were taken and the samples analyzed by LC-MS/MS as previously described (Finn et al., 2008). Pharmacokinetic parameters were calculated using a non-compartmental model (WinNonLin v4.1 (Pharsight, Munich, Germany)).
RESULTS

Phenotype of BCN mice- To establish the in vivo role(s) of microsomal cytochrome b\(_5\) we generated a global deletion of this gene in the mouse. Mice lacking microsomal cytochrome b\(_5\) were unexpectedly viable, born at expected Mendelian ratios and exhibited no gross anatomical abnormalities. BCN mice of both sexes were fertile, indicating that cytochrome b\(_5\) was not necessary for sexual maturation. The BCN mice however did display dry skin and an interesting fur condition where pups and young adult mice had a characteristically dull, sparse coat, with marked pilo-erection, along with a degenerative nurturing phenotype probably related to changes in milk composition. This phenotype was reflected in low neonatal body weights, which improved when the pups were weaned and became normal when the animals reached 6-8 weeks of age. A manuscript documenting these characteristics in detail is currently in preparation.

Analysis of cytochrome P450-dependent monooxygenase components – The cytochrome b\(_5\) content was measured in wild-type liver (253.7 ± 47.9 pmol/mg) (Figure 1), kidney (208.4 ± 15.1 pmol/mg) and small intestine microsomes (161.4 ± 16.8 pmol/mg). Cytochrome b\(_5\) levels were not detectable in the equivalent BCN samples. Total hepatic cytochrome P450 levels were significantly increased (2-fold) in the BCN mice as measured by Fe\(^{2+}\)-CO versus Fe\(^{2+}\) difference spectra (0.4 ± 0.07 vs. 0.9 ± 0.4 nmol P450/mg microsomal protein (\(p<0.05\)) (Figure 2A). To confirm that cytochrome b\(_5\) protein had been deleted, microsomes from liver, lung, kidney, small intestine and testes from 10 week old male mice were analyzed by Western blotting. Cytochrome b\(_5\) protein was undetectable in all tissues of the BCN mice studied (Figure 2B and 6D). To explain the increase in P450 content we carried out Western blotting analysis in order to establish which P450s isoenzymes were changed; microsomes of each genotype were analyzed for the expression of P450s, POR and cytochrome b\(_5\) reductase (Figure 1B,C). Interestingly, hepatic P450 expression was increased in BCN for members of
the Cyp2a, Cyp2b, Cyp2e and Cyp3a subfamily members with Cyp2b10 showing the most pronounced induction (17-fold; p<0.001) (Figure 2B,C). BCN hepatic POR levels showed a small (25%) but statistically significant increase which was reflected in an increase in the rate of reduction of the POR substrate cytochrome c as compared to wild-type (130.2 ± 9.7 vs. 74.8 ± 17.3 nmol cytochrome c reduced/min/mg respectively; p=0.008). The increase in cytochrome c reduction was greater than the increase in POR protein. The reason for this is unclear.

In extra-hepatic tissues, some changes in the levels of cytochrome P450 expression were observed; namely an 80% decrease in the expression of Cyp2e1 was observed in the kidney (p= 0.035), a 35% increase in the expression of Cyp2b (p= 0.004) in the lung, and in the small intestine a 70 % decrease (p= 0.003) in Cyp2d and 40% decrease in Cyp2e (p= 0.03) (Figure 2B). No changes in testicular P450 levels were observed (data not shown). The increases in hepatic cytochrome P450 expression were reflected in the mRNA levels for Cyp2a4, Cyp2b10, Cyp3a11 and Cyp4a10 (Figure 2D), indicating that the observed increases in protein expression were due to increases in gene transcription, or mRNA stabilization. In order to determine if the changes in P450 expression observed in BCN liver were present from birth hepatic P450 levels were measured in one day old pups (two from each genotype). As in the adult mice, Cyp2a, Cyp2b, Cyp3a levels were increased in neonatal BCN liver at this early time-point, while Cyp2c and Cyp2d protein was marginally repressed (Figure 2E).

In vitro cytochrome P450 activities – In vitro cytochrome P450 activities in microsomes from kidney, liver, lung and small intestine of wild-type and BCN mice were determined using a panel of probe substrates comprising alkoxy-resorufin and alkoxy-4-trifluoromethylcoumarin derivatives (Table 1). In the case of the alkoxy-resorufins, no differences in the hepatic metabolism of ER or MR between BCN and wild-type mice were observed, whereas the BCN samples exhibited a significantly higher rate of metabolism of BR (4.1-fold) and PR (2.4-
fold). These increases in turnover of PB and BR probably reflect the marked increase of Cyp2b10 expression, which is known to metabolise these compounds. Hepatic alkoxy-4-trifluoromethylcoumarin metabolism was significantly lower in the BCN samples (BFC 4.2-fold; EFC 5.5-fold and MFC 5.5-fold lower respectively). The changes in hepatic metabolism of MR and BFC were also reflected in the kinetic parameters (Table 2). All data was monophasic and followed standard Michaelis Menten kinetics, and in the case of MR metabolism there was little difference in either $K_m$ or $V_{\text{max}}$ between wild-type and BCN samples. However, deletion of cytochrome $b_5$ caused a 4.5-fold increase in $K_m$ and a 4.6-fold decrease in $V_{\text{max}}$ for the metabolism of BFC.

In the lung, metabolism of the alkoxy-resorufins was reduced to undetectable levels in the BCN samples, and with the three 4-trifluoromethylcoumarins metabolism was also profoundly reduced (BFC 29.8-fold; EFC 5.5-fold and MFC 115-fold lower). In the small intestine, BR, PR, BFC, EFC and MFC metabolism was detected in wild-type mice, but in BCN mice metabolism of BR and BFC was markedly reduced and the metabolism of EFC, MFC, MR and ER was reduced to undetectable levels. In contrast, the rate of PR metabolism in the intestine was increased in the BCN samples. No activity towards these probe substrates could be measured using either wild-type or BCN kidney microsomes. In liver microsomes from wild-type and BCN mice, NADH-dependent metabolism of BFC, EFC and MFC was approximately 25-30% that of the equivalent NADPH reactions in wild-type mice (BFC: 57 vs. 275 pmol/min/mg; EFC: 61 vs. 245 pmol/min/mg and MFC: 55 vs. 181 pmol/min/mg). However, no NADH-dependent metabolism could be detected in the BCN.

Effect of the addition of cytochrome $b_5$ – In order to establish whether the changes in monooxygenase activity could be directly attributable to the absence of cytochrome $b_5$, E. coli membranes containing recombinant murine cytochrome $b_5$ were added back to BCN liver microsomes. This caused a concentration-dependent increase in BFC activities, with the
highest concentration of added cytochrome \(b_5\) giving approximately a 12-fold higher turnover than BCN microsomes alone and a 2.3 fold increase above wild-type levels (Figure 3A). Addition of the highest concentration of cytochrome \(b_5\) to wild-type microsomes caused a comparatively small (19%) increase in activity. When this experiment was carried out using BR as a substrate (Figure 3B), no increase in wild-type activity was observed; however the activity in BCN microsomes (which was already approximately 2-fold more active towards BR than wild-type) was increased by 4.5-fold by cytochrome \(b_5\) addition, achieving a 7.8-fold increase over the control levels. When cytochrome \(b_5\) was added to wild-type lung microsomal incubations, activities were increased 1.5- and 7-fold for BFC and BR respectively (Figure 3C and 3D). Addition of the same amount of cytochrome \(b_5\) back to BCN lung microsomes resulted in a 7.4- fold increase in BFC turnover (Figure 3C) and the appearance of BR activity (Figure 3D). For both substrates these activities were equal to those observed when cytochrome \(b_5\) was added back to wild-type membranes. Similar experiments were performed using kidney microsomes with chlorzoxazone as substrate; in this case addition of cytochrome \(b_5\) to BCN microsomes brought the activity back approximately the levels seen in wild-type microsomes, while it did not increase the activities of wild-type microsomes (Rates (pmol/min/mg) – WT: 110 ± 5; BCN: 14 ± 1; WT + cytochrome \(b_5\): 96 ± 1 and BCN + cytochrome \(b_5\): 91 ± 2).

Metabolism of probe drugs – In addition to the use of model substrates, \textit{in vitro} assays were also performed with probe drug substrates (Figure 4). Chlorzoxazone 6-hydroxylation was significantly reduced in liver, lung and kidney by 4-, 2- and 5.5-fold respectively (Figure 4A). In the case of midazolam, the rate of production of the 1'- and 4-hydroxy-midazolam metabolites was also much lower in the BCN samples, reaching statistical significance in the liver and kidney for 1'-hydroxy-midazolam production, and in the liver for midazolam 4-hydroxylation (Figure 4B). Metoprolol O-demethylation was significantly reduced in
cytochrome \( b_5 \) null liver and kidney samples, while no effect was observed in the lung or small intestine samples. 1'-Hydroxy-metoprolol was detected in liver, kidney and small intestine incubations but not in the lung samples. Similarly to O-desmethyld metoprolol production, formation of this metabolite was significantly decreased in the liver and kidney samples of BCN mice (Figure 4C). In contrast to the above substrates, metabolism of phenacetin to acetaminophen was significantly increased in the livers of BCN mice (p<0.05) while in the kidney and lung, ablation of cytochrome \( b_5 \) had no effect on activity (Figure 4D). In the case of tolbutamide hydroxylation, activity was only detected in liver and kidney microsomes; however, in both cases BCN samples showed lower activity compared to controls. The reduction being significant in the kidney (Figure 4E).

*In vivo Metoprolol pharmacokinetics* – In order to determine if the observed decreases in *in vitro* cytochrome P450 metabolism were reflected *in vivo*, the pharmacokinetics of orally administered metoprolol were determined in wild-type and BCN mice. The elimination profiles of metoprolol disappearance were significantly different between wild-type and BCN mice (Figure 5). The AUC and terminal half-life of metoprolol were significantly increased (3.5- and 2.4-fold respectively) and clearance decreased (3.5-fold) in the BCN animals compared to wild-type. Furthermore, although not statistically significant, the metoprolol \( C_{\text{max}} \) values were also increased in the BCN (4-fold). Further studies have been carried out which show a clear effect of cytochrome \( b_5 \) deletion on the pharmacokinetics of a range of drugs. These data will form the basis of a further manuscript.

*Steroid hormone metabolism and testosterone levels* – Cytochrome \( b_5 \) has been reported to be essential for the conversion of 17\( \alpha \) hydroxy-progesterone to androstenedione (Akhtar et al., 2005). In order to study the pathway of testosterone biosynthesis, we measured progesterone metabolism using testicular microsomes from wild-type and BCN mice. A significant 2-fold reduction in the rate of metabolism was measured in the absence of microsomal cytochrome
When $17\alpha$ hydroxy-progesterone was used as a substrate to investigate the effect of cytochrome $b_5$ deletion on Cyp17-lyase activities, testicular BCN microsomes had almost no activity, being 100-fold lower than wild-type controls ($p < 0.001$) (Figure 6A). Since Cyp17 and $17\beta$-hydroxysteroid dehydrogenase ($17\beta$HSD) are both microsomal NADPH-dependent enzymes (Fig 6C), incubations with $17\alpha$ hydroxy-progesterone also yielded the metabolite testosterone (produced by the metabolism of androstenedione by $17\beta$HSD (Figure 6C)). Testicular microsomes from wild-type mice were 66-fold more active than BCN in the production of testosterone from androstenedione formed by the Cyp17 (Fig 6A). A reflection of this reduction in levels of testosterone was also observed in vivo, as when testes from wild-type and BCN mice were analyzed for testosterone content, BCN samples contained significantly less testosterone than wild-type (approximately 1.8 fold) (Figure 6B). There were no compensatory alterations in the levels of Cyp17 protein expressed in the testes of cytochrome $b_5$ null mice (Figure 6D).
DISCUSSION

Due to the large number of functions associated with cytochrome $b_5$, which include fundamental processes such as steroid hormone biosynthesis and haemoglobin homeostasis (Porter, 2002; Schenkman and Jansson, 2003), it would be predicted that the deletion of this enzyme could be lethal. Unexpectedly, this was not the case, with BCN pups produced at expected Mendelian ratios with no gross anatomical abnormalities. In addition, no overt reduction in fertility was observed in either sex. Interestingly however, several phenotypic characteristics were identified including skin defects closely resembling the human condition Ichthyosis Vulgaris, which were exacerbated in breeding females on the birth of successive litters.

Until the publication of our recent paper detailing the conditional hepatic deletion of microsomal cytochrome $b_5$ (Finn et al., 2008), investigation of the role of cytochrome $b_5$ in drug metabolism was based on in vitro studies (Gruenke et al., 1995; Yamazaki et al., 1996; Yamazaki and Shimada, 2006) or heterologous over-expression of proteins (Patten and Koch, 1995). The BCN model therefore allows the role of cytochrome $b_5$ in the in vitro as well as in vivo metabolism of drugs to be established.

Complete deletion of cytochrome $b_5$ as evidenced by spectral analysis (Figure 1) and Western blotting (Figure 2B) and, resulted in significant increase in hepatic expression of Cyp2a, Cyp2b and Cyp3a proteins. The presence of multiple bands in the Western blots for Cyp2a, Cyp2b, Cyp2c and Cyp4a reflect the multiplicity of the proteins within each gene sub-family in the mouse, and it would appear that cytochrome $b_5$ deletion differentially affects the expression of these multiple forms. Which specific members of these gene sub-families are upregulated is the subject of ongoing investigations. The increases in cytochrome P450 content were already apparent from birth and were mirrored by increases in mRNA expression suggesting a transcriptional mechanism. A similar increase in P450 expression
was also observed when hepatic POR was conditionally (Gu et al., 2003; Henderson et al., 2003) which in this latter case, appears to be due to activation of the constitutive androstane receptor and, to a lesser degree, the pregnane X receptor by accumulated polyunsaturated fatty acids (Finn et al., 2009). No such increases were found in BCN mice which unlike the HRN mice, did not have a fatty liver, indicating that the activation is the result of an alternative metabolic pathway.

Investigation of the in vitro metabolism of cytochrome P450 substrates produced some unexpected and interesting results (Table 1). NADH-mediated metabolism was undetectable in BCN mice, demonstrating that cytochrome \( b_5 \) is an unequivocal electron donor to cytochrome P450s. For NADPH-dependent metabolism, BCN hepatic microsomes exhibited the same magnitude of decrease in the metabolism of the alkoxy-trifluoromethylcoumarins as previously observed in HBN mice (Finn et al., 2008); however, there were some differences between these models with alkoxy-resorufin metabolism. Turnover of MR and ER in BCN hepatic microsomes was essentially unchanged from the rates observed in wild-type, while in the HBN microsomes, ER O-deethylase activity was approximately 26% lower (Finn et al., 2008). This difference could be explained by the finding that compensatory changes in cytochrome P450 gene expression in BCN mice were not observed in HBN animals, therefore the ratio of different P450s to each other as well as their absolute levels is different between the lines. These data suggest that cytochrome \( b_5 \) plays little role in the metabolism of Cyp1a substrates. In support of this, the metabolism of BR and PR, traditionally attributed to Cyp2b enzymes, was increased 4.1- and 2.4-fold, respectively, correlating very well with the markedly higher levels of hepatic Cyp2b expression in BCN samples (Figure 2B,C). In the HBN model, BR was metabolized significantly slower (30%) of the wild-type rate (Finn et al., 2008). Indeed, Cyp2b10 levels were elevated 17-fold in BCN liver which would therefore equate to a predicted maximal 5.1-fold increase in turnover (17 x 30% activity), close to the
figure in this study (4.1-fold). The above data are interesting in that in the conditional HBN model the effects of cytochrome b₅ deletion are reflected in changes in the activities of constitutively expressed cytochrome P450 enzymes, while in the BCN mice the consequence of this deletion are observed in both constitutive and inducible enzymes. It will be interesting to establish the impact of cytochrome b₅ deletion following pre-treatment with P450 inducers such as phenobarbital, where it may be possible to normalize the level of P450 expression.

The activities of BCN lung microsomes towards the fluorogenic probes were more markedly reduced by cytochrome b₅ deletion than the hepatic samples, showing either a complete ablation or profound reduction in turnover. Cytochrome b₅ therefore appears to differentially affect the activity of cytochrome P450s in different tissues. Adding back exogenous cytochrome b₅ to hepatic and pulmonary BCN incubations restored turnover to wild-type levels and then further stimulated activities in a concentration-dependent manner for both BR and BFC activity, resulting in activities 2.3- and 7.8-fold higher than wild-type for BFC and BR, respectively. This “super-activation” of turnover can be attributed to the increased levels of Cyp2b (BR and BFC activity), and Cyp2a and Cyp3a (BFC) in the livers of BCN animals (Figure 2B,C). Furthermore, addition of exogenous cytochrome b₅ to wild-type lung microsomes, unlike hepatic microsomes, significantly stimulated turnover; most markedly during BR O-dealkylation (6.8-fold). Together, these data suggest that, in the lung, cytochrome b₅ is a key component of the cytochrome P450 monooxygenase system (Croft et al., 1986), the constitutive levels of which for the metabolism of some substrates are rate limiting.

When the effect of cytochrome b₅ deletion was investigated using a set of probe drugs, hepatic activities for chlorzoxazone (Cyp2e), midazolam (Cyp2c, Cyp3a), metoprolol (Cyp2d) and tolbutamide (Cyp2c) were markedly reduced (Figure 4), indicating that cytochrome b₅ is important for maximal turnover of these substrates. The metabolism of
phenacetin, which is chiefly mediated by Cyp1a2, was either unaffected (kidney, lung) or increased (liver) in response to cytochrome b\textsubscript{5} deletion again suggesting that cytochrome b\textsubscript{5} is not important in Cyp1a2 function. Interestingly, cytochrome b\textsubscript{5} deletion in the small intestine also caused a reduction in turnover of midazolam and metoprolol, indicating that perturbations in gut cytochrome b\textsubscript{5} levels may have a bearing on the bioavailability of orally-dosed drugs.

To investigate more fully the mechanism of cytochrome b\textsubscript{5} interaction with cytochrome P450, the kinetic parameters of MR, which is metabolized by Cyp1a2, and is unaffected by cytochrome b\textsubscript{5} (Table 1) and BFC, which is metabolized by multiple P450s (Cyp3a, Cyp2a Cyp2b, Cyp2c) and is significantly affected by cytochrome b\textsubscript{5}, were determined. The $K_m$ and $V_{max}$ for MR were not markedly different between BCN and wild-type, while for BFC there was a 5-fold increase in $K_m$ and a 4.5-fold decrease in $V_{max}$ in the BCN samples (Table 2). The alteration in $K_m$ for BFC provides evidence to support theory that cytochrome b\textsubscript{5} effects P450 activity through allosteric interactions with the metabolising cytochrome P450s, causing a conformational shift in the active site, which affects substrate binding (Bridges et al., 1998; Hlavica and Lewis, 2001). Alternatively, the change in $K_m$ observed for BFC could be attributable to multiple P450s, differentially upregulated by the deletion of cytochrome b\textsubscript{5} contributing to the metabolism of this substrate. There is evidence to support the binding of cytochrome b\textsubscript{5} to mammalian CYP1A2 (Shimada et al., 2005), therefore the lack effect on the $K_m$ for MR in the BCN samples suggests that cytochrome b\textsubscript{5} binding does not significantly alter Cyp1a2 active site conformation. This is supported by evidence which indicates that unlike enzymes such as CYP3A4 and CYP2B4, CYP1A2 has an extremely stable and inflexible active site (Anzenbacher and Hudecek, 2001).

The generation of global and liver-specific models for cytochrome b\textsubscript{5} deletion now allows the contribution of this protein in extra-hepatic and hepatic P450 drug metabolism to
be assessed in greater detail. We have previously shown that hepatic deletion of cytochrome $b_5$ did not make a significant difference to the *in vivo* metabolism of metoprolol (Finn et al., 2008). In contrast, determination of the *in vivo* pharmacokinetic parameters of metoprolol in BCN mice provides evidence that extra-hepatic tissues play a key role in its metabolism, as in this model the AUC and $C_{\text{max}}$ are increased 3.5- and 4-fold, respectively, and clearance is decreased by 3.5-fold compared to wild-type mice (Figure 5). In support of this, it has recently been shown that in mouse, intestinal P450s play important roles in drug pharmacokinetics (van Herwaarden et al., 2009). These data reinforce our previous conclusions that the level and/or the activity of cytochrome $b_5$ can play a significant role in the metabolism, disposition and therapeutic effectiveness of clinically used drugs.

A particularly well-characterised role for cytochrome $b_5$ is in steroid hormone biosynthesis, where it enhances the lyase activity of Cyp17 through an allosteric mechanism while having little effect on the hydroxylase activity (Akhtar et al., 2005; Auchus et al., 1998; Naffin-Olivos and Auchus, 2006). Testicular Cyp17 activity was severely compromised in BCN samples not only for the lyase reaction (1% of wild-type), but also in progesterone hydroxylation (50% of the wild-type rate) (Figure 6A). In addition BCN mice had a significantly lower concentration of testicular testosterone (Figure 6B), with no change in levels of Cyp17 protein observed (Figure 6D). It appears therefore, that although Cyp17 lyase activity is almost completely cytochrome $b_5$ dependent, the presence of cytochrome $b_5$ is not an obligatory requirement for successful testosterone biosynthesis.

In summary, we have generated a mouse model in which microsomal cytochrome $b_5$ is completely deleted, and characterised the effect on cytochrome P450 expression and metabolism both *in vitro* and *in vivo*. The data presented provides evidence that cytochrome $b_5$ can have a marked effect on the rate of substrate turnover, and particularly in extra-hepatic in tissues such as the small intestine and lung, where it can be rate-limiting. Cytochrome $b_5$
deletion profoundly alters the *in vitro* activity of Cyp17; however, this results in only a halving of testicular testosterone levels, which has no detrimental effect on reproductive ability. The BCN model provides an important tool which, in conjunction with the HBN mouse (Finn et al., 2008), will further our understanding of the many and varied physiological roles of this protein. In addition, it is known that levels of cytochrome *b*$_5$ in man are subject to significant variation (Forrester et al., 1992) which could contribute to individual differences in drug pharmacokinetics.
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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Quantitation of microsomal cytochrome b₅ by difference spectrophotometry
Traces illustrate typical cytochrome b₅ difference spectra obtained using hepatic microsomal fractions from Cytb₅⁺/⁺ (WT) and Cytb₅⁻/⁻ (BCN) mice. Assays were performed as described in Materials and Methods.

Figure 2. Characterization of cytochrome P450 monooxygenase components in cytochrome b₅ null mice
A. CO difference spectra in hepatic microsomal fractions of Cytb₅⁺/⁺ (WT) (solid line) and Cytb₅⁻/⁻ (BCN) (dashed line) hepatic microsomes. Samples were run as described in Materials and Methods. B. Immunoblot analysis of liver, kidney, lung and small intestine microsomes (20 μg protein per lane) from Cytb₅⁺/⁺ (WT) and Cytb₅⁻/⁻ (BCN) mice, as detailed in Materials and Methods. C. Densitometric analysis of liver immunoblot data (from B). Values are expressed as fold change normalised to wild-type expression. Bars are mean ± S.D. from three samples of each genotype. Hatched bars represent wild-type and white bars BCN values. D. Quantification of hepatic mRNA changes by Taqman analysis. Data is normalised to 18s expression and expressed as fold change relative to wild-type levels. Bars are mean ± S.D. from three samples of each genotype. Hatched bars represent Cytb₅⁺/⁺ (wild-type) and white bars Cytb₅⁻/⁻ (BCN) values. E. Immunoblot analysis of cytochrome P450-dependent monooxygenase components in liver microsomes (20 μg protein per lane) from one day old Cytb₅⁺/⁺ (WT) and Cytb₅⁻/⁻ (BCN) pups as detailed in Materials and Methods. (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001)
Figure 3. The effect of exogenous cytochrome b₅ addition on microsomal cytochrome P450 activities

A and B. Activities of Cytb₅⁺/⁺ (WT) and Cytb₅⁻/- (BCN) liver microsomes towards 7-benzyloxy-4-trifluoromethylcoumarin (A) and benzyloxy-resorufin (B) in the absence and presence of increasing concentrations of recombinant cytochrome b₅ (3.3, 6.6, 13.2 and 26.4 pmol of cytochrome b₅ per incubation respectively). Samples were treated as detailed in Materials and Methods, values are presented as mean ± SD from triplicate incubations. C and D. Activities of Cytb₅⁺/⁺ (WT) and Cytb₅⁻/- (BCN) lung microsomes towards 7-benzyloxy-4-trifluoromethylcoumarin (C) and benzyloxy-resorufin (D) in the absence and presence of recombinant cytochrome b₅ (26.4 pmol per incubation). Samples were treated as described in Materials and Methods, and data presented as mean ± S.D. from triplicate incubations.

Figure 4. Probe drug metabolism in microsomes from wild-type and cytochrome b₅ null mice.

Assays were performed in triplicate on microsomes from three mice of each genotype, as described in Experimental Procedures. A. Chlorzoxazone 6-hydroxylase, B. Midazolam 1'- (1'-OH Midaz) and 4-hydroxylase (4-OH midaz), C. Metoprolol O-demethylase (O-DM Metop) and 1-hydroxylase (1-OH Metop), D. Phenacetin O-deethylase and E. Tolbutamide hydroxylase activities. Hatched bars represent Cytb₅⁺/⁺ (wild-type) and white bars Cytb₅⁻/- (BCN) samples. Mean values are presented ± S.D. Lv = liver; Ln = lung; K = kidney and SI = small intestine. (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001)

Figure 5. In vivo pharmacokinetic profiles and parameters of the Cytochrome P450 substrate metoprolol in wild-type and cytochrome b₅ null mice
Metoprolol was administered orally to *Cytb*<sup>+/−</sup> (wild-type) and *Cytb*<sup>−/−</sup> (BCN) mice at a dose of 2mg/kg. The data shown represents the mean ± S.E.M. of metoprolol blood concentrations at the individual time points with 5 animals per group (Wild type: black circles and BCN: white circles). Samples were analyzed and pharmacokinetic parameters were calculated as described in Methods. (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001)

**Figure 6. The effect of cytochrome b<sub>5</sub> deletion on steroid hormone metabolism**

A. Assays were performed in triplicate using testicular microsomes from three mice of each genotype as described in Experimental Procedures. Activities were determined using either progesterone (Prog) or 17α hydroxy progesterone (17α OH prog) as substrates. Hatched bars (*Cytb*<sup>+/−</sup> (WT)) and white bars (*Cytb*<sup>−/−</sup> (BCN)) represent the respective levels of metabolite production (Andro = androstenedione and Test = testosterone). Each bar represents mean ± S.D. (* = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001). B. Intra-testicular testosterone content of *Cytb*<sup>+/−</sup> (wild-type) (hatched bars) and *Cytb*<sup>−/−</sup> (BCN) (white bars) mice. Bars represent the respective levels (mean ± SD) for three mice of each genotype expressed per mg weight of tissue. C. Pathway of NADPH-dependent testosterone formation from progesterone. D. Analysis of Cytochrome b<sub>5</sub> and Cyp17 levels by western blotting (20 μg protein per lane) from *Cytb*<sup>+/−</sup> (WT) and *Cytb*<sup>−/−</sup> (BCN), for the expression of Cyp17.
Table 1. Effect of cytochrome b5 deletion on cytochrome P450 activities

For liver and small intestine samples, assays were performed in triplicate on three individual mouse samples and data shown is mean ± S.D. for three mice. Due to the poor yield of microsomes from mouse lung, assays were performed in triplicate on a pool of three lung preparations where data presented is mean ± S.D. of the triplicate determinations, precluding statistical analysis. Percentage change is shown in brackets for the BCN samples Kidney microsomes were also tested but no activities were detected.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Liver Activity (pmol/min/mg)\textsuperscript{a}</th>
<th>Lung Activity (pmol/min/mg)\textsuperscript{a}</th>
<th>Small intestine Activity (pmol/min/mg)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR</td>
<td>3.8±1.2</td>
<td>15.7±1.7 (413%\textsuperscript{***})</td>
<td>15.8±2.3 n.d.</td>
</tr>
<tr>
<td>ER</td>
<td>10.1±1.9</td>
<td>9.3±3.7 (92%)</td>
<td>2.4±0.3 n.d.</td>
</tr>
<tr>
<td>MR</td>
<td>52.5±14.4</td>
<td>51.0±27.0 (97%)</td>
<td>0.4±0.2 n.d.</td>
</tr>
<tr>
<td>PR</td>
<td>2.2±0.2</td>
<td>5.2±1.2 (240%\textsuperscript{*})</td>
<td>0.6±0.4 n.d.</td>
</tr>
<tr>
<td></td>
<td>BFC</td>
<td>EFC</td>
<td>MFC</td>
</tr>
<tr>
<td>---</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
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<tr>
<td></td>
<td>274.9±46.0</td>
<td>244.6±59.0</td>
<td>181.3±47.6</td>
</tr>
<tr>
<td></td>
<td>65.1±6.2 (24%)**</td>
<td>30.5±5.4 (12%)**</td>
<td>32.9±6.9 (18%)*</td>
</tr>
<tr>
<td></td>
<td>44.7±4.4</td>
<td>45.7±10.7</td>
<td>89.7±4.0</td>
</tr>
<tr>
<td></td>
<td>1.5±1.7 (3%)</td>
<td>8.2±1.2 (18%)</td>
<td>0.78±1.3 (1%)</td>
</tr>
<tr>
<td></td>
<td>17.5±20.0</td>
<td>3.8±6.6</td>
<td>20.1±25.4</td>
</tr>
<tr>
<td></td>
<td>0.05± (0.3%)</td>
<td>n.d</td>
<td>n.d</td>
</tr>
</tbody>
</table>

*For BR, ER, MR and PR, metabolite formed is resorufin, and for BFC, EFC and MFC is 7-hydroxy-4-trifluoromethylcoumarin.*

**= p ≤ 0.05, ***= p ≤ 0.01; n.d. = not detected
Table 2. Kinetic parameters for hepatic microsomal methoxyresorufin (MR) and 7-benzyloxy-4-trifluoromethylcoumarin (BFC) metabolism in $Cytb_5^{+/+}$ (wild-type) and $Cytb_5^{-/-}$ (BCN) liver microsomes.

Assays were performed using triplicate samples for each concentration of substrate as described in the Experimental Procedures section. Standard deviations given are from the fit of the curve as calculated using the Michaelis Menten equation (GraFit version 5 (Erithacus Software, Horley, UK)).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pmol/min/mg)</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MR</td>
<td>0.039 ± 0.001</td>
<td>35.5 ± 0.3</td>
<td>0.054 ± 0.003</td>
<td>31.9 ± 0.5</td>
</tr>
<tr>
<td>BFC</td>
<td>3.4 ± 0.9</td>
<td>410 ± 26</td>
<td>15.8 ± 2.4</td>
<td>91.8 ± 4.7</td>
</tr>
<tr>
<td>BCN</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
Figure 5

The figure shows a graph plotting concentration (μg/ml) against time (min) for different conditions. The graph includes error bars to indicate variability.

A table below summarizes the pharmacokinetic (PK) parameters for Wild-Type and BCN conditions:

<table>
<thead>
<tr>
<th>PK Parameter</th>
<th>Wild-Type</th>
<th>BCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (min*μg/ml)</td>
<td>0.22 ± 0.05</td>
<td>0.76 ± 0.24 *</td>
</tr>
<tr>
<td>Cmax (μg/ml)</td>
<td>0.002 ± 0.001</td>
<td>0.008 ± 0.003</td>
</tr>
<tr>
<td>Clearance (L/min/kg)</td>
<td>8.37 ± 0.95</td>
<td>2.36 ± 0.53 ***</td>
</tr>
<tr>
<td>Terminal half-life (min)</td>
<td>146 ± 30</td>
<td>350 ± 66 *</td>
</tr>
</tbody>
</table>

Values represent the mean ± S.E.M. where n = 5.

* indicates a statistically significant difference compared to the Wild-Type condition.

*** indicates a statistically significant difference compared to the Wild-Type condition.
Figure 6
Correction to “Deletion of Microsomal Cytochrome b₅ Profoundly Affects Hepatic and Extrahepatic Drug Metabolism”

In the above article [McLaughlin LA, Ronseaux S, Finn RD, Henderson CJ, and Wolf CR (2010) Mol Pharmacol 78:269–278; doi:10.1124/mol.110.064246], the metoprolol pharmacokinetic data in the BCN mouse line described in this article was generated from a protocol involving the administration of a cocktail of P450 probe substrates rather than the single drug alone. The corrected information, from the Materials and Methods section, appears below.

Five 10-week-old male mice of each genotype were orally gavaged with a five-drug cocktail comprising phenacetin (5 mg/kg), tolbutamide (5 mg/kg), metoprolol (2 mg/kg), chlorzoxazone (5 mg/kg), and midazolam (5 mg/kg) dissolved in a vehicle consisting of 5% ethanol, 5% DMSO, 35% polyethylene glycol 200, 40% phosphate-buffered saline, and 15% water) as previously described (Finn et al., 2008). Blood samples were taken, and the samples were analyzed by LC/MS-MS, as described previously (Finn et al., 2008). Pharmacokinetic parameters were calculated using a noncompartmental model (WinNonLin version 4.1; Pharsight, Munich, Germany).

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The authors regret any inconvenience this error may cause.