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Characterization of Mice Lacking the Multidrug Resistance Protein Mrp2 (Abcc2)

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Mrp2 knockout mice

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ABC-transporter, ATP Binding Cassette transporter; MRP, multidrug resistance protein; DBSP, dibromosulfophthalein; DJS, Dubin-Johnson syndrome; TR^- , Transporter deficient rats; EHBR, Eisai hyperbilirubinemic rats; EROD, ethoxyresorufin *O*-dealkylation; PROD, pentoxyresorufin *O*-dealkylation; HT, hydroxytestosterone.

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Abstract

The multidrug resistance protein Mrp2 is an ABC-transporter mainly expressed in liver, kidney, and intestine. One of the physiological roles of Mrp2 is to transport bilirubin glucuronides from liver into the bile. Current *in vivo* models to study Mrp2 are the TR^- and EHBR rat strains. Previous reports showed hyperbilirubinemia and induction of Mrp3 in the hepatocyte sinusoidal membrane in the mutant rats. In addition, differences in liver cytochrome P450 and UGT1a levels between wild type and mutant rats were detected. To study whether these compensatory mechanisms were specific to rats, we characterized $Mrp2^{-/-}$ mice. Functional absence of Mrp2 in the knockout mice was demonstrated by showing increased levels of bilirubin and bilirubin glucuronides in serum and urine, reduction in biliary excretion of bilirubin glucuronides and total glutathione, and reduction in the biliary excretion of the Mrp2 substrate dibromosulfophthalein. To identify possible compensatory mechanisms in $Mrp2^{-/-}$ mice, the expression level of 98 phase I, -II and transporter genes was compared in liver, kidney and intestine of male and female $Mrp2^{-/-}$ and control mice. Unlike in Mrp2 mutant rats, no induction of Mrp3 in $Mrp2^{-/-}$ mice was detected. However, Mrp4 mRNA and protein in liver and kidney were increased ~6- and 2-fold, respectively. Phenotypic analysis of major cytochrome P450-mediated activities in liver microsomes did not show differences between wild type and $Mrp2^{-/-}$ mice. In conclusion, $Mrp2^{-/-}$ mice are a new valuable tool to study the role of Mrp2 in drug disposition.

Introduction

Several members of the ATP-binding cassette (ABC) superfamily of transporters are involved in the transport of both endogenous and xenobiotic compounds from major organs in the body (Borst and Oude Elferink, 1992). An example is the multidrug resistance protein 2 (MRP2 or ABCC2). MRP2 protein has mainly been detected in the apical plasma membrane of hepatocytes, kidney proximal tubules and intestine (Paulusma et al., 1997; Mottino et al., 2001; Schaub et al., 1999). In addition, MRP2 is localized in lung, gallbladder, and placental trophoblasts (St-Pierre et al., 2000; Rost et al., 2001; Konig et al., 2003).

The substrate specificity of MRP2 is broad and includes glutathione-, glucuronide- and sulfate conjugates of many drugs, some non-conjugated organic anions, and various neutral or positively charged drugs (Konig et al., 2003). Known high affinity physiological substrates of MRP2 are glucuronides of the hemoglobin breakdown product bilirubin (Kamisako et al., 1999).

In humans, mutations in the MRP2 gene can result in the autosomal recessive Dubin-Johnson syndrome (DJS). The primary defect in patients with DJS is a mild conjugated hyperbilirubinemia, caused by the impaired hepatobiliary transport system of non-bile salt organic anions across the canalicular membrane (Oude Elferink and Groen, 2002). Although in general DJS is an extremely rare disorder, it is relatively frequent in Iranian Jews, where the prevalence in 1:1300. Several mutations have been reported in patients with DJS, resulting in absence of MRP2 or intracellular trafficking defects (Suzuki and Sugiyama, 2002).

The animal models for DJS are the Groningen Yellow (GY)/Transporter deficient (TR^-) and Eisai Hyperbilirubinemic (EHBR) rat strains, spontaneous mutants from the Wistar and Sprague-Dawley strains, respectively (Jansen et al., 1985; Hosokawa et al., 1992). The Mrp2 mutation in the TR^- rats is a single nucleotide deletion leading to a

frameshift and a stop codon (Paulusma et al., 1996). In EHBR rats, a one nucleotide substitution results in a stop codon (Ito et al., 1997). Both mutant rat strains have been extensively characterized and main differences between the mutant and wild type rats are a decreased bile flow, increased plasma levels of bilirubin glucuronides, a strong reduction in the biliary secretion of reduced glutathione, and a range of glutathione-, glucuronide-, and sulfate conjugates, and unconjugated compounds (Oude Elferink et al., 1995; Konig et al., 2003).

The phenotype of patients with DJS and Mrp2 deficient rats is relatively mild and life expectancy is not significantly affected (Oude Elferink et al., 1995). This is most likely explained by the strong induction of MRP3 (ABCC3) in hepatocytes under conditions where the function of MRP2 is impaired (Hirohashi et al., 1998; Konig et al., 1999). MRP3 is localized in the sinusoidal membrane and, recently, it has been shown that bilirubin glucuronides are substrates for human MRP3 (Lee et al., 2004). In the absence of a functional MRP2, MRP3 compensates for the lack of MRP2-mediated transport by exporting at least part of the intra-hepatically formed bilirubin glucuronides into the blood. In normal human liver, MRP3 protein was mainly detected in the bile ducts and not in hepatocytes (Scheffer et al., 2002). In addition, MRP3 protein was found in adrenal gland, kidney and intestine.

Analysis of TR^- and EHBR rats has resulted in a wealth of information regarding the function and substrate specificity of Mrp2 and has demonstrated the importance of this transporter in the biliary and renal elimination of many drugs, like for example pravastatin and SN38 (an active metabolite of irinotecan) (Chu et al., 1997; Yamazaki et al., 1997). Interpretation of data obtained with TR^- and EHBR rats was in some cases complicated by the induction of Mrp3, UGT1a and significant differences in cytochrome P450 enzyme levels between wild type and mutant strains (Jager et al., 1998; Newton et al., 2005; Johnson et al., 2005). The availability of additional *in vivo* models to study the

role of Mrp2 in the pharmacokinetics of drugs would therefore be desirable. The goal of this report was to characterize mice with a homozygous disruption of the Mrp2 gene. Differences between the knockout mice and TR^- and EHBR rat strains are discussed.

Methods

Animals

Mrp2$^{-/-}$ mice were generated by Deltagen, Inc. (San Carlos, CA). Briefly, the Mrp2 gene was inactivated by homologous recombination in ES cells derived from the 129/OlaHsd mouse substrain by deletion of nucleotides 1886-1897 of the coding sequence of the Mrp2 gene and introduction of a *LacZ-Neo* cassette. F1 mice were generated by breeding with C57BL/6 females. Heterozygous offspring of the F1 generation was used for re-derivation by embryo transfer at Taconic Farms (Germantown, NY). Post-derivation, mice were backcrossed for eight generations to a Taconic C57BL/6 background. Mice used in this manuscript were from heterozygote x heterozygote crosses of F2 mice. All mice were maintained at Taconic in a sterile isolator and monitored to verify their germfree/defined flora condition. Animals were kept in a temperature controlled environment with a 12 h light/12 h dark cycle. Animals received food and water *ad libitum*. All animal handling was according to Animal Procedure Statements approved by the Merck Rahway Institutional Animal Care and Use Committee.

Mice were genotyped by PCR analysis of purified tail DNA. Wild type Mrp2 was detected with oligo nucleotides GS(E) 5'gttcagcacctaacagaggtgattg3' and GS(E, T) 5'tcctgaggaagagctggaaagcaag3', and the knock-out allele was detected with oligonucleotides NEO(T) 5'gggtgggattagataaatgcctgctct3' and GS(E, T), resulting in fragments of 237 and 502 bp, respectively (see Figure 1A).

Analysis of serum and urine

Male and female Mrp2$^{-/-}$ and wild type mice (between 20-24 weeks of age) were used in this study. The animals were fasted overnight with free access to water. Blood samples were taken by cardiac puncture. The serum samples obtained were used for analysis. Urine samples were collected overnight in metabolic cages with three mice per

cage with three to four groups for each study. During urine collection, samples were kept on ice and in the dark. Serum and urine chemistry parameters listed in Tables 1 and 2 were determined using a Hitachi 911 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN). Sodium, potassium and chloride levels were determined using ion-specific electrodes. Other tests were performed by standard biochemical methods.

Analysis of bilirubin glucuronides and total glutathione

Male Mrp2$^{-/-}$ and wild type mice (between 20-24 weeks of age) were used in this study. The animals were fasted overnight with free access to water. Mice were anesthetized via isoflurane inhalation, administered at a rate of 2% in oxygen at 0.5 L/min during the surgical procedure. The bile duct was catheterized and the gallbladder was ligated and removed. After a recovery period of 1-3 hrs, the bile samples were collected on ice at 0-15, 15-30, 30-45, and 45-60 min. For measurement of bilirubin glucuronides, the bile samples were collected in the dark and immediately immersed in liquid nitrogen until analysis. Direct bilirubin (bilirubin glucuronides) in bile were analyzed with a Stanbio direct bilirubin assay kit (Stanbio Laboratory, Boerne, Texas). Total glutathione (reduced plus oxidized) in bile was measured using a Glutathione Assay Kit (Sigma Chemical Co., St. Louis, MO). Glutathione in liver extracts and plasma was determined using the same kit.

Western blotting

Crude plasma membranes were prepared from mouse liver and kidney homogenates as described previously (Kobayashi et al., 1990). Protein was separated in a 7.5% denaturing polyacrylamide gel. Gels were immuno-blotted onto nitrocellulose membranes. Blotting efficiency was assessed by staining blots with Ponceau S. Mouse Mrp2 was detected with monoclonal antibody M_2III-5 (Kamiya Biomedical Company, Seattle, WA), and mouse Mrp4 with M_4I-10 (Alexis Corp., San Diego, CA). Anti-mouse and anti-rat HRP-labeled secondary antibodies were from Amersham Corp. (Arlington

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Heights, IL). Blots were developed with the enhanced chemiluminescence (ECL) kit (Amersham Corp., Piscataway, NJ). ECL signals were detected with a GeneGnome System (Syngene, Frederick, MD), and signals were quantified using Gene Tools Software version 3.04 (Syngene).

Quantitative RT-PCR

RNA was isolated from tissues of Mrp2$^{-/-}$ and wild type male and female mice (20-24 weeks of age) with three animals per group. RNA was purified with a Qiagen RNeasy Midi kit (Qiagen Inc., Valencia, CA) according to instructions provided by the manufacturer. A two-step RT-PCR reaction was conducted by reverse transcribing an aliquot of total RNA (~500 ng) to cDNA using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). cDNA reactions from three animals in each group were pooled. PCR reactions were then prepared by adding cDNA to a reaction mixture containing TaqMan 2x Universal PCR Master Mix (Applied Biosystems). Low-Density Microarrays (Microfluidic Cards) were custom made by Applied Biosystems and contained probes in duplicate for the detection of 95 genes (see Tables 4-1 and 4-2). Samples were applied to Low-Density Microarrays by centrifugation twice for 1 min at 1200 g. RT products from the pooled cDNAs of three animals were analyzed in three independent experiments. Real-time quantitative PCR was performed using an ABI PRISM 7900 Sequence Detector instrument and Sequence Detector 2.1 Software (Perkin Elmer Inc. Shelton, CT). Quantitation of the target cDNAs in all samples was normalized to 18S ribosomal RNA ($Ct_{target} - Ct_{18S} = \Delta Ct$), and the difference in expression for each target cDNA in the Mrp2$^{-/-}$ mice was expressed to the amount in the wild type mice ($\Delta Ct_{wildtype} - \Delta Ct_{Mrp2-/-} = \Delta\Delta Ct$). Fold changes in target gene expression were determined by taking 2 to the power of this number ($2^{-\Delta\Delta Ct}$).

Liver microsomal cytochrome P450 enzyme activities

Microsomes from mouse livers were prepared as described previously (Newton et al., 2005), resuspended in 10 mM potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose, and stored at -80^{o}C until use. Protein concentrations were measured using the bicinchoninic acid procedure. Ethoxyresorufin *O*-dealkylation (EROD) and pentoxyresorufin *O*-dealkylation (PROD) activities were measured as described with some modifications (Newton et al., 2005). Testosterone hydroxylation activity was determined by incubating microsomes (0.25 mg/ml) with substrate (250 μM) in potassium phosphate buffer (100 mM, pH 7.4) with $MgCl_2$ (3 mM), and an NADPH-generating system. Incubations were performed for 10 min at 37 ^{o}C. Reactions were quenched by adding equal volume methanol. Samples were centrifuged for 10 min at 14000 g, and the supernatants were injected directly for reversed-phase HPLC analysis. The HPLC system used consisted of a Shimadzu SCL 10A system controller (Shimadzu, Columbia, MD), two LC 10AT pumps, a SIL 10AD automatic sample injector, a SPD-M10A UV-VIS spectrophotometric detector. Aliquots of the supernatant (50 μl) were injected onto a Zorbax SB-C8 column (4.6x75 mm, 3.5 um, Agilent Technologies, Palo Alto, CA) and eluted at a flow rate of 2 ml/min. The mobile phase consisted of a mixture of buffer A (10 mM ammonium acetate) and buffer B (10 mM ammonium acetate in 90% acetonitrile and 10% methanol). A linear gradient elution with buffer B was run from 20 to 60% in 16 min and chromatographic peaks of testosterone and its metabolites were monitored at 254 nm at the following retention times: 6β-hydroxytestosterone at 5.3 min, 7α-hydroxytestosterone at 4.5 min, 16α-hydroxytestosterone at 6.4 min, and 2α-hydroxytestosterone at 7.1 min.

Biliary excretion and pharmacokinetics of dibromosulfophthalein (DBSP)

Male Mrp2$^{-/-}$ and wild type mice (between 20-24 weeks of age) were used. The animals were fasted overnight with free access to water. DBSP (Laboratoire SERB,

Paris, France) was dissolved in saline. For biliary excretion studies, after intravenous administration of DBSP (5 mg/kg), bile samples were collected over a total of 90 minutes in pre-weighted tubes on ice. For the pharmacokinetic study, after intravenous dosing of DBSP (5 mg/kg), 20 µl of blood samples were taken from the tail vein at designated time points and blood was added to 60 µl of sodium citrate. All samples were kept at -80^{o}C until further analysis.

Quantification of DBSP in bile and blood

Briefly, aliquots of bile (10 µl) were diluted with 50 mM Tris-HCl buffer (pH 7.4), and then made alkaline by the addition of 0.1 M NaOH (100 µl). Concentrations of DBSP in bile samples were determined in a spectrophotometer (Spectramax M2, Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm (Sathirakul et al., 1993).

Blood samples were thawed on ice. Two volumes of acetonitrile containing formic acid (0.1% (v/v)) were added to the samples and standard solutions. Labetolol (Sigma Co., St Louis, MO) was added as an internal standard. After mixing, the contents were centrifuged at 1800 g for 10 min. The supernatant was then transferred to a 96-well plate containing an equal volume of water and analyzed using LC-MS/MS. Chromatography was performed on a Beta Basic Phenyl Dash column (10 x 1 mm, 5 µm) and an HPLC system consisting of PerkinElmer Series 200 Micro Pumps (PerkinElmer Instruments, Shelton, CT) and a Leap HTS PAL Autosampler (LEAP Technologies, Carrboro, NC), using a gradient mobile phase of water (A) and acetonitrile (B). The HPLC flow rate was 0.6 ml/min. The gradient was started with 5% B, increased linearly to 95% B in 0.7 min, and hold at 95% B for 0.5 min. Detection of the analyte was performed using a Sciex API 4000 mass spectrometer (MDS-Sciex, Toronto, Canada) in the negative ion mode using the Turbo-Ion Spray source. Mass transition (m/z) monitoring for DBSP was 316.90 → 294.85. The concentration of DBSP in blood samples were determined by comparing the analyte to internal standard peak area ratios against a standard curve.

Determination of DBSP pharmacokinetic parameters

Pharmacokinetic parameters of DBSP were calculated using Watson software (version 6; Watson Software System) with non-compartmental models.

Statistical analysis

Student t-Tests were used to determine the significance of differences between groups of animals. Differences with P values <0.05 or <0.01 were considered significant.

Results

Generation of Mrp2 $^{-/-}$ mice

Constitutive Mrp2$^{-/-}$ mice were generated by Deltagen by inserting a *Lac-Neo* cassette into the Mrp2 gene, resulting in deletion of bp 1886-1897. PCR analysis of purified tail DNA allowed the detection of the wild type and mutant alleles (Figure 1A). To confirm the absence of Mrp2 protein in the knockout animals, crude membrane fractions were isolated from livers from Mrp2$^{-/-}$ and control animals and Mrp2 was detected by Western blotting using monoclonal antibody M_2III-5. No Mrp2$^{-/-}$ protein was detectable in membranes of knockout mice (Figure 1B, lane 2), whereas a strong signal was observed in the membranes isolated from wild type mice (lane 1). Overall, no gross abnormalities were observed in the Mrp2$^{-/-}$ mice, their life span was normal and mice were born at the expected Mendelian ratio.

Serum analysis of Mrp2 $^{-/-}$ mice

Table 1 shows the values for several clinical chemical parameters measured in serum. No significant increases were observed in ALT and AST between Mrp2$^{-/-}$ and wild type mice, suggesting no hepatocellular damage in livers of knockout mice. Total bilirubin levels were 5-fold higher in knockout animals. In Mrp2$^{-/-}$ mice, 60% of total (conjugated plus unconjugated) bilirubin was conjugated (direct) bilirubin, whereas the level of conjugated bilirubin in serum of wild type mice was below the limit of quantitation. Cholesterol levels were 1.5-fold higher in male knockout animals, but no significant change was observed between female Mrp2$^{-/-}$ and control mice. The increased cholesterol level in male Mrp2$^{-/-}$ mouse serum did not result in changes in the biliary excretion of cholesterol (data not shown). No significant differences were found in any of the other parameters measured, except for a slightly decreased alkaline phosphatase level in Mrp2$^{-/-}$ compared to control female mice.

Urine analysis of Mrp2$^{-/-}$ mice

Part of conjugated bilirubin was excreted into the urine in TR^{-} rats (Jansen et al., 1985). To determine whether this was also the case in mice, total and direct bilirubin amounts in urine were measured (Table 2). In urine collected over a 12 h time period, levels of direct bilirubin were 6.7-fold higher in Mrp2$^{-/-}$ mice. The other urine parameters measured were not changed significantly.

Glutathione levels in Mrp2 $^{-/-}$ mice

Paulusma et al. (1999) found previously that the biliary excretion of glutathione was reduced strongly in TR^{-} rats, suggesting that Mrp2 was involved in the transport of glutathione. Therefore, the biliary excretion of glutathione in mice was measured (Figure 2A). The biliary excretion rate of glutathione was 16-fold lower in Mrp2$^{-/-}$ than in controls (25 nmol/kg/min in Mrp2$^{-/-}$ *versus* 400 nmol/kg/min in wild type mice). Some very low glutathione export was still detectable in knockout mice, suggesting the presence of other compensatory (unknown) canalicular glutathione transporters. The reduced excretion of glutathione in the knockout mice correlated with a 3-fold increased glutathione level in Mrp2$^{-/-}$ liver extracts (Figure 2B). Differences in plasma levels of glutathione between Mrp2$^{-/-}$ and control mice were not statistically significant (Figure 2C).

Bilirubin glucuronide levels in Mrp2$^{-/-}$ mice

As physiologically important substrates of Mrp2, biliary secretion of endogenous bilirubin glucuronides in Mrp2$^{-/-}$ mice was evaluated. As shown in Figure 2D, the biliary excretion rate of bilirubin glucuronides was about 2-fold lower in Mrp2$^{-/-}$ mice than that in control mice. This is in agreement with reports that the biliary excretion of bilirubin and bilirubin glucuronides in EHBR and TR^{-} rats is only decreased mildly (Jansen et al., 1985; Kurisu et al., 1991; Paulusma et al., 1999).

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Decreased biliary secretion of dibromosulfophthalein in Mrp2 $^{-/-}$ mice

To study the *in vivo* disposition of a non-metabolized model Mrp2 substrate, dibromosulfophthalein (DBSP; 5 mg/kg) was administered intravenously to wild type and Mrp2$^{-/-}$ mice. DBSP blood elimination was impaired in Mrp2$^{-/-}$ mice (Figure 3D), resulting in a significant increase in the area under the curve (AUC) and a decrease in blood clearance (CL_b) in the knockout mice (Table 3). In line with these findings, in bile duct catheterized mice, the biliary excretion rate of DBSP (5 mg/kg, i.v.) was slower in knockout than in wild type mice (Figure 3A). Cumulative excretion of DBSP in bile indicated that almost 100% of the dose was excreted at t = 50 min in wild type animals, whereas only 25% was recovered in the Mrp2$^{-/-}$ mice at the same time point. This percentage increased to 70% at 90 min. Bile flow was somewhat variable between the different time points (Figure 3C), but on average ~25% lower in Mrp2$^{-/-}$ than in wild type mice. Taken together, these data demonstrated that Mrp2 was contributing significantly to the elimination of DBSP. Most likely, the delayed appearance of DBSP in Mrp2$^{-/-}$ mice relates to the fact that sinusoidal efflux is enhanced (see discussion).

Gene expression profiling in Mrp2 $^{-/-}$ liver, kidney, and intestine

A general concern in the use of transporter knockout animals for pharmacokinetic studies is the potential for induction of compensatory mechanisms which may complicate the interpretation of data obtained with such animals. Using TaqMan Low-Density Microarrays (Microfluidic Cards), we therefore determined the expression level of a total of ninety five Phase I, Phase II, transporter and a number of other genes relevant to drug metabolism in liver, intestine and kidney of wild type and Mrp2$^{-/-}$ female and male mice (Tables 4-1 and 4-2). Student T tests were performed between $\Delta Ct_{wildtype}$ and $\Delta Ct_{Mrp2-/-}$ from triplicate TaqMan analyses performed with the same reverse transcription reaction products to determine whether changes observed were statistically significant.

The Mrp2 mRNA level was 30-1000-fold lower in liver, kidney and intestine from Mrp2$^{-/-}$ than in the corresponding organs from control mice, confirming that the Mrp2 gene was inactivated in the knockout mice. The low RNA signal detected in tissues from Mrp2$^{-/-}$ mice was probably due to the presence of an unstable RNA transcript, which still may be formed as the Mrp2 promoter was not disrupted in these mice.

In humans and rats in which MRP2/Mrp2 activity is impaired or absent, MRP3/Mrp3 mRNA is induced in the liver (Hirohashi et al., 1998; Konig et al., 1999). In contrast, no induction of Abcc3 (Mrp3) mRNA was observed in liver of either female or male Mrp2$^{-/-}$ mice (Table 4-1). Interestingly, Mrp4 (Abcc4) mRNA was induced 6-7-fold in liver of male and female Mrp2$^{-/-}$ mice. In females, a 2.5-fold lower level of Slc22a7 (Oat2) was found in the liver of knockout versus wild type mice, whereas no significant differences were detected for any of the other transporter genes analyzed.

In kidney, expression of Mrp4 was increased 2.5-fold in male and female Mrp2$^{-/-}$ mice. Expression of Abcb9, Slco1a1 (Oatp1) and Slco1a6 (Oatp5) was higher in males than in females. In female kidney, expression of Abcc3 was higher than in male kidney.

Of the cytochrome P450 genes analyzed, Cyp2b13 was expressed at a 10-fold lower level in liver of female Mrp2$^{-/-}$ than in wild type mice (Table 4-2). Cyp2b9 was expressed at a 2-fold lower level in female liver of the knockout mice. The cholesterol-7α-hydroxylase Cyp7a1 gene was expressed 2.2-fold higher in male Mrp2$^{-/-}$ liver, and Cyp4a14 in male Mrp2$^{-/-}$ liver was expressed 2.5-fold lower than in liver from control mice. Cyp4a14 in male Mrp2$^{-/-}$ kidney was expressed 2-fold lower than in wild type kidney. Expression of Cyp2d9, and Cyp4a12 was higher in male than in female liver, whereas expression of Cyp2b13, Cyp2b9, Cyp3a16 and Cyp3a41 was higher in female liver. In kidney, expression of Cyp2d9, and Cyp4a12 was higher in males than in females.

For the UDP-glucuronosyltransferase (UGT), glutathione *S*-transferase and sulfotransferase (SULT) genes analyzed, no significant differences were found between wild type and knockout mice (Table 4-2). Expression of Ugt1a1 was higher in female than in male kidney, and expression of Ugt8 was higher in female then in male kidney.

As nuclear receptors play an important role in the regulation of gene expression, the expression of several nuclear receptors, including the pregnane X receptor (Pxr, Nr1i2), the constitutive androstane receptor (Car, Nr1i3), the liver X receptor (Lxr, Nr1h3), and the farnesoid X receptor (Fxr, Nr1h4) were evaluated. No significant differences were detected for these genes in the liver, kidney and intestine of knockout versus control mice (Table 4-2).

Mrp4 protein levels in liver and kidney

Since Mrp4 mRNA was increased in both liver and kidney of Mrp2$^{-/-}$ mice, protein levels were determined in crude membrane extracts of male mice by Western blotting. Mrp4 was detected with monoclonal antibody M_4I-10. By comparing the Western blot signal after loading various amounts of protein, Mrp4 protein was found to be increased 7-10-fold in liver of Mrp2$^{-/-}$ mice (Figure 4A). In kidney the Mrp4 level was increased 2-fold in Mrp2$^{-/-}$ mice (Figure 4B, lanes 3-6). The Mrp4 level in wild type kidney was ~12-fold higher than in wild type liver (Figure 4B, compare lanes 1 and 3).

Bile salt levels in bile and plasma

Since Mrp4 is localized to the basolateral membrane in hepatocytes and has been demonstrated to transport bile salts and conjugated bile acids *in vitro* (Rius et al., 2003), total bile salt levels were measured in serum of male Mrp2$^{-/-}$ and control mice. Although bile salt levels were higher in serum of some Mrp2$^{-/-}$ mice, variation was high and not statistically different from control mice (data not shown). Bile salt levels were also measured in bile. Over a 60 min sampling period, the excretion rate was somewhat

higher in Mrp2$^{-/-}$ compared to control mice at all time points (average of 1.5 ± 0.1 μmol/kg/min in Mrp2$^{-/-}$ *versus* 0.9 ± 0.1 μmol/kg/min in control mice, n = 3).

Liver microsomal cytochrome P450 enzyme activities

To investigate whether the reduced expression of Cyp2b13 in liver of female Mrp2$^{-/-}$ mice resulted in a phenotypic effect and to confirm the lack of clear induction or suppression in the level of expression of any of the other P450 isoforms tested (Table 4-2), we investigated P450-mediated enzyme activities in liver microsomes isolated from Mrp2$^{-/-}$ and wild type male and female mice. While the specificity of probe substrates for all of the mouse P450 enzymes have not been clearly identified, it has been inferred that in mouse liver microsomes EROD and PROD activities are mediated by Cyp1A and Cyp2B, respectively, whereas testosterone 6β-hydroxylase (HT) activity is mediated by Cyp3A, 2α HT activity by Cyp2C, 16α HT activity by Cyp2D9 and Cyp2B, 7α HT activity by Cyp2A, and 16β HT by Cyp2B (Sapone et al., 2003). Table 5 shows that no significant differences were detected in liver microsomes from wild type *versus* knockout mice. Some gender different activities were detected: 16α HT activity was higher in male than in female mouse liver microsomes, and PROD activity was lower in male than in female liver microsomes.

Taken together, these data suggest no significant differences in major P450 enzyme activities between the Mrp2$^{-/-}$ and wild type mice.

Discussion

In this report, we describe the characterization of Mrp2 knockout mice. These mice are healthy and show no phenotypic abnormalities. Western blotting of liver membranes demonstrated that Mrp2 protein was not detectable in liver of knockout mice. In serum and urine, significantly increased levels of conjugated and/or unconjugated bilirubin were found in the $Mrp2^{-/-}$ mice. The functional absence of Mrp2 was demonstrated by showing a delayed excretion and incomplete recovery of the Mrp2 substrate DBSP in bile of the $Mrp2^{-/-}$ mice, and a significant reduction in the excretion of glutathione and bilirubin glucuronides. In general, lack of Mrp2 may result in decreased biliary secretion, decreased renal elimination, and reduced intestinal secretion of Mrp2 substrates.

Unlike in EHBR/TR^- rats, Mrp3 mRNA was not induced in liver of $Mrp2^{-/-}$ mice (Table 4-1). This was unexpected as strong induction of Mrp3/MRP3 has been demonstrated in livers of EHBR/TR^- rats, and in humans with DJS (Hirohashi et al., 1998; Konig et al., 1999). A plausible explanation for this discrepancy is that under normal conditions the level of Mrp3 in mouse hepatocytes is much higher than in rats and humans and consequently mice have a constitutive mechanism to compensate for the inhibition or absence of Mrp2. This is in accord with a recent report by Belinsky et al. (2005), who demonstrated that Mrp3 protein was detectable at good levels in normal wild type mouse liver. Our TaqMan data showed that Mrp2 was expressed at a similar level as Mrp3 in wild type mouse liver. This is in contrast to normal Wistar rats, in which the hepatic mRNA level of Mrp2 was approximately 60-fold higher than of Mrp3 (data not shown). The above findings suggest that $Mrp2^{-/-}$ mice are not a good animal model to study human DJS.

The relatively strong Induction of Mrp4 protein in liver (Figure 4A) of $Mrp2^{-/-}$ mice was unexpected as this has not been found in TR^- rats (Chen et al., 2005). MRP4 is

localized in the basolateral membrane in hepatocytes (Rius et al., 2003) and in the apical membrane of renal proximal tubule cells (van Aubel et al., 2002). Mrp4 therefore may act as a compensatory transporter for the lack of Mrp2 in mice. Although, Mrp2 and Mrp4 show overlap in substrate specificity (Borst and Oude Elferink, 2002), it is not known whether Mrp4 transports bilirubin glucuronides. Since it has been demonstrated that human MRP4 transports bile salts *in vitro* (Ruis et al., 2003), we tested bile acid levels in serum of Mrp2$^{-/-}$ and control mice. Although bile salt levels were elevated in some Mrp2$^{-/-}$ mice, inter-animal variation was high and differences to controls were not significant.

The mechanism explaining induction of Mrp4 in Mrp2$^{-/-}$ mice is not clear. Mrp4 is induced in mice lacking the nuclear receptor Fxr (Nrih4), whereas levels of Bsep (Abcb11) are reduced in these mice (Schuetz et al., 2001). Since the level of Fxr and Abcb11 mRNA was not changed in Mrp2$^{-/-}$ mice (Table 4-1, 4-2), and the excretion rate of total bile salts in the knockout was similar to control mice, downregulation of Fxr or Abcb11 is an unlikely explanation for the induction of Mrp4. A possible mechanism may be that absence of Mrp2 results in increased levels of bilirubin in hepatocytes and as a consequence activation of the constitutive androstane receptor (CAR; Huang et al., 2003). Since Mrp4 is a target gene of CAR, this may explain the induction of this transporter (Assem et al., 2004). We did not find activation of other CAR target genes, however, like for instance Cyp2b10 (Table 4-2).

In the Mrp2$^{-/-}$ mice, total and conjugated bilirubin in serum were increased 5- and >3-fold, respectively (Table 1), and in urine conjugated bilirubin was increased 6.7-fold (Table 2). In TR^{-}/EHBR rats the level of bilirubin conjugates in plasma was 50-100 times higher than in Wistar or SD rats, respectively (Hosokawa et al., 1992; Jansen et al., 1985). Jansen et al. also showed that bilirubin and bilirubin conjugates are excreted via the urine in TR^{-} rats, whereas no excretion was detected in Wistar rats. It was

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concluded, however, that the urinary excretion of total bilirubin was quantitatively low. At least four hypotheses could explain the relatively low increase in bilirubin plasma concentration in Mrp2$^{-/-}$ mice: (i) A mechanism for Mrp2-independent biliary excretion of bilirubin glucuronides may be present in mice and this mechanism might be more potent than in rats**.** Quantitative comparison of bilirubin glucuronides secretion between Mrp2$^{-/-}$ mice and TR^{-}/EHBR rats would be needed to confirm this hypothesis. (ii) The induction of Mrp3 in TR^{-}/EHBR rats might result in higher protein levels than the endogenous level detected in mice, causing more efficient export of bilirubin glucuronides from the liver into the circulation. (iii) Johnson et al. (2005) indicated that expression of UGT1a protein was increased ~ 3.5-fold in TR^{-} rats compared to controls. Therefore, increased bilirubin glucuronidation in the liver of TR^{-} rats could contribute to the higher plasma bilirubin glucuronide levels in TR^{-} rats. (iv) Like in TR^{-} rats, urinary excretion of bilirubin glucuronides is increased in Mrp2$^{-/-}$ mice (Table 2). If more efficient in mice than in rats (e.g., via Mrp4), this could result in lower plasma levels.

Differences in cytochrome P450 activities have been found between TR^{-}/EHBR and wild type rats (Jager et al., 1998; Newton et al., 2005). One of the reasons to characterize the Mrp2$^{-/-}$ mice was to use these animals to study the role of Mrp2 in the pharmacokinetics of drugs. It therefore was important to investigate whether differences in results between knockout and control mice could not only be explained by the lack of Mrp2 but potentially also by differences in phase I enzymatic activities. Based on the phenotypic and mRNA expression analysis (Tables 4-2 and 5), no significant differences were found in P450 enzyme activities or mRNA levels in male mice. In female mice Cyp2b13 was 10-fold lower in Mrp2$^{-/-}$ than in control liver. Based on PROD and 16α-HT activities this did not translate into a phenotypic effect, however.

Bile duct cannulation studies with the Mrp2 model substrate DBSP were performed to demonstrate that Mrp2 was functionally absent in the knockout mice

(Figure 3). DBSP was chosen as it is a non-metabolized Mrp2 substrate (Klaassen, 1970), and it has been shown previously that the biliary excretion rate and plasma clearance of DBSP in both TR^- and EHBR rats was impaired (Jansen et al., 1987; Sathirakul et al., 1993). Residual transport was detected, however, in the mutant rats which suggested that other transporters in the canalicular membrane transport DBSP. In the $Mrp2^{-/-}$ mice, we observed a delayed and reduced biliary excretion rate of DBSP in bile (Figure 3A). As in EHBR/TR^- rats, the residual excretion of DBSP in the knockout mice is likely explained by another low affinity/low capacity transporter in the canalicular membrane. At present, it is not clear what the molecular identity is of this transporter. A possible candidate is the breast cancer resistance protein (Bcrp or Abcg2), which is expressed in liver and able to transport some negatively charged compounds (Doyle and Ross, 2003).

We found that in the knockout mice the biliary excretion of DBSP started to decrease after 50 min (Figure 3A), although at that time point only 30% of the dose had been excreted (Figure 3B). As the bile flow was relatively constant over this time frame (Figure 3C), this suggested that after the intrahepatic DBSP concentration reached a certain threshold, DBSP was transported back into the blood. This may be via Mrp3, Mrp4, or via an Oatp as it has been shown for rat Oatps that they can act bidirectionally (Li et al., 2000).

In normal rats, excretion of glutathione into bile is high and this transport is almost absent in TR^- rats (Paulusma et al., 1999). In line with this finding, we also found that excretion of glutathione was reduced significantly in $Mrp2^{-/-}$ mice compared to control mice (Figure 2A). In our measurements, we detected both reduced and oxidized glutathione, but levels of the latter are usually very low (Paulusma et al., 1999). Uptake experiments with membrane vesicles have suggested that GSH is a substrate for human and rat MRP2/Mrp2 with an apparent K_m in the mM range (Paulusma et al., 1999). GSH

probably can be transported by MRP2/Mrp2 directly as a low affinity substrate or its transport can be associated with the transport of other compounds, like for instance arsenite, vinblastine, etoposide and sulfinpyrazone (Ballatori et al., 2005). We also found that biliary excretion of bilirubin glucuronides was only mildly decreased in Mrp2$^{-/-}$ mice (Figure 2D), suggesting the involvement of other transporters on biliary excretion of bilirubin glucuronides.

In summary, we have characterized Mrp2$^{-/-}$ mice. These mice did not show differences in P450 enzyme levels in liver or induction of Mrp3. Mrp4 was increased in the Mrp2$^{-/-}$ mice, however, especially in liver. Since the Mrp2$^{-/-}$ mice show differences with TR^{-}/EHBR rats, we expect that these mice will be a valuable additional tool to study the role of Mrp2 in the absorption and disposition of drugs.

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Legends for Figures

Figure 1

Targeted disruption of the Mrp2 gene in mice. Panel A. PCR analysis of purified tail DNA isolated from wild type (lanes 3, 4), Mrp2$^{-/-}$ (lanes 5, 6), and Mrp$^{+/-}$ (lanes 7, 8) mice. Two PCR reactions with different sets of primers were performed for each mouse tail DNA as described in Materials and Methods. The wild type allele and targeted allele generate fragments of 237 and 502 bp, respectively. Panel B. Western blot analysis of Mrp2 in liver of wild type (lane 1) and Mrp2$^{-/-}$ (lane 2) mice. Ten µg of crude plasma membranes from wild type and Mrp2$^{-/-}$ mouse liver were separated in a 7.5% denaturing polyacrylamide gel. Gels were immuno-blotted onto nitrocellulose membranes. Mouse Mrp2 was detected with M_2III-5, an anti-MRP2 monoclonal antibody.

Figure 2

Biliary excretion of glutathione and bilirubin glucuronides in wild type and Mrp2$^{-/-}$ mice. Panel A. Biliary excretion rate of total glutathione in male Mrp2$^{-/-}$ (open squares) and wild type (closed squares) mice were measured using a Glutathione Assay Kit as described in Materials and Methods. Panels B and C. GSH levels in liver and plasma, respectively. Panel D. Biliary excretion rate of bilirubin glucuronides in male Mrp2$^{-/-}$ (open squares) and wild type (closed squares) mice. All data represent the mean ± SE for 3 animals.

Figure 3

In vivo disposition of DBSP in wild type and Mrp2$^{-/-}$ mice. Panels A and B. Biliary excretion rate (A) and cumulative biliary excretion (B) of DBSP after intravenous administration of DBSP (5 mg/kg) in wild type (closed squares) and Mrp2$^{-/-}$ (open squares) mice. Panel C. Bile flow rate in wild type (closed squares) and Mrp2$^{-/-}$ (open squares) mice. Panel D. Blood DBSP concentration time profile after intravenous administration of DBSP (5 mg/kg) in wild type (closed squares) and Mrp2$^{-/-}$ mice (open squares). All the data represent the mean ± SE of 3-4 animals.

Figure 4

Detection of Mrp4 protein in liver and kidney extracts. Panel A. Various amounts of crude membranes from liver of Mrp2-/- and control mice were separated in a 7.5% polyacrylamide gel. After Western blotting, Mrp4 was detected using monoclonal antibody M_4I-10. Protein amounts loaded are indicated above the lanes. Panel B. Same as A, but crude kidney membranes were analyzed (lanes 3-6). To compare relative protein levels, liver membranes were loaded onto the same gel (lanes 1 and 2).

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TABLE 1 Serum chemistry parameters of wild-type and Mrp2 $^{-/-}$ mice

Data represent means ± SE determined from 5 to 6 animals

Parameter		Males				Female			
		Wild type		Mrp2 $^{-/-}$		Wild type		Mrp2 $^{-/-}$	
Glucose	**(mg/dl)**	128 ± 10		145 ± 21		139 ± 6		119 ± 9	
Urea nitrogen	**(mg/dl)**	26.1 ± 1.5		30 ± 2.9		25.0 ± 2.8		19.8 ± 1.8	
Total protein	**(g/dl)**	6.2 ± 0.2		6.0 ± 0.1		5.7 ± 0.1		6.1 ± 0.04	
Albumin	**(g/dl)**	4.1 ± 0.2		4.1 ± 0.1		4.3 ± 0.1		4.5 ± 0.1	
Total bilirubin	**(mg/dl)**	0.1 ± 0.02		0.5 ** ± 0.02		0.1 ± 0.02		0.5 ** ± 0.1	
Direct bilirubin	**(mg/dl)**	< 0.1 [a]		0.3 ± 0.02		< 0.1 [a]		0.3 ± 0.03	
Alkaline phosphatase	**(U/l)**	89.2 ± 5.0		81.8 ± 8.7		183 ± 14		132 ** ± 15	
Alanine aminotransferase	**(U/l)**	40.3 ± 5.4		34.7 ± 3.8		56.6 ± 10.1		36.8 ± 5.4	
Aspartate aminotransferase	**(U/l)**	234 ± 41		164 ± 42		217 ± 45		142 ± 20	
Lactate dehydrogenase	**(U/l)**	543 ± 127		334 ± 67		403 ± 56		275 ± 43	
Cholesterol	**(mg/dl)**	83.5 ± 8.2		130 ** ± 9		80.8 ± 6.4		95.2 ± 4.6	
Calcium	**(mg/dl)**	10.1 ± 0.1		10.3 ± 0.2		10.0 ± 0.1		10.3 ± 0.1	
Phosphorus	**(mg/dl)**	7.5 ± 0.2		7.9 ± 0.4		7.6 ± 0.7		7.3 ± 0.4	
Sodium	**(mEq/l)**	154 ± 1		155 ± 1		154 ± 2		154 ± 1	
Potassium	**(mEq/l)**	7.6 ± 0.4		7.4 ± 0.2		7.5 ± 0.3		6.9 ± 0.5	
Chloride	**(mEq/l)**	114 ± 1		114 ± 1		113 ± 2		111 ± 1	
Triglycerides	**(mg/dl)**	62.8 ± 5.3		75.0 ± 9.2		62.2 ± 8.4		64.6 ± 3.8	
Creatine kinase	**(U/l)**	676 ± 101		661 ± 197		484 ± 63		430 ± 152	
Magnesium	**(mg/dl)**	2.7 ± 0.1		2.7 ± 0.2		2.8 ± 0.2		2.8 ± 0.2	
Osmolality	**(mOs/kg)**	304 ± 5		316 ± 1		309 ± 3		309 ± 2	

[a]: Below the detection limit

**: P<0.01, significantly different from wild type mice

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TABLE 2 Urine chemistry parameters of wild-type and Mrp2 $^{-/-}$ mice

The parameters tested were normalized to urine volume (ml).

Data represent means ± SE determined from urine samples obtained from 5 groups of animals; each group included 3 animals as described in Materials and Methods.

Parameter		Males		Female	
		Wild type	Mrp2 $^{-/-}$	Wild type	Mrp2 $^{-/-}$
Glucose	**(mg/dl/ml)**	3.6 ± 1.6	4.0 ± 1.1	2.1 ± 0.4	3.8 ± 1.0
Urea nitrogen	**(mg/dl/ml)**	496 ± 223	508 ± 145	252 ± 49	415 ± 110
Creatinine	**(mg/dl/ml)**	8.7 ± 4.0	9.4 ± 2.4	3.8 ± 0.6	6.5 ± 1.7
Total protein	**(g/dl/ml)**	0.3 ± 0.1	0.3 ± 0.1	0.04 ± 0.01	0.1 ± 0.01
Total bilirubin	**(mg/dl/ml)**	0.7 ± 0.3	0.7 ± 0.2	0.4 ± 0.1	0.7 ± 0.2
Direct bilirubin	**(mg/dl/ml)**	0.06 ± 0.01	0.4* ± 0.1	0.03 ± 0.003	0.2* ± 0.04
Phosphorus	**(mg/dl/ml)**	36.2 ± 14.2	39.1 ± 9.3	21.2 ± 3.4	29.8 ± 6.8
Sodium	**(mEq/l/ml)**	19.4 ± 5.0	19.4 ± 4.8	11.2 ± 1.7	16.1 ± 4.1
Potassium	**(mEq/l/ml)**	52.7 ± 25.8	52.4 ± 15.2	21.1 ± 5.2	36.4 ± 9.8
Chloride	**(mEq/l/ml)**	17.0 ± 4.3	16.8 ± 4.3	10.9 ± 1.9	16.3 ± 4.5

*: $P<0.05$, significantly different from wild type mice

TABLE 3 Pharmacokinetic parameters of DBSP following intravenous administration (5 mg/kg) to wild type and Mrp2 $^{-/-}$ mice

Data represent means ± SE obtained from 4 animals

AUC, area under the curve; $CL_{b,}$ blood clearance; $T_{1/2}$, terminal-phase half life; V_{dss}, volume of distribution at steady-state

Parameters		Wild type	Mrp2 $^{-/-}$
AUC	µM.min	4.4 ± 1.0	8.2* ± 1.7
CL_b	l/kg/min	1.9 ± 0.3	0.7* ± 0.3
$T_{1/2}$	min	6.6 ± 1.2	26.5 ± 14.2
V_{dss}	l/kg	17.8 ± 5.2	21.5 ± 4.4

*: P<0.05, significantly different from wild type mice

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Table 4-1 Gene expression in Mrp2 $^{-/-}$ mouse liver, kidney, and intestine

The data were expressed as fold change in Mrp2 $^{-/-}$ compared to wild type mice

Data represent means ± SE determined from three independent experiments

Gene	Accession number	Liver		Kidney		Intestine	
		Males	Females	Males	Females	Males	Females
Abca1	NM_013454	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.9 ± 0.1	1.0 ± 0.2
Abca13	NM_178259	b	b	0.7 ± 0.2	0.8 ± 0.2	b	a
Abca2	NM_007379	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.6 ± 0.2	0.6 ± 0.02	1.2 ± 0.2
Abca3	NM_013855	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.7 ± 0.1	0.9 ± 0.1
Abca4	NM_007378	a	a	1.0 ± 0.2	0.8 ± 0.2	b	a
Abca5	NM_147219	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.7 ± 0.3	a	a ±
Abca6	NM_147218	0.7 ± 0.1	0.8 ± 0.1	a	a	a	a
Abca7	NM_013850	0.5 ± 0.1	0.6 ± 0.1	0.9 ± 0.2	0.5 ± 0.2	0.8 ± 0.1	0.9 ± 0.0
Abca8a	NM_153145	0.7 ± 0.05	1.5 ± 0.1	0.7 ± 0.1	1.0 ± 0.2	a	0.9 ± 0.2
Abca8b	NM_013851	0.8 ± 0.1	0.7 ± 0.1	a	a	a	a
Abca9	NM_147220	a	a	a	a	a	1.0 ± 0.3
Abcb10	NM_019552	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
Abcb11	NM_021022	0.6 ± 0.1	0.6 ± 0.2	b	b	a	a
Abcb1a	NM_011076	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.7 ± 0.3	1.2 ± 0.2	1.3 ± 0.2
Abcb1b	NM_011075	1.0 ± 0.3	0.9 ± 0.4	0.5 ± 0.1	0.7 ± 0.2	a	a
Abcb4	NM_008830	0.5 ± 0.1	0.8 ± 0.1	a	a	a	a ±
Abcb6	NM_023732	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	1.0 ± 0.1
Abcb9	NM_019875	a	a	0.9[c] ± 0.1	0.6 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
Abcc1	NM_008576	a	a	1.0 ± 0.3	0.7 ± 0.2	a	0.8 ±
Abcc10	NM_170680	a	a	1.5 ± 0.5	0.6 ± 0.2	1.0 ± 0.2	0.7 ± 0.1
Abcc2	NM_013806	0.001 ± 0.0001*	0.004 ± 0.001*	0.003 ± 0.001*	0.004 ± 0.001*	0.03 ± 0.001*	0.03 ± 0.004*
Abcc3	NM_029600	0.9 ± 0.1	1.0 ± 0.1	0.6 ± 0.2	0.6[d] ± 0.2	1.0 ± 0.3	1.0 ± 0.1
Abcc4	GI: 39771467	6.4 ± 0.9*	7.0 ± 1.7*	2.5 ± 0.2*	2.3 ± 0.3*	0.9 ± 0.2	1.3 ± 0.1*
Abcc5	NM_013790	a	a	0.7 ± 0.1	0.7 ± 0.2	a	a
Abcc6	NM_018795	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.5 ± 0.2	a	a
Abcc8	NM_011510	b	b	a	a	a	a
Abcc9	NM_011511	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.6 ± 0.2	0.9 ± 0.3
Abcg1	NM_009593	0.9 ± 0.1	0.8 ± 0.0	0.8 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	0.6 ± 0.1
Abcg2	NM_011920	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	0.9 ± 0.1
Abcg3	NM_030239	a	a	a	a	a	a
Abcg4	NM_138955	b	b	b	a	a	a
Abcg5	NM_031884	0.8 ± 0.1	1.1 ± 0.1	a	b	1.1 ± 0.1	1.2 ± 0.1
Abcg8	NM_026180	0.8 ± 0.1	0.9 ± 0.1	a	b	1.0 ± 0.1	1.0 ± 0.1
Cftr	NM_021050	a	a	a	a	0.9 ± 0.1	0.8 ± 0.1
Slc10a1	NM_011387	0.7 ± 0.1	0.9 ± 0.2	a	a	a	a
Slc10a3	NM_145406	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.7 ± 0.1	0.8 ± 0.2
Slc15a2	NM_021301	a	a	0.9 ± 0.1	0.6 ± 0.2	a	a
Slc22a1	NM_009202	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.0	0.7 ± 0.2	0.6 ± 0.3	0.6 ± 0.3
Slc22a12	NM_009203	a	b	0.9 ± 0.2	0.9 ± 0.3	b	b
Slc22a13	NM_133980	b	b	0.7 ± 0.1	0.8 ± 0.2	b	b
Slc22a17	NM_021551	a	a	0.7 ± 0.1	0.8 ± 0.2	a	a
Slc22a18	NM_008767	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	0.9 ± 0.1	0.9 ± 0.1
Slc22a2	NM_013667	b	b	0.7 ± 0.1	0.7 ± 0.2	b	b
Slc22a3	NM_011395	0.6 ± 0.1	0.6 ± 0.1	a	a	a	a
Slc22a4	NM_019687	1.1 ± 0.2	0.8 ± 0.3	0.6 ± 0.2	0.8 ± 0.2	1.1 ± 0.2	0.9 ± 0.04
Slc22a5	NM_011396	0.6 ± 0.3	1.4 ± 0.6	0.7 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	0.6 ± 0.2
Slc22a6	NM_008766	b	b	0.8 ± 0.3	0.7 ± 0.2	b	b
Slc22a7	NM_144856	1.3 ± 0.2	0.4 ± 0.02*	0.7 ± 0.1	0.7 ± 0.2	b	b
Slc22a8	NM_031194	b	b	0.7 ± 0.1	0.7 ± 0.2	a	b
Slc22a9	NM_019723	a	a	a	a	0.8 ± 0.1	1.1 ± 0.2
Slco1a1	NM_013797	0.6 ± 0.1	0.6 ± 0.1	0.8[c] ± 0.3	b	a, b	b
Slco1a4	NM_030687	1.2 ± 0.1	1.2 ± 0.2	a	a	a	b
Slco1a5	NM_130861	b	b	b	b	b	b
Slco1a6	NM_023718	b	b	0.9[c] ± 0.2	0.8 ± 0.3	b	b
Slco1b2	NM_020495	0.7 ± 0.1	0.9 ± 0.2	b	b	b	b
Slco1b2	NM_020495	0.6 ± 0.1	0.5 ± 0.2	b	b	b	b
Slco1c1	NM_021471	a	a	a	a	b	b
Slco2a1	NM_033314	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	0.8 ± 0.2
Slco3a1	NM_023908	a	a	0.7 ± 0.2	0.9 ± 0.2	1.4 ± 0.4	0.9 ± 0.02
Slco4a1	NM_148933	b	b	a	b	b	b
Tap1	NM_013683	0.5 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.2	0.7 ± 0.1	0.8 ± 0.1
Tap2	NM_011530	0.5 ± 0.04	0.5 ± 0.2	0.6 ± 0.1	0.7 ± 0.3	0.7 ± 0.1	1.1 ± 0.2

[a]: Low gene expression (C_t value > 32)

[b]: The gene was not amplified

[c]: Gene expression in male was higher than in female mice

[d]: Gene expression in female was higher than in male mice

*: P< 0.05, ΔCt value significantly different from wild type mice

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Table 4-2 Gene expression in Mrp2 $^{-/-}$ mouse liver, kidney, and intestine

The data were expressed as fold change in Mrp2 $^{-/-}$ compared to wild type mice
Data represent means ± SE determined from three independent experiments

Gene	Accession number	Liver		Kidney		Intestine	
		Males	Females	Males	Females	Males	Females
Acat2	NM_009338	0.6[e]	0.7[e]	0.9 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	1.4 ± 0.2
Cyp1a1	Y00071,K02588	a	a	a	a	a	a
Cyp1a1	NM_009992	b	b	a	b	b	a
Cyp1a2	NM_009993	0.9 ± 0.1	1.1 ± 0.2	a	b	a	b
Cyp1b1	NM_009994	a	a	0.5 ± 0.1	0.6 ± 0.2	0.8 ± 0.1	a
Cyp27a1	NM_024264	0.8 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.7 ± 0.3	1.1 ± 0.2	0.9 ± 0.1
Cyp2b10	NM_009998	a	a	a	b	a	a
Cyp2b13	NM_007813	a	0.1[d] ± 0.02*	b	b	b	b
Cyp2b19	NM_007814	a	a	a	a	a	b
Cyp2b9	NM_010000	a	0.5[d] ± 0.02*	b	b	b	b
Cyp2d9	NM_010006	0.7[C] ± 0.2	0.6 ± 0.1	0.3[C] ± 0.1	0.5 ± 0.2	a	a
Cyp2e1	NM_021282	0.7 ± 0.2	0.8 ± 0.2	0.7 ± 0.1	0.7 ± 0.2	b	1.1 ± 0.05
Cyp39a1	NM_018887	0.9 ± 0.1	0.9 ± 0.1	a	a	a	a
Cyp3a11	NM_007818	1.1 ± 0.1	1.3 ± 0.3	a	a	1.2 ± 0.04	1.3 ± 0.3
Cyp3a13	NM_007819	1.7 ± 0.2	1.1 ± 0.2	a	a	1.2 ± 0.2	1.0 ± 0.02
Cyp3a16	NM_007820	a	1.0[d] ± 0.2	b	b	a	b
Cyp3a41	NM_017396	a	1.2[d] ± 0.2	b	b	b	b
Cyp4a12	NM_172306	0.7[C] ± 0.1	a	0.6[C] ± 0.1	0.6 ± 0.1	b	b
Cyp4a14	NM_007822	0.4 ± 0.1*	0.8 ± 0.1	0.5 ± 0.1*	0.8 ± 0.2	a	a
Cyp51	NM_020010	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1
Cyp7a1	NM_007824	2.2 ± 0.2*	1.5 ± 0.1	b	b	a ±	b
Fasn	NM_007988	0.4[e]	0.5[e]	1.2 ± 0.3	0.7 ± 0.2	0.9 ± 0.1	0.9 ± 0.1
Gclc	NM_010295	0.5 ± 0.1	1.6 ± 0.3	0.7 ± 0.1	0.8 ± 0.2	1.6 ± 0.2	1.0 ± 0.1
Gss	NM_008180	0.9 ± 0.1	1.1 ± 0.1	0.9 ± 0.2	0.8 ± 0.3	0.8 ± 0.1	1.1 ± 0.04
Gsta2	NM_008182	b	b	b	b	b	b
Gstp1	NM_181796	0.8 ± 0.1	a	0.9 ± 0.2	0.8 ± 0.2	1.1 ± 0.1	0.9 ± 0.03
Mvk	NM_023556	0.5[e]	0.6[e]	0.9 ± 0.02	0.6 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
Nr1i2	NM_010936	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.3	1.2 ± 0.1	1.5 ± 0.3	1.6 ± 0.5
Nr1i3	NM_009803	1.0 ± 0.2	1.5 ± 0.04	0.7 ± 0.1	1.5 ± 0.1	1.1 ± 0.2	1.4 ± 0.1
Nr1h3	NM_013839	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.2
Nr1h4	NM_009108	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	1.6 ± 0.1	0.9 ± 0.1	1.4 ± 0.2
Pmvk	NM_026784	0.6[e]	0.7[e]	0.6 ± 0.2	0.8 ± 0.2	0.7 ± 0.1	0.8 ± 0.1
Sult1a1	NM_133670	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.2	1.1 ± 0.2	0.8 ± 0.1
Sult1d1	NM_016771	1.2 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	0.8 ± 0.2	1.3 ± 0.1	1.1 ± 0.1
Ugt1a1	NM_013701	1.1 ± 0.2	1.2 ± 0.1	1.9 ± 0.3	1.0[d] ± 0.3	b	b
Ugt2a1	NM_053184	b	a	b	b	b	b
Ugt8	NM_011674	a	a	0.8[C] ± 0.1	0.6 ± 0.1	a	b

[a]: Low gene expression (C_t value > 32)
[b]: The gene was not amplified
[c]: Gene expression in male was higher than in female mice
[d]: Gene expression in female was higher than in male mice
[e]: data were expressed as average value of duplicated experiments
*: P< 0.05, ΔCt value significantly different from wild type mice

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Table 5 Comparision of Phase I metabolic enzyme activities between wild type and Mrp2 $^{-/-}$ mice

The data were expressed in nmol/min/mg protein; Data represent means ± SE determined from four liver microsome preparations, each preparation consisting of a pool of 3 to 4 livers. No statistically significant difference were found between wild type and Mrp2 $^{-/-}$ mice at P<0.05.

Substrate	Males		Females	
	Wild type	Mrp2 $^{-/-}$	Wild type	Mrp2 $^{-/-}$
EROD	81.56 ± 11.72	64.83 ± 6.86	96.39 ± 3.83	85.44 ± 8.28
PROD	18.49 ± 0.92**	17.31 ± 0.84**	33.11 ± 2.58	28.16 ± 0.78
6β-HT	1.25 ± 0.19	1.39 ± 0.19	1.42 ± 0.09	1.23 ± 0.15
2α-HT	0.24 ± 0.03	0.28 ± 0.03	0.26 ± 0.01	0.28 ± 0.04
16α-HT	0.50 ± 0.06**	0.41 ± 0.04**	0.15 ± 0.02	0.15 ± 0.02
7α-HT	0.30 ± 0.04	0.28 ± 0.05	0.28 ± 0.02	0.33 ± 0.05

**: P < 0.05, significantly different from female mice

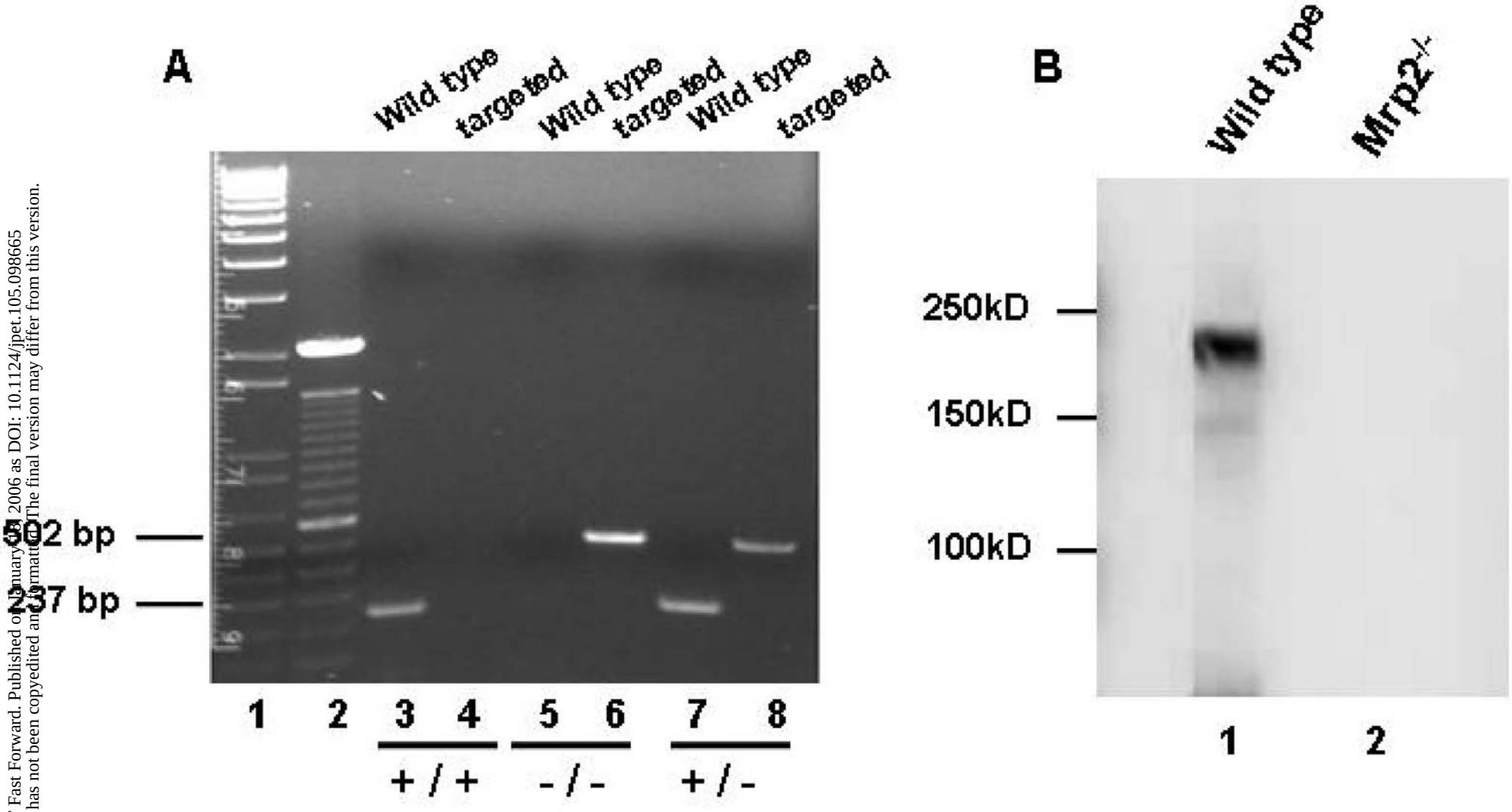


Figure 1

Figure 2

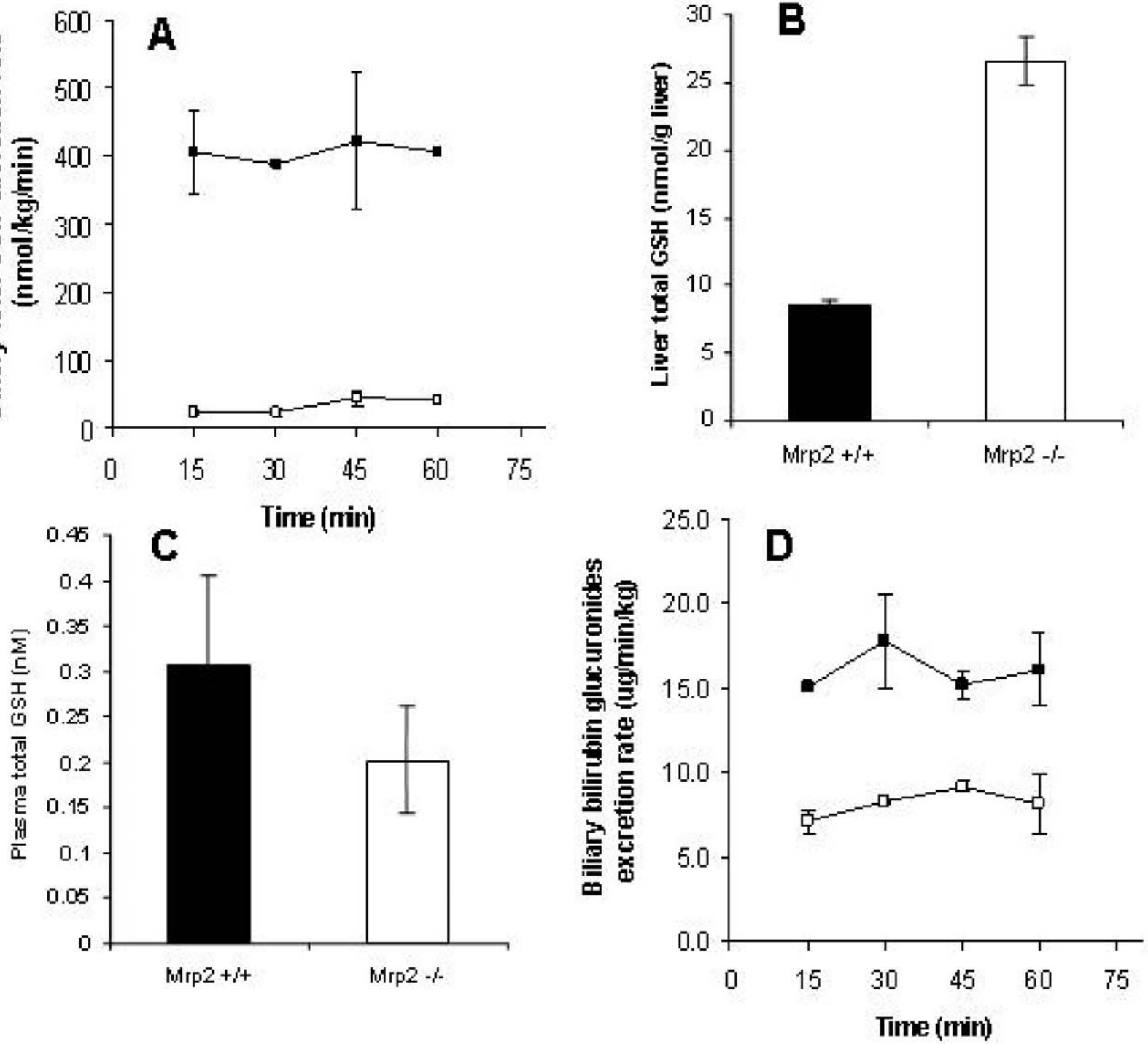
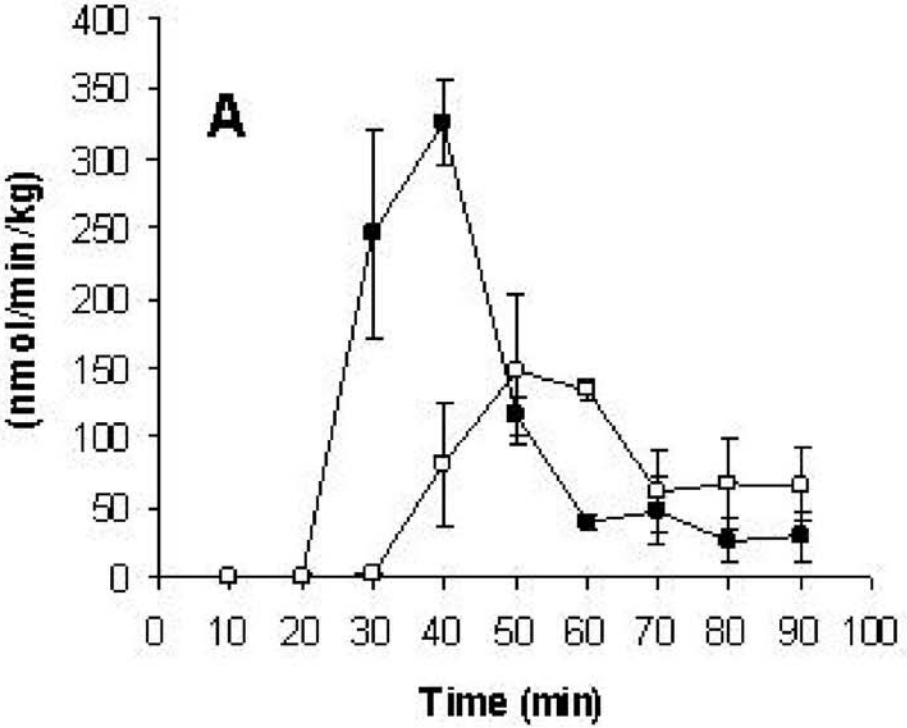
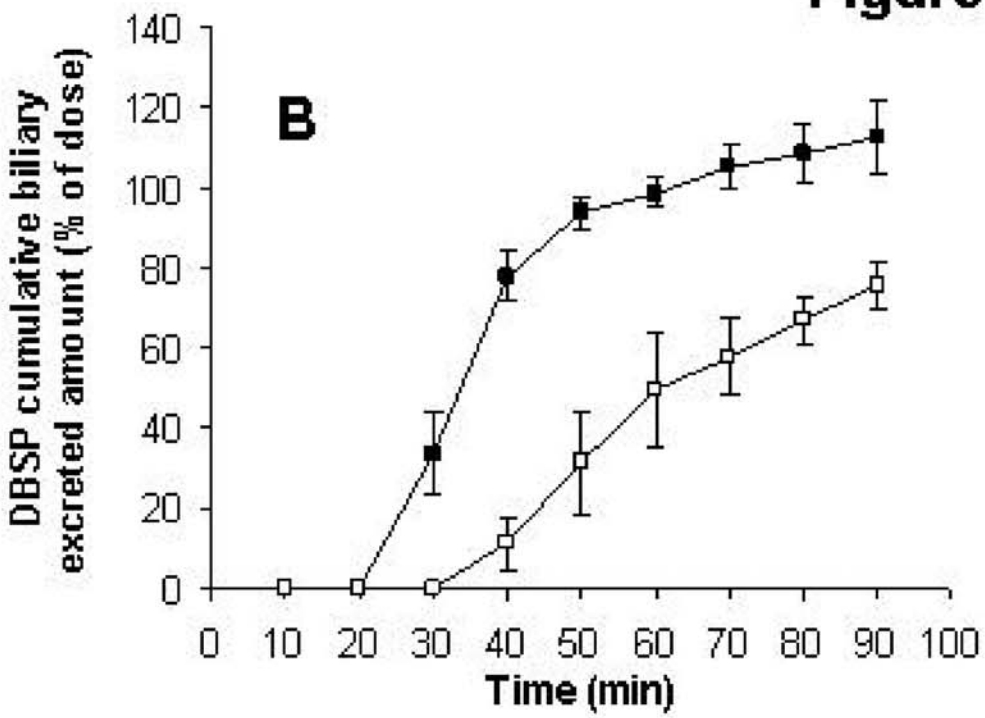
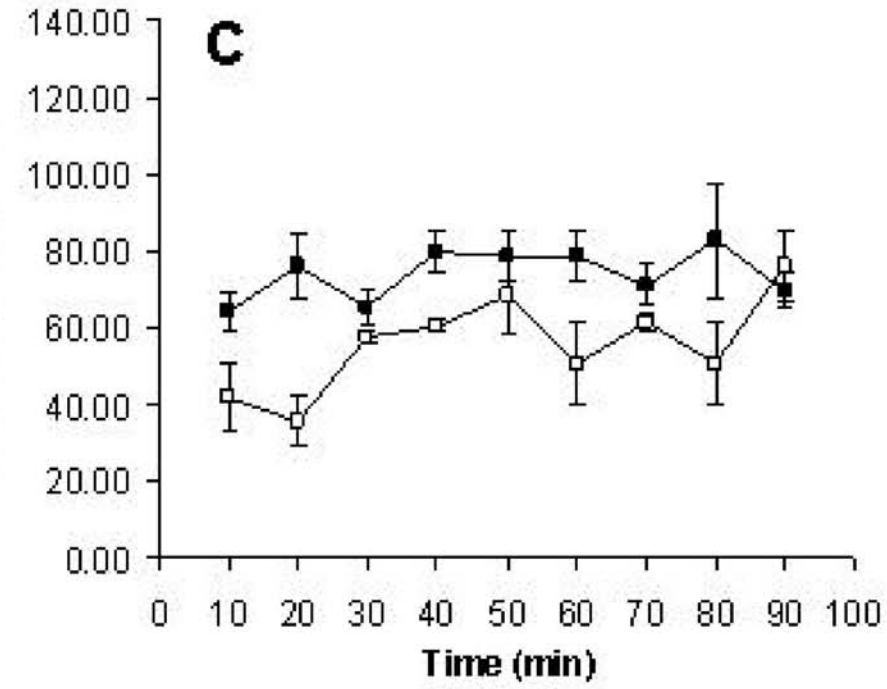
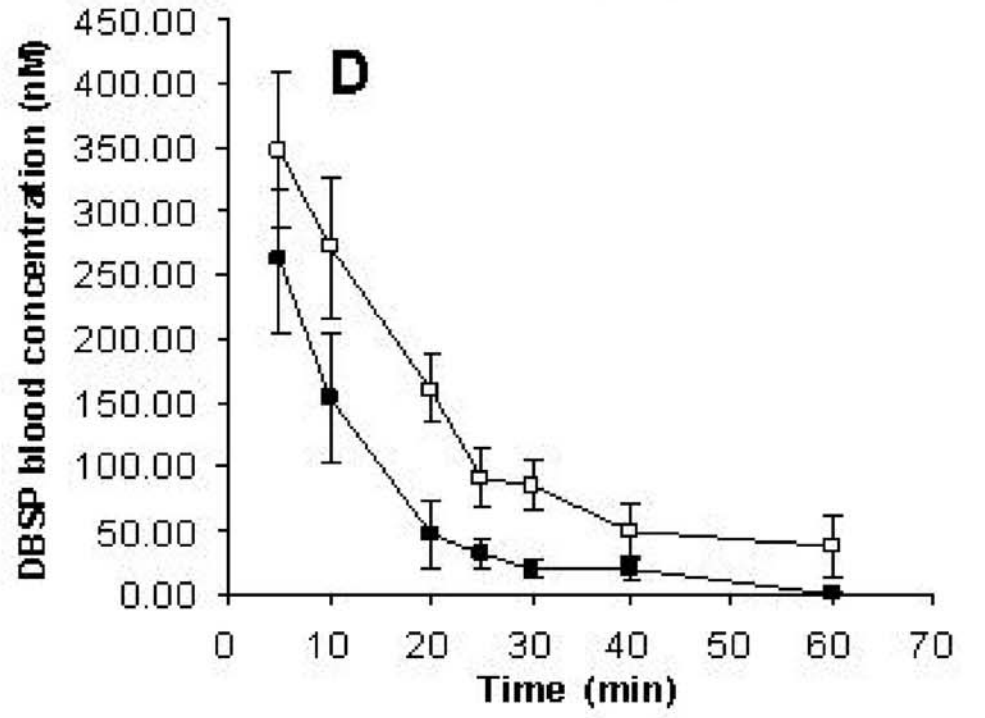


Figure 3









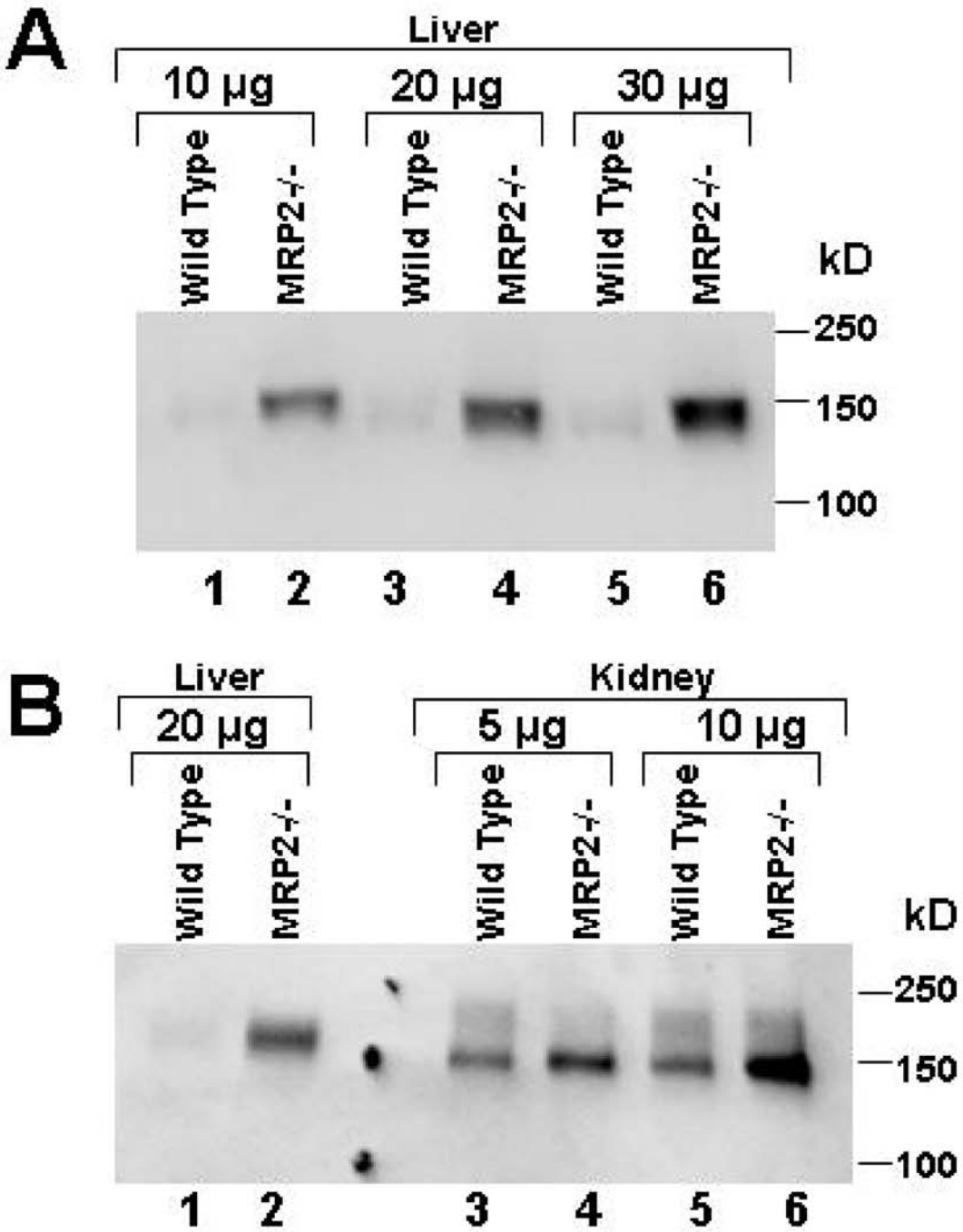


Figure 4