Carcinogen and anti-cancer drug transport by Mrp2 in vivo: studies using Mrp2 (Abcc2) knockout mice


Running title: Mrp2 (Abcc2) knockout mice

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Number of text pages: 35 (excluding title pages)

Number of tables: 0

Number of figures: 7

Number of references: 40

Number of words in Abstract: 250

Number of words in Introduction: 583

Number of words in Discussion: 1495

Abbreviations: ABC, ATP-binding-cassette; Bcrp, breast cancer resistance protein; EHBR, Eisai hyperbilirubinemic rat; GSH, glutathione; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; MRP, multidrug resistance protein; MTX, methotrexate; P-gp, P-glycoprotein; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; SD rat, Sprague Dawley rat; SN-38, 7-ethyl-10-hydroxycamptothecin.

Recommended section: Absorption, Distribution, Metabolism, and Excretion
ABSTRACT

The ABC transporter MRP2 (ABCC2) forms a natural barrier and efflux system for various (conjugates of) drugs, other xenotoxins and endogenous compounds. To obtain insight in the pharmacological and physiological functions of Mrp2, we generated Mrp2 knockout mice, which were viable and fertile but suffered from mild hyperbilirubinemia due to impaired excretion of bilirubin monoglucuronides into bile. The mice also had an 80-fold decreased biliary glutathione excretion and a 63% reduced bile flow. Levels of Mrp3 (Abcc3) in liver and Mrp4 (Abcc4) in kidney of Mrp2-/- mice were about 2-fold increased. After oral administration of the food-derived carcinogens [14C]PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and [14C]IQ (2-amino-3-methylimidazo[4,5-f]quinoline) plasma values were 1.9-fold and 1.7-fold higher in Mrp2-/- mice vs. wild-type mice respectively, demonstrating the role of Mrp2 in restricting exposure to these compounds. At a high dose of 50 mg/kg of the drug [3H]methotrexate, the plasma AUC for i.v. administration was 1.8-fold higher in Mrp2-/- mice (1345 ± 207 min·µg/ml vs. 734 ± 81 min·µg/ml). No clear plasma concentration difference arose at low dose (1 mg/kg). Subsequently Mdr1a/b/Mrp2 knockout mice were generated. Their biliary excretion of doxorubicin after i.v. administration (5 mg/kg) was 54-fold decreased (0.32 ± 0.13 nmol/gr liver vs. 17.30 ± 6.59 nmol/gr liver in wild-type), and a role for both Mdr1a/b and Mrp2 in this process was revealed. Our results demonstrate that the Mrp2-/- mouse provides a valuable tool for studies of the impact of Mrp2 on behavior of drugs and other toxins, especially when combined with other ABC transporter knockout mice.
The multidrug transporter MRP2 (ABCC2, cMOAT), member of the ATP-binding-cassette (ABC) superfamily, confers resistance to a range of anti-cancer drugs (Borst and Oude Elferink, 2002; Chan et al., 2004). The protein is mainly present in the apical membranes of polarized cells in liver, small intestine and kidney and mediates active transport of both endogenous and xenobiotic compounds to bile, urine or feces (Chan et al., 2004). MRP2 is functionally deficient in patients with the Dubin-Johnson syndrome (Zimniak, 1993), in TR rats (Jansen et al., 1985) and EHBRs (Eisai hyperbilirubinemice rats) (Hosokawa et al., 1992), which all show impaired secretion of bilirubin glucuronides into the bile and as a consequence suffer from conjugated hyperbilirubinemia (Hosokawa et al., 1992; Jansen et al., 1985; Zimniak, 1993). The mutant rat strains also show substantially reduced biliary excretion of glutathione and glutathione conjugates (Paulusma et al., 1999).

Besides its role in transport of endogenous compounds, MRP2 plays an important role in the transport of various drugs and their metabolites. MRP2 is expressed in many solid human tumors originating from kidney, colon, breast, lung and ovary (Sandusky et al., 2002), and it was shown that the protein actively transports many types of anti-cancer and other drugs like vinca alkaloids, anthracyclines, protease inhibitors, antibiotics, etoposide, cisplatin, methotrexate and irinotecan in vitro (Bakos et al., 2000; Borst and Oude Elferink, 2002; Chan et al., 2004; Luo et al., 2002). Mrp2 is involved in vivo in the excretion of methotrexate and irinotecan and its metabolites into the bile, as was demonstrated in Mrp2 deficient rats (Chu et al., 1997; Masuda et al., 1997). Experiments with Mrp2 deficient rats indicate that the protein furthermore plays a role in reducing the
oral availability and biliary and intestinal excretion of the heterocyclic amine PhIP, a food-derived carcinogen (Dietrich et al., 2001a; Dietrich et al., 2001b).

To obtain a more complete view of the relative role of the various ABC transporters that perform related functions in vivo, it will be of great interest to establish the precise role of Mrp2 in physiology and pharmacology of drugs and other toxic compounds. The in vivo roles of other ABC transporters, such as P-gp (Abcb1), Bcrp1 (Abcg2), Mrp1 (Abcc1) and Mrp3 (Abcc3) have been studied using previously generated knockout mice for these proteins (Belinsky et al., 2005; Jonker et al., 2002; Schinkel et al., 1997; Wijnholds et al., 1997, Zhou et al., 2002). MRP2 shows substantial overlap in substrate specificity with these members of the ABC transporter family (Borst and Oude Elferink, 2002; Chan et al., 2004). Mrp2 knockout mice will therefore be very useful tools to study the in vivo role of Mrp2 and compare this with that of other ABC transporters. Importantly, the existence of Mrp2 knockout mice allows generation of compound knockout mice by crossing this strain with the other ABC transporter knockout strains, permitting systematic analysis of the relative role of each ABC transporter in physiology, drug resistance, and pharmacokinetics of drugs and xenotoxins.

In the present study we generated and subsequently characterized Mrp2−/− mice by analysis of basic physiological parameters, comparing our results with previous results from mutant rats and humans deficient in Mrp2. We used the generated Mrp2−/− mice to analyze the effect of murine Mrp2 deficiency on in vivo behavior of the anti-cancer drugs methotrexate and irinotecan and of the dietary carcinogens PhIP and IQ. The Mrp2−/− mice were subsequently crossed with Mdr1a/b−/− mice to determine the relative roles of both ABC transporters in the biliary excretion of the anti-cancer drug doxorubicin.
METHODS

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. The animals used were Mrp2−/− and wild-type mice of a comparable mixed genetic background (approximately 50% 129/Ola, 50% FVB), between 9-14 weeks of age. For the doxorubicin experiment Mrp2−/− mice were backcrossed to 99% FVB background and subsequently crossed with Mdr1a/b−/− mice to obtain Mdr1a/1b/Mrp2 triple knockout mice (Lagas et al., in preparation). Animals were kept in a temperature-controlled environment with a 12-hour light/12-hour dark cycle. They received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water ad libitum.

Chemicals. [3H]MTX (7.0 Ci/mmol) was obtained from Amersham (Little Chalfont, UK). PhIP, [14C]PhIP (10 Ci/mol), IQ and [14C]IQ (10 Ci/mol) were obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Doxorubicin-HCl (Doxorubin 0.2%), irinotecan (Campto® 20 mg/ml) and MTX (Emthexate PF® 25 mg/ml) were obtained from Pharmachemie (Haarlem, The Netherlands). Ketamine was obtained from Parke-Davis (Hoofddorp, The Netherlands), xylazine from Sigma Chemical Co. (St. Louis, MO) and methoxyflurane (Metofane) from Medical Developments Australia Pty. Ltd. (Springvale, Victoria, Australia). M2III-5 was a kind gift of Dr. G.L. Scheffer (Free University Hospital, Amsterdam, The Netherlands). Sf-9 vesicles containing MRP4, C219, MRPr1, M3-18 and M4I-10 were kind gifts of Dr. N. Zelcer and Dr. K. van de Wetering in our institute. BXP-53 was described before (Jonker et al., 2002).
Generation of Mrp2\(^{-/-}\) and Mdr1a/b/Mrp2 knockout mice. Using Mrp2 cDNA probes, a 129/Ola mouse genomic sequence containing exons 3-7 of Mrp2 was identified (Fig. 1A). A 1.7 kb fragment containing exons 4-6 was deleted and replaced by a 2.0 kb \(pgk\)-hygro cassette in reverse transcriptional orientation. Deletion of exons 4-6 results in removal of amino acids 111 to 209, encoding the transmembrane (TM) regions 3-5 and part of the large intracellular loop between TM regions 5 and 6, and in a frame shift. Electroporation of the targeting construct and selection for recombinant E14 embryonic stem cells was done as described (Jonker et al., 2003). Of 262 hygromycin-resistant clones, 14 were targeted correctly as judged by Southern analysis of BclI-digested genomic DNA with 3’ and 5’ Mrp2 probes (Fig. 1A). The absence of additional \(pgk\)-hygro cassettes elsewhere in the genome was confirmed by hybridization with a hygro-specific probe. Chimeric mice were generated by microinjection of two independently targeted embryonic stem cell clones into blastocysts. Chimeric offspring were backcrossed to FVB mice. By using this approach, two independent Mrp2\(^{-/-}\) mouse lines of a mixed genetic background (approximately 50% 129/Ola, 50% FVB) were established. One of these Mrp2\(^{-/-}\) mouse lines was backcrossed to 99% FVB background and subsequently crossed with Mdr1a/b\(^{-/-}\) mice (Schinkel et al., 1997) to obtain Mdr1a/b/Mrp2 triple knockout mice of a 99% FVB background.

Bile flow measurements and analysis. Gall bladder cannulations in male wild-type and Mrp2\(^{-/-}\) mice (n = 5 for each group) were performed as described (van Herwaarden et al., 2003). Bile of the mice was collected in 15 min fractions in pre-weighed tubes for 60 min. At the end of the experiment, blood was collected by cardiac puncture. Also, urine
of the mice was collected. Bile flow was determined by weighing the collected bile samples, assuming a density of 1.0 g/ml. All samples were frozen immediately and stored at -80°C. Concentrations of bilirubin monoglucuronides, bilirubin diglucuronides and unconjugated bilirubin in bile and urine were determined as described (Spivak and Carey, 1985). GSH in the bile samples was determined as described (Tietze, 1969).

**Pharmacokinetic experiments.** For oral administration of [\(^{14}\)C]PhIP and [\(^{14}\)C]IQ, 5 µl/g body weight of a 0.2 mg/ml [\(^{14}\)C]PhIP or [\(^{14}\)C]IQ solution in 20% (v/v) DMSO and 5% (w/v) D-glucose was dosed by gavage into the stomach of female mice. After 30 min, blood samples were collected by cardiac puncture and levels of radioactivity were determined by scintillation counting. \[^{3}\text{H}]\text{MTX}\) was i.v. administered by injecting 5 µl of drug solution (appropriate concentration in saline)/g body weight into the tail vein of female mice lightly anesthetized with methoxyflurane. Animals were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia and organs were removed and homogenized in 4% BSA using a Polytron homogenizer. Intestinal contents (feces) were separated from the intestinal tissue. Levels of radioactivity in homogenized organs, feces and blood were determined by liquid scintillation counting. For i.v. administration of irinotecan a 2 mg/ml drug solution in 5% (w/v) D-glucose was prepared and 5 µl/g body weight was injected into the tail vein of female mice lightly anesthetized with methoxyflurane. Blood samples were collected by cardiac puncture under methoxyflurane anesthesia and livers were isolated. Of each plasma sample (250 µl) obtained after centrifugation at 5,000 rpm for 5 min at 4°C, 50 µl was frozen in dry ice immediately after harvesting and used for determination of the total irinotecan and SN-38...
concentrations in the blood. The remaining 200 µl of the plasma was used for the separate determination of lactone and carboxylate forms of irinotecan and SN-38 and was processed as described (Herben et al., 1998). Livers were homogenized in human plasma (5 ml/liver) and frozen in liquid nitrogen. Samples were stored at -80°C until HPLC analysis was performed. Measurements of total irinotecan and SN-38 and the lactone and carboxylate forms of irinotecan and SN-38 were based on the HPLC-technology as described previously (Herben et al., 1998). Doxorubicin-HCl was administered i.v. to female mice by injecting 2.5 µl/g body weight of a 2 mg/ml solution directly after gall bladder cannulation, which was performed as described (van Herwaarden et al., 2003). Bile was collected for 60 minutes and mice were subsequently killed by terminal bleeding through cardiac puncture. Plasma samples, liver, small intestine and small intestinal contents were collected and stored at −80°C until HPLC analysis, which was performed as described (van Asperen et al., 1998).

Clinical-chemical analysis of plasma. Standard clinical chemistry analyses on plasma were performed on a Hitachi 917 analyzer to determine levels of total and conjugated bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatinine, urea, Na+, K+, Cl−, Ca2+, phosphate, total protein and albumin.

Hematological analysis. Hemoglobin, hematocrit, mean corpuscular volume, red blood cells, white blood cells, lymphocytes, monocytes, granulocytes and platelets were
determined in EDTA blood on a Beckman Coulter Ac-T Diff analyzer. This analysis was performed 2 times within a time span of 2 months.

**Western analysis.** Crude membrane fractions from tissues were prepared as described (Ogihara et al., 1996). Western blotting was performed as described (Jonker et al., 2003), the ECL signal was detected by film. Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer (not shown). In the dilution series for Mrp3 and Mrp4 expression, both Ponceau S and India ink staining of the membranes could readily reveal 2-fold protein loading differences. P-gp was detected with mAb C219 (dilution 1:1000) and Mrp1 with mAb MRPr1 (1:1000). For detection of Mrp2 the primary antibody M_{2III}-5 was used (dilution 1:50). Mrp3 and Mrp4 were detected with mAbs M_{3-18} (dilution 1:50) and M_{4I-10} (dilution 1:5) respectively. Bcrp1 was detected using the mAb BXP-53 (dilution 1:400). The primary antibodies were detected by incubating the blot either with rabbit anti-mouse IgG (for M_{2III}-5 and C219) or rabbit anti-rat IgG (for MRPr1, M_{3-18}, M_{4I-10} and BXP-53) (both 1:1000, DAKO).

**Histological analysis.** Tissues were fixed in 4% phosphate-buffered formalin and embedded in paraffin. Sections were cut at 4 μm from the paraffin blocks and stained with hematoxylin and eosin according to standard procedures. The sections were examined by a mouse pathologist.

**Pharmacokinetic calculations and statistical analysis.** The two-sided unpaired Student's t-test was used throughout the study to assess the statistical significance of
differences between two sets of data. Results are presented as the means ± standard deviations (SD). Differences were considered to be statistically significant when $P < 0.05$. Averaged concentrations for each time-point were used to calculate the area under the plasma concentration versus time curve (AUC) from $t = 0$ to the last sampling point by the linear trapezoidal rule; standard errors (SE) were calculated by the law of propagation of errors (Bardelmeijer et al., 2000). Results of AUC measurements are presented as means ± standard error of the mean (SEM).
RESULTS

Generation and analysis of \textit{Mrp2}^\text{−/−} mice. \textit{Mrp2} knockout mice were generated as outlined in Materials and Methods and Fig. 1A. Complete absence of Mrp2 protein in \textit{Mrp2}^\text{−/−} mice was confirmed with Western blot analysis using M2III-5, an antibody that binds to an epitope in amino acids 1339-1541 at the C-terminus of rat Mrp2 (Fig. 1B). \textit{Mrp2}^\text{−/−} mice were fertile and had normal life spans and body weights. Crosses of \textit{Mrp2}^{+/−} mice yielded progeny with all Mrp2 genotypes at the expected Mendelian ratio.

Macroscopic and microscopic histological and pathological analysis did not reveal any obvious specific aberrations in tissues of the \textit{Mrp2}^\text{−/−} mice, including the liver. However, adult \textit{Mrp2}^\text{−/−} mice did have a 20-25% increased liver weight compared to wild-type mice (males: 5.71 ± 0.29% of the total body weight in \textit{Mrp2}^\text{−/−} mice vs. 4.52 ± 0.34% in wild-types, \(n = 5\), \(P = 0.0003\); females: 5.43 ± 0.28% of the total body weight in \textit{Mrp2}^\text{−/−} mice vs. 4.32 ± 0.56% in wild-types, \(n = 7-8\), \(P = 0.0003\)).

Expression of other ABC multidrug transporter proteins in tissues of \textit{Mrp2}^\text{−/−} mice. Since many multidrug transporters have overlapping substrate specificities, absence of Mrp2 could result in (compensatory) induction of expression of other ABC multidrug transporters. Therefore the levels of P-gp, Bcrp1, Mrp1, Mrp3 and Mrp4 protein were determined by Western blot analysis of tissues from male and female \textit{Mrp2}^\text{−/−} mice. There was no up-regulation of P-gp and Bcrp1 in the liver of \textit{Mrp2}^\text{−/−} mice (data not shown). Mrp1 was not detectable in livers of wild-type and \textit{Mrp2}^\text{−/−} mice, but Mrp1 expression in the kidneys of the \textit{Mrp2}^\text{−/−} mice was unchanged (data not shown). In contrast, the level of Mrp3 in the liver of \textit{Mrp2}^\text{−/−} mice was ~2 fold increased compared to wild-type (Fig. 2,
upper panel). Mrp4 in male liver was low and therefore hard to quantify in both wild-type and Mrp2<sup>-/-</sup> mice. Since liver Mrp4 expression in female mice is higher than in males (Mahler et al., 2005), we analyzed Mrp4 expression in liver of Mrp2<sup>-/-</sup> females. Mrp4 protein levels were not substantially different between wild-type and Mrp2<sup>-/-</sup> liver (Fig. 2, middle panel). Mrp4 levels in kidney however, were ~2-fold increased in Mrp2<sup>-/-</sup> mice (Fig. 2, lower panel).

**Analysis of plasma composition of Mrp2<sup>-/-</sup> mice.** Clinical chemical analysis of the plasma of Mrp2<sup>-/-</sup> mice showed a moderately increased (~2-fold in males and ~3-fold in females) level of total bilirubin compared to wild-type mice (Fig. 3A). This difference could be attributed mainly to elevated levels of conjugated bilirubin in the Mrp2<sup>-/-</sup> mice (Fig. 3B). The absolute increase could not be quantified, because conjugated bilirubin levels of wild-type plasma were below the detection limit of the analyzer (<1 µM). The other clinical-chemical parameters measured (see Materials and Methods) showed no significant differences between wild-type and Mrp2<sup>-/-</sup> mice.

**Hematological analysis of Mrp2<sup>-/-</sup> mice.** Extensive hematological analysis (see Materials and Methods) was performed on blood from wild-type and Mrp2<sup>-/-</sup> mice (n = 12, 6 males and 6 females). Within two months two separate sets of analyses were performed. The only measured parameter that consistently showed significant differences between wild-type and Mrp2<sup>-/-</sup> mice was hemoglobin concentration, which was lower in Mrp2<sup>-/-</sup> mice. In the first analysis the hemoglobin levels were 8.7 ± 0.4 mM in wild-type mice and 8.1 ± 0.6 mM in Mrp2<sup>-/-</sup> mice (n = 12, P = 0.007), which was qualitatively
confirmed in the second analysis. Hemoglobin levels did not differ significantly between sexes in either wild-type or Mrp2−/− mice.

**Analysis of bile and urine composition of Mrp2−/− mice.** Mrp2 is involved in the excretion of many conjugated and non-conjugated anionic compounds into bile. TR− rats and EHBRs therefore suffer from impaired biliary secretion of glutathione, glutathione conjugates, and bilirubin glucuronides and also have a reduced bile flow (Jansen et al., 1985; Paulusma et al., 1999). We measured the bile flow and the excretion of GSH and bilirubin glucuronides into the bile of male Mrp2−/− mice after gall bladder cannulation. The bile flow in the Mrp2−/− mice was reduced quite constantly to approximately 37% of wild-type bile flow over a 1 hour period (Fig. 4A). Analysis of the collected bile showed a significant decrease in total bilirubin output (75 ± 29 pmol/min per g liver for Mrp2−/− mice and 135 ± 22 pmol/min per g liver for wild-type mice), which was mainly due to a decrease in bilirubin monoglucuronide output (46 ± 18 pmol/min per g liver for Mrp2−/− mice and 120 ± 16 pmol/min per g liver for wild-type mice) (Fig. 4B). The Mrp2−/− mice also showed a dramatic decrease in glutathione output into the bile (~80 fold) to only 1.2% of wild-type output (0.5 ± 0.2 nmol/min per g liver for Mrp2−/− mice and 41.2 ± 6.7 nmol/min per g liver for wild-type mice) (Fig. 4C). Analysis of the urine of the two mouse strains showed a 7-fold increase of total bilirubin concentration in the Mrp2−/− mice (Fig. 4D). This was primarily due to a dramatic increase (47-fold) in the bilirubin monoglucuronide concentration in the urine of the Mrp2−/− mice. This suggests that in Mrp2−/− mice excretion via the urine is used as an alternative route for diminished bilirubin monoglucuronide elimination via the liver.
Mouse Mrp2 restricts plasma levels of the dietary carcinogens PhIP and IQ. Many heterocyclic amines formed in cooked meat are highly mutagenic and carcinogenic (Gooderham et al., 1996). MDCKII cells expressing human MRP2 have been shown to transport one of these heterocyclic amines, PhIP, thereby providing a possible mechanism to protect the body from this dietary carcinogen (Dietrich et al., 2001b). To test whether Mrp2 affects availability of PhIP in the mouse, we administered 1 mg/kg $[^{14}C]$PhIP orally to female wild-type and $Mrp2^{-/-}$ mice and measured radioactivity in plasma after 30 minutes. This experiment showed a 1.9-fold increased plasma value of $[^{14}C]$PhIP in $Mrp2^{-/-}$ compared to wild-type mice ($202 \pm 73$ ng/ml versus $107 \pm 27$ ng/ml, $n = 8$, $P = 0.0037$), as shown in Fig. 5. This indicates that murine Mrp2 contributes to protection of the body from the dietary carcinogen PhIP.

We further tested the role of murine Mrp2 in the oral availability of another heterocyclic amine dietary carcinogen, IQ. 1 mg/kg $[^{14}C]$IQ was orally administered to wild-type and $Mrp2^{-/-}$ mice and plasma radioactivity was measured after 30 minutes. The plasma levels in $Mrp2^{-/-}$ mice were 1.7-fold higher than in wild-type mice ($346 \pm 72$ ng/ml versus $209 \pm 46$ ng/ml, $n = 5$, $P = 0.0071$) (Fig. 5), indicating a significant role for Mrp2 in limiting exposure of the body to IQ as well.

Dose-dependent role of Mrp2 in elimination of the anti-cancer drug MTX from blood. Mrp2 is known to play a role in transport of, and resistance to, the drug MTX in vitro (Bakos et al., 2000; Borst and Oude Elferink, 2002). It was also shown using Mrp2 deficient rats that Mrp2 excretes MTX into bile (Masuda et al., 1997). To test whether Mrp2 in mice plays a role in the elimination of MTX, $[^{3}H]$MTX was administered i.v. to
female wild-type and Mrp2⁻/⁻ mice at dosages of 1, 10 and 50 mg/kg. The results show that the influence of Mrp2 on [³H]MTX elimination was dose-dependent (Fig. 6A). At 1 mg/kg, no difference in the plasma concentration of [³H]MTX between wild-type and Mrp2⁻/⁻ mice was observed at t = 30 min (163 ± 39 ng/ml for Mrp2⁻/⁻ and 152 ± 29 ng/ml for wild-type mice, n = 6, P = 0.57). At 10 mg/kg of [³H]MTX, a moderate (1.6-fold) but significant difference was found (2.30 ± 0.29 µg/ml for Mrp2⁻/⁻ and 1.47 ± 0.37 µg/ml for wild-type mice, n = 3-4, P = 0.021), whereas for 50 mg/kg the difference in plasma level between Mrp2⁻/⁻ and wild-type mice was 2.2-fold (14.5 ± 4.3 µg/ml versus 6.49 ± 1.92 µg/ml, n = 3-4, P = 0.019). This indicates that Mrp2 in mice becomes relatively more important in elimination of [³H]MTX when the administered dose and thereby the plasma level increases. The amount of [³H]MTX in the contents of the small intestine at 30 min after i.v. administration of 50 mg/kg [³H]MTX was significantly lower in the Mrp2⁻/⁻ mice (4.87 ± 4.51% versus 11.40 ± 1.84% in wild-type mice) (Fig. 6B), which suggests that Mrp2 is important in hepatobiliary and/or direct intestinal excretion of [³H]MTX into the small intestinal lumen. This difference is reflected by a lower amount of [³H]MTX in the small intestinal wall in Mrp2⁻/⁻ compared to wild-type mice (1.50 ± 0.78% of the dose versus 3.94 ± 1.28%), as shown in Fig. 6B. There was no difference in radioactivity in the livers of both mouse strains 30 min after [³H]MTX administration (Fig. 6B).

Plasma samples at different time points after i.v. administration of 50 mg/kg [³H]MTX were obtained to generate a plasma concentration versus time curve and investigate the time-dependence of [³H]MTX elimination by Mrp2 (Fig. 6C). The difference in elimination between wild-type and Mrp2⁻/⁻ mice was most pronounced during the first 15 min after administration, when plasma levels were high. Already at 7.5
min after administration there was a significantly higher plasma level of $[^3\text{H}]\text{MTX}$ in $Mrp2^{-/-}$ mice. The area under the curve between 7.5 and 120 minutes was 1.8-fold increased in $Mrp2^{-/-}$ mice ($1345 \pm 104 \text{ min} \cdot \mu\text{g/ml}$ versus $734 \pm 37 \text{ min} \cdot \mu\text{g/ml}$ in wild-type mice, $n = 3-6$, $P = 0.032$, means ± SEM), indicating that Mrp2 plays a significant role in the plasma elimination (and thus exposure) of MTX in mice.

**Murine Mrp2 does not affect irinotecan and SN-38 pharmacokinetics after i.v. administration of 10 mg/kg irinotecan.** In Mrp2 deficient rats (EHBRs) the plasma clearance of total and especially the carboxylate forms of irinotecan and SN-38 after i.v. administration was found to be much slower than in wild-type rats (Chu et al., 1997). We therefore tested whether this was similar in Mrp2 deficient mice. After i.v. administration of irinotecan at a 10 mg/kg dose to female mice, no differences in plasma clearance were measured for total irinotecan and SN-38 in $Mrp2^{-/-}$ mice (Supplementary Figure 1). Also, no differences in plasma values of the separate lactone or carboxylate forms of irinotecan and SN-38 were measured (data not shown). Apparently, in contrast to rats, Mrp2 in mice does not substantially impact on the clearance of irinotecan and its metabolites, at least not when the drug is administered at a dose of 10 mg/kg.

**Mrp2 and Mdr1a/b play distinct roles in the biliary excretion of doxorubicin.**

Recently we have obtained $Mdr1a/b/Mrp2$ triple knockout mice by crossing the above-described $Mrp2^{-/-}$ mice with previously generated $Mdr1a/b$ double knockout mice (Lagas et al., in preparation). With this model, which is readily amenable to pharmacological analysis, we can determine the relative roles of both P-gp and Mrp2 in the elimination
and excretion of shared substrates. To determine the relative roles of Mrp2 and Mdr1a/b in biliary and direct intestinal excretion of the anti-cancer drug doxorubicin, which is a substrate for both ABC transporters (Borst and Oude Elferink, 2000; Chan et al., 2004), we performed gall bladder cannulations and administered doxorubicin i.v. to female wild-type, Mrp2−/−, Mdr1a/b−/− and Mdr1a/b/Mrp2−/− mice (all in 99% FVB background) at a dose of 5 mg/kg. After 60 minutes, there were no significant differences in plasma doxorubicin levels between wild-type and (combination) knockout mice (not shown, see Supplementary Table 1). Similarly, there were no significant differences in doxorubicin levels of liver, nor in small intestine and small intestinal contents, indicating that neither Mdr1a/b, nor Mrp2 play a substantial role in direct intestinal excretion of doxorubicin (Supplementary Table 1), or that an alternative intestinal transporter can compensate for absence of Mrp2 and Mdr1a/b.

The biliary doxorubicin output in 60 minutes is shown in Figure 7. In Mrp2−/− mice the biliary doxorubicin excretion was about 2-fold (51%) decreased (8.45 ± 1.85 nmol/g liver for Mrp2−/− mice versus 17.30 ± 6.59 nmol/g liver for wild-type mice, n = 4-5, P = 0.041), indicating that Mrp2 can transport doxorubicin into the bile. In Mdr1a/b−/− mice the biliary excretion of doxorubicin is about 10-fold (90%) decreased (1.68 ± 0.64 nmol/g liver for Mdr1a/b−/− versus 17.30 ± 6.59 nmol/g liver for wild-type mice, n = 3-4, P = 0.010), indicating a more substantial role for Mdr1a/b than for Mrp2 in the biliary excretion of doxorubicin. It was previously shown in single Mdr1a−/− mice that the biliary excretion was about 5-fold (80%) reduced compared to wild-type mice after 90 minutes (van Asperen et al., 2000). Our data suggest that additional removal of Mdr1b in mice doubles the difference compared to wild-type mice, suggesting that both Mdr1a and
Mdr1b have a separate role in the biliary excretion of doxorubicin. When Mrp2 and Mdr1a/b are all absent, the biliary excretion of doxorubicin is even further reduced to less than 2% of wild-type biliary excretion (0.32 ± 0.13 nmol/g liver for Mdr1a/b/Mrp2 knockout mice versus 17.30 ± 6.59 nmol/g liver for wild-type mice, n = 4-5, P = 0.0006). The excretion of doxorubicin in the Mdr1a/b/Mrp2 knockout mice is 5-fold lower than in the Mdr1a/b+/− mice (n = 3-5, P = 0.0028) and 26-fold lower than in the Mrp2+/− mice (n = 4-5, P = 2.2 * 10⁻⁵), indicating that Mrp2 and Mdr1a/b can partly compensate for the absence of the other ABC transporter. From these data we conclude that both Mrp2 and Mdr1a/b can transport doxorubicin into the bile, although the roles of Mdr1a/b are more pronounced.
DISCUSSION

Here we present the generation and characterization of an Mrp2 knockout mouse. These mice display several modest aberrations, like mild hyperbilirubinemia due to impaired secretion of conjugated bilirubin into the bile, and a reduction in bile flow and biliary GSH excretion. Mrp2/− mice also display a 20-25% increased liver size. Nevertheless, they appear quite healthy overall and therefore provide an excellent model to study the role of Mrp2 in physiology and pharmacokinetics of drugs and other toxic compounds. While this study was prepared for submission, Chu et al. (2006) published a basic characterization of an independently generated Mrp2/− strain, against a different genetic background (C57BL/6). Like us, also based on their complementary analysis of (limited) expression changes in detoxifying genes in Mrp2/− mice, Chu et al. conclude that these mice provide valuable tools to study the role of Mrp2 in drug disposition. Whereas many physiological changes in the two knockout strains are similar (e.g. reduced bile flow and reduced biliary output of conjugated bilirubin and GSH), there are also some differences, as discussed below.

Although the generated Mrp2/− mice look quite healthy overall, the liver weight of these mice is 20-25% increased. This could be caused by accumulation of Mrp2 substrates in the liver, possibly promoting liver proliferation. An increased liver weight has also been shown in the EHBR previously (Hosokawa et al., 1992). Whether liver weight was increased in the Mrp2/− strain analyzed by Chu et al. (2006) was not reported.

In our Mrp2/− mouse model the protein levels of Mrp3 in liver and Mrp4 in kidney are about 2-fold induced. Elevated levels of Mrp3 in the liver have also been found in Mrp2-deficient rats and Dubin-Johnson patients (Donner and Keppler, 2001),
although in rats and humans the Mrp3 induction is more pronounced. It is noteworthy that Chu et al. (2006) found no induction of hepatic Mrp3 expression, in contrast to Mrp2-deficient rats, humans and our Mrp2\textsuperscript{\textminus}\textminus mice. Recently, Nezasa et al. (2006) did find elevated hepatic Mrp3 protein levels in the same Mrp2\textsuperscript{\textminus}\textminus mouse strain as analyzed by Chu et al. (2006). This indicates that the differences could be due to inter-laboratory variation (e.g. food composition differences).

Consistent with our results, 2-fold higher Mrp4 protein levels in kidney but not in liver were recently detected in TR\textsuperscript{\textplus} rats (Chen et al., 2005). In contrast, Chu et al. (2006) did find clear induction of Mrp4 expression in liver of their Mrp2\textsuperscript{\textminus}\textminus mice. Mrp4 regulation in liver is clearly dependent on the genetic background, as we did see substantial Mrp4 induction in liver of the Mrp2\textsuperscript{\textminus}\textminus mice in 99\% FVB background we recently obtained (not shown). Induction of Mrp3 in the liver and Mrp4 in the kidney as a result of Mrp2 deficiency suggests that compensatory expression mechanisms exist for these genes, which are activated due to the absence of Mrp2. The availability of Mrp2\textsuperscript{\textminus}\textminus mice will allow systematic analysis of such compensatory elimination mechanisms by studying separate and compound (Mrp3, Mrp4) knockout mice.

Even though the transport of bilirubin monoglucuronides into the bile is significantly decreased in Mrp2\textsuperscript{\textminus}\textminus mice, bilirubin monoglucuronides are still present in their bile. This suggests that another anion transporter, possibly Bcrp1 (Abcg2) or Bsep (Abcb11), in the apical membrane of hepatocytes is capable of transporting conjugated bilirubin into the bile.

Others have shown transport of the heterocyclic amine PhIP by human MRP2 \textit{in vitro} (Dietrich et al., 2001b) and rat Mrp2 has been shown to protect the body against this
carcinogen by biliary excretion and reducing the bioavailability of PhIP (Dietrich et al., 2001a; Dietrich et al., 2001b). In this study we show that mouse Mrp2 also plays a clear role in protecting the body from this carcinogen upon oral administration. We further show that murine Mrp2 limits the plasma levels of another food-derived carcinogen, IQ, after oral administration. The results suggest that Mrp2 could be involved in protecting the body from these and many more (food-derived) carcinogens.

For the drug MTX we found that in mice Mrp2 plays a significant role in the elimination from the body, but only at a comparatively high dose (50 mg/kg) and not (or much less) at lower doses (1 or 10 mg/kg). Already 7.5 min after high dose (50 mg/kg) i.v. administration a clear difference between plasma MTX values in wild-type and $Mrp2^{-/-}$ mice was observed (Fig. 6C), indicating a role for Mrp2 in the elimination of MTX at high plasma concentrations. This is consistent with the idea that Mrp2 is a transporter with a low affinity, but high capacity for MTX (Bakos et al., 2000; Zelcer et al., 2003a), causing a relatively large impact of Mrp2 on the total MTX elimination only at high plasma concentrations. At lower dosages (and plasma concentrations) other MTX clearance mechanisms appear to predominate. Accordingly, beyond 20-30 min after MTX administration (Fig. 6C), when the plasma levels of MTX had decreased, MTX clearance was no longer higher in wild-type than in $Mrp2^{-/-}$ mice. A similar result was found earlier when the role of mouse Bcrp1 in MTX plasma clearance was investigated (Breedveld et al., 2004). Interestingly, also in humans a role for MRP2 in elimination of high-dose MTX has been suggested. A patient with a heterozygous loss of function mutation in ABCC2 who received high-dose MTX i.v. had a three-fold reduced
methotrexate elimination rate, leading to severe over-dosing and nephrotoxicity (Hulot et al., 2005). Investigations in humans and rats suggest that MTX metabolism is quite limited (Brunton et al., 2005, Masuda et al., 1997). Our data presenting radioactivity plasma levels are quite comparable to data from the study in male mice by Li et al. (2004), who determined unchanged MTX plasma levels by HPLC. We conclude that the amount of radioactivity we measure in the plasma probably accounts mainly for unchanged \[^3\text{H}\]MTX.

The reduced levels of MTX in the intestinal wall of the Mrp2\^/- mice likely reflect the reduced intestinal content values, which indicate that Mrp2 is involved in biliary and/or direct intestinal excretion of MTX. MTX levels in the liver were not different in Mrp2\^/- mice at $t = 30$ min. A possible explanation is the up-regulation of Mrp3 in the liver of Mrp2\^/- mice. Since Mrp3 can transport MTX (Chan et al., 2004), Mrp3 in the hepatocytes of Mrp2\^/- mice may compensate for reduced biliary MTX excretion by transporting accumulated MTX back into the circulation of Mrp2\^/- mice. It could also be that the small amount of radioactivity in the liver ($<5\%$) mainly accounts for metabolites of \[^3\text{H}\]MTX that are not Mrp2 substrates.

We investigated the role of Mrp2 in the elimination of irinotecan and/or its active metabolite SN-38 using our Mrp2\^/- mice. In a previous study on the role of rat Mrp2 in elimination of irinotecan and SN-38 (Chu et al., 1997), there was a clear difference between SD rats and Mrp2 deficient EHBRs in the biliary excretion of irinotecan and SN-38. In our study, no role for murine Mrp2 in the elimination of irinotecan or SN-38 was found, showing a clear difference between Mrp2-deficient mice and rats.
mean that murine Mrp2 is a comparatively poor irinotecan transporter compared to rat Mrp2, or possibly that other ABC transporters, like Bcrp1 and P-gp, of which the human homologues have been shown to transport irinotecan in vitro (Luo et al., 2002; Maliepaard et al., 1999), play a more pronounced role in the elimination of this drug in mice.

Crossing the generated Mrp2−/− mice with other ABC transporter knockout mice enables us to study the relative roles of the various ABC transporters in transport of shared substrates in vivo, as we show here with Mdr1a/b/Mrp2 triple knockout mice. Our experiment with the anti-cancer drug doxorubicin shows that Mdr1a, Mdr1b and Mrp2 can all transport this shared substrate into the bile, but the influence of Mdr1a and Mdr1b is more substantial than that of Mrp2. From our results it seems that both Mrp2 and Mdr1a/b can take over at least part of each other’s functions. Of course differences between humans and mice could exist, so translation to the human situation must be done cautiously. We also show with this experiment that neither Mrp2, nor Mdr1a/b play a detectable role in the direct intestinal excretion of doxorubicin. This suggests that another intestinal transporter is involved in this process.

We conclude that murine Mrp2 plays an important role in oral availability and elimination of drugs and carcinogens, most likely by biliary excretion and possibly by decreasing uptake from the small intestine. The Mrp2−/− mice allow us to generate many different combination knockout mice by crossing them with other ABC transporter knockout mice. These will provide important tools to define the relative contributions of the different ABC transporters to drug resistance, drug pharmacokinetics and protection against endogenous and exogenous toxins. This will eventually lead to a better
understanding of the role of ABC-transporters in intra- or inter-individual variation in response to drugs and thus improve the possibility of optimizing drug treatment for individual patients, and minimizing side effects.
ACKNOWLEDGEMENTS

We thank our colleagues for critical reading of the manuscript, Rob Lodewijks and Hans Tensen for excellent technical assistance, Martin van der Valk for histological analysis, and George Scheffer, Koen van de Wetering and Noam Zelcer for kindly providing the antibodies and the Sf-9 vesicles.
REFERENCES


FOOTNOTES

a) This work was supported by grant NKI 2003-2940 from the Dutch Cancer Society.


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LEGENDS FOR FIGURES

Figure 1
Generation and validation of Mrp2\(^{-/-}\) mice. A, in the targeting construct, a 1.7 kb fragment containing exons 4-6 was deleted and replaced with an inverted 2.0 kb Pgk-hygro cassette. Only relevant restriction sites are indicated and only part of the Mrp2 gene is shown. For Southern analysis, 5’ and 3’ probes as indicated were used on BclI digested genomic DNA. Exons are indicated by closed boxes (exact sizes of exons are not drawn to scale) and the sizes of diagnostic restriction fragments for wild-type and targeted alleles are indicated by double-headed arrows (drawn to scale). B, detection of Mrp2 protein in crude membrane fractions of Mrp2\(^{-/-}\) and wild-type mouse liver samples (10 µg/lane). Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer. Lysates of MDCKII cells expressing human MRP2 (Evers et al., 1998) (10 µg) were used as a positive control.

Figure 2
Protein levels of Mrp3 in crude membrane fractions of male liver (upper panel) and Mrp4 protein in crude membrane fractions of female liver (middle panel) and male kidney (lower panel) from two independent wild-type and Mrp2\(^{-/-}\) mice, as detected by Western blot analysis. The wild-type band for Mrp3 is representative for independent wild-type samples tested with the anti-Mrp3 mAb on other blots. In the middle panel, the lane with the positive control (Sf-9 vesicles containing MRP4 (Zelcer et al., 2003b)) is indicated with C, and the marker lane with M. Equal protein loading was confirmed by Ponceau S.
staining of the membranes after transfer. The amount of protein loaded in each lane (in μg) is noted above the respective lanes.

**Figure 3**

Bilirubin levels in plasma of Mrp2−/− mice. A, plasma values of total bilirubin in wild-type (open bars) and Mrp2−/− mice (closed bars) (means ± SD, n = 5-6, *** P < 0.001). B, plasma values of conjugated bilirubin in Mrp2−/− mice (closed bars) (means ± SD, n = 5-6). The values for wild-type mice were below the detection limit of the analyzer (<1 µM). In previous studies (Jonker et al., 2002), male wild-type mice displayed unconjugated bilirubin levels of 1.5 ± 0.55 µM.

**Figure 4**

Analysis of bile and urine from male wild-type (open bars) and Mrp2−/− mice (closed bars) after gall bladder cannulations. Bile was collected in 15 min fractions over 1 hr. Bile collected in the first 15 min was analyzed. A, bile flow in wild-type and Mrp2−/− mice. B, output of total bilirubin, bilirubin monoglucuronide (BMG), bilirubin diglucuronide (BDG) and unconjugated bilirubin (UCB) in bile of wild-type and Mrp2−/− mice. The output of bilirubin diglucuronide in wild-type mice was below the detection limit of the analyzer (5 µM). C, glutathione output in bile of wild-type and Mrp2−/− mice. D, total bilirubin, bilirubin monoglucuronide (BMG), bilirubin diglucuronide (BDG) and unconjugated bilirubin (UCB) concentration in urine of wild-type and Mrp2−/− mice (n.q., not quantifiable, the detection limit of the analyzer for BDG in urine was 5 µM). Data are means ± SD (n = 5, ** P < 0.01, *** P < 0.001).
Figure 5

Plasma concentrations of $[^{14}\text{C}]\text{PhIP}$ and $[^{14}\text{C}]\text{IQ}$ 30 minutes after oral administration of 1 mg/kg of the compounds to female wild-type (open bars) and $\text{Mrp2}^{-/-}$ (closed bars) mice (means ± SD, n = 5-8, ** P < 0.01).

Figure 6

Effect of Mrp2 on elimination of $[^{3}\text{H}]\text{MTX}$ in female mice. A, plasma concentrations of $[^{3}\text{H}]\text{MTX}$ 30 minutes after i.v. administration of different dosages of MTX (1, 10, 50 mg/kg) to wild-type (open bars) and $\text{Mrp2}^{-/-}$ (closed bars) mice (means ± SD, n = 3-6, * P < 0.05). After administration of 1 mg/kg $[^{3}\text{H}]\text{MTX}$ the plasma concentrations were 152 ± 29 ng/ml in wild-type and 163 ± 39 ng/ml in $\text{Mrp2}^{-/-}$ mice (n = 6, P = 0.57). B, $[^{3}\text{H}]\text{MTX}$ in small intestinal tissue, small intestinal contents and liver 30 minutes after i.v. administration of 50 mg/kg $[^{3}\text{H}]\text{MTX}$ to wild-type (open bars) and $\text{Mrp2}^{-/-}$ mice (closed bars) (means ± SD, n = 3-4, * P < 0.05). C, plasma concentration versus time curves after i.v. administration of 50 mg/kg $[^{3}\text{H}]\text{MTX}$ to wild-type and $\text{Mrp2}^{-/-}$ mice (means ± SEM, n = 3-6, P = 0.032 for area under the curves, * P < 0.05).

Figure 7

Hepatobiliary excretion of doxorubicin in wild-type, $\text{Mrp2}^{-/-}$, $\text{Mdr1a/b}^{-/-}$ and $\text{Mdr1a/b/Mrp2}^{-/-}$ mice (means ± SD, n = 3-5, * P < 0.05, *** P < 0.001). Doxorubicin (5 mg/kg) was administered i.v. to mice with a cannulated gall bladder. Bile fractions were collected over a 60 min period. After 60 min, plasma, liver, small intestine and small intestinal contents were collected. All samples were analyzed by HPLC. Biliary
doxorubicin output in 60 min as % of the administered dose in the different strains: wild-type 9.43 ± 3.05 %; Mrp2\(^{-/-}\) 6.18 ± 1.31 % (n = 4-5, P = 0.067); Mdr1a/b\(^{-/-}\) 0.89 ± 0.30 % (n = 3-4, P = 0.005); Mdr1a/b/Mrp2\(^{-/-}\) 0.23 ± 0.11 % (n = 4-5, P = 0.0002). (Doxorubicin levels in plasma, liver small intestine and small intestinal contents are shown in Supplementary Table 1.)
Figure 1

A

1 kb

13.9 kb

BclI Apal

exon 3

exon 3 4 5 6 7

Sall BclI

wild-type allele

Asp718 BclI

exon 3

PGK-Hygro

Apal

7

Sall BclI

targeting construct

BclI Apal

exon 3

PGK-Hygro

7

Sall BclI

mutant allele

5' probe

9.1 kb

3' probe

5.1 kb

B

MDCK-MRP2

Mrp2-/-

Wild-type

- 216 kD

- Mrp2

male female male female male female male female
Figure 2

Wild-type  Mrp2<sup>−/−</sup>

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Figure 3

A

Plasma total bilirubin (µM)

Wild-type

Mrp2-/-

Male

Female

***

B

Plasma conjugated bilirubin (µM)

Wild-type

Mrp2-/-

Male

Female

< 1
Figure 4

A

Bile flow (µl/min,g liver⁻¹)

0 1 2 3 4

0-15 15-30 30-45 45-60

Time (min)

*** *** *** ***

B

Bile bilirubin output (pmol/min,g liver⁻¹)

Total bilirubin BMG BDG UCB

Wild-type Mrp2⁻/⁻

<10

C

Bile GSH output (pmol/min,g liver⁻¹)

0 10 20 30 40 50

Wild-type Mrp2⁻/⁻

***

D

Urine bilirubin concentration (µM)

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4

Total bilirubin BMG BDG UCB

*** *** n.q.

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Figure 5

![Chemical structures](image)

Plasma level (ng/ml)

- **Wild-type**
- **Mrp2**

- PhIP
- IQ

**P** values:
- **PhIP**: **P** < 0.01
- **IQ**: **P** < 0.01
Figure 6

A

$[^3]H$MTX plasma concentration (μg/ml)

1 mg/kg 10 mg/kg 50 mg/kg

Wild-type Mrp2$^{-/-}$

* indicates significant difference

B

$[^3]H$MTX (% of dose)

contents tissue liver

Wild-type Mrp2$^{-/-}$

* indicates significant difference

C

$[^3]H$MTX plasma concentration (μg/ml)

Wild-type Mrp2$^{-/-}$

* indicates significant difference

Time (min.)
Figure 7

Biliary doxorubicin output (nmol/g liver)

fvb    Mrp2 ko    Mdr1a/b ko    Mdr1a/b/Mrp2 ko
Supplementary Fig. 1. Plasma concentration versus time curves for total irinotecan (A) and total SN-38 (B) after i.v. administration of 10 mg/kg irinotecan to female wild-type and Mrp2"/" mice (means ± SD, n = 3). Note the differences in X- and Y-axes for both graphs. CPT-11 plasma concentrations at t = 4 hrs and t = 8 hrs were not detectable and therefore are not added to this graph.
Supplementary Figure 1

A

CPT-11 plasma level (ng/ml)

Time (hrs)

B

SN-38 plasma level (ng/ml)

Time (hrs)

Mrp2-/-
Wild-type

Mrp2-/-
Wild-type