**Functionally Overlapping Roles of Abcg2 (Bcrp1) and Abcc2 (Mrp2) in the Elimination of Methotrexate and Its Main Toxic Metabolite 7-Hydroxymethotrexate In vivo**

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**Abstract**

**Purpose:** ABCC2 (MRP2) and ABCG2 (BCRP) transport various endogenous and exogenous compounds, including many anticancer drugs, into bile, feces, and urine. We investigated the possibly overlapping roles of Abcg2 and Abcc2 in the elimination of the anticancer drug methotrexate (MTX) and its toxic metabolite 7-hydroxymethotrexate (7OH-MTX).

**Experimental Design:** We generated and characterized Abcc2;Abcg2−/− mice, and used these to determine the overlapping roles of Abcc2 and Abcg2 in the elimination of MTX and 7OH-MTX after i.v. administration of 50 mg/kg MTX.

**Results:** Compared with wild-type, the plasma areas under the curve (AUC) for MTX were 1.6-fold and 2.0-fold higher in Abcg2−/− and Abcc2−/− mice, respectively, and 3.3-fold increased in Abcc2;Abcg2−/− mice. The biliary excretion of MTX was 23-fold reduced in Abcc2;Abcg2−/− mice, and the MTX levels in the small intestine were dramatically decreased. Plasma levels of 7OH-MTX were not significantly altered in Abcg2−/− mice, but the areas under the curve were 6.2-fold and even 12.4-fold increased in Abcc2−/− and Abcc2;Abcg2−/− mice, respectively. This indicates that Abcc2 compensates for Abcg2 deficiency but that Abcg2 can only partly compensate for Abcc2 absence. Furthermore, 21-fold decreased biliary 7OH-MTX excretion in Abcc2;Abcg2−/− mice and substantial 7OH-MTX accumulation in the liver and kidney were seen. We additionally found that in the absence of Abcc2, Abcg2 mediated substantial urinary excretion of MTX and 7OH-MTX.

**Conclusions:** Abcc2 and Abcg2 together are major determinants of MTX and 7OH-MTX pharmacokinetics. Variations in ABCC2 and/or ABCG2 activity due to polymorphisms or coadministered inhibitors may therefore substantially affect the therapeutic efficacy and toxicity in patients treated with MTX.

**ABCG2 (BCRP) and ABCC2 (MRP2), both members of the ATP-binding cassette (ABC) transporter superfamily, are present in the apical membranes of hepatocytes and epithelial cells of the small intestines and kidney, where they play a role in the excretion of both endogenous and exogenous compounds into bile, feces, and urine. They protect the body from a wide range of potentially toxic compounds and influence the pharmacokinetics of many drugs (1). ABCG2 and ABCC2 have very broad and substantially overlapping substrate specificities. Examples of xenobiotics that are transported by both ABCG2 and ABCC2 are the anticancer drugs doxorubicin, methotrexate (MTX), SN-38, and the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (1–3). Furthermore, they can both transport a range of glucuronide and sulphate conjugates of drugs and endogenous compounds (1, 3–5). Many polymorphisms and mutations of ABCG2 and ABCC2 have been identified, often leading to differences in expression and activity (2, 6, 7). These may partly explain the high interpatient variability in drug response and the occurrence of adverse drug reactions, a major concern in the treatment of cancer patients receiving high doses of potentially toxic drugs (2, 6, 7). Also, effective ABCG2 and/or ABCC2 inhibitors (e.g., elacridar and cyclosporin A) are tested in clinical trials to improve treatment (8). It is therefore important to define the specific and possibly overlapping effects of the different ABC transporters on the pharmacokinetics of (anticancer) drugs. Recently, knockout mouse models for both Abcg2 and Abcc2 have been generated (9–12), which have been used extensively...
significant urinary excretion of MTX and 7OH-MTX. As well as overlapping functions in determining the pharmacokinetics of both transporters on the pharmacokinetics (16), we additionally investigated the relative impact of Abcc2 in determining the i.v. pharmacokinetics of MTX, a widely used anticancer drug and substrate of both transporters Abcc2;Abcg2-/-. Abcc2;Abcg2-/ mice, we show here that Abcc2 and Abcg2 have overlapping in vivo functions in the elimination of MTX and especially 7OH-MTX. Our results suggest that patients with mutations or (heterozygous) polymorphisms in ABCB2 and/or ABCC2 resulting in altered transport activity might be at increased risk during treatment with MTX.

Translational Relevance

The ATP-binding cassette (ABC) transporters ABCC2 and ABCG2 have very broad and substantially overlapping substrate specificities, and many polymorphisms and mutations of ABCB2 and ABCG2 have been identified. A well-known shared substrate of ABCC2 and ABCG2, the anticancer drug methotrexate (MTX), can cause severe and even lethal toxicity in treated patients. Its toxic metabolite 7-hydroxymethotrexate (7OH-MTX) is found in the urine of patients after MTX treatment and is associated with kidney failure. We have shown previously that the ABC transporter Abcc2 has a major impact on the pharmacokinetics of MTX and its toxic metabolite 7OH-MTX in vivo. Using Abcc2-/-, Abcg2-/-, and Abcc2;Abcg2-/- mice, we show here that Abcc2 and Abcg2 have overlapping in vivo functions in the elimination of MTX and especially 7OH-MTX. Our results suggest that patients with mutations or (heterozygous) polymorphisms in ABCB2 and/or ABCC2 resulting in altered transport activity might be at increased risk during treatment with MTX.

Materials and Methods

Animals. The mice were housed and handled according to institutional guidelines complying with Dutch legislation. The generation of Abcc2- (12) and Abcg2- (9) mice has been described. Abcc2;Abcg2- mice were generated by crossbreeding the single knockout strains. All animals were of >99% FVB background and between 9 to 14 wk of age. The animals were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle. They received a standard diet (AM-III; Hope Farms) and acidified water ad libitum.

Chemicals. MTX (Emethexate PF; 25 mg/mL) was from Pharmachemie, 7OH-MTX from Toronto Research Chemicals Inc., and [3H]MTX from GE Healthcare. Ketamine was from Parke-Davis and methoxylfluorane (Metofane) from Medical Developments Australia Pty. Ltd. MRP1, M1I-18, M1I-80, and M1I-54 were kind gifts of Dr. George L. Scheffer (Free University Hospital, Amsterdam, the Netherlands). K12 was kindly provided by Dr. Bruno Stieger (University of Zürich, Zürich, Switzerland). Real-time PCR primers were from Qiagen. All other chemicals and reagents were from Sigma-Aldrich.

Western analysis. Preparation of crude membrane fractions and western blotting were done as described (21, 22). Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer (not shown). Abcc1, Abcc4, Abcc5, and Abch11 were detected as described (16). Abcc3 was detected with monoclonal antibody M3II-18 (dilution, 1:100). Bound primary antibodies were detected as described (12, 16). Densitometric analysis was done with the use of the TINA 2.09 software program (Raytest).

Real-time PCR analysis. RNA isolation, cDNA synthesis, and real-time PCR analysis on livers of female mice (n = 3) were done as described (23).

Vesicular transport assays. ATP-dependent transport of [3H]MTX into inside-out vesicles prepared from S9 cells overproducing human or murine ABCB2/Abcc2 (24) and control S9 cells was determined as described (15).

Histologic, clinical-chemical, and hematologic analysis. Histologic analysis of male and female mouse tissues (n = 6), clinical chemistry analyses (including total and conjugated bilirubin, alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase) on serum of male and female mice (n = 6), and standard hematologic analyses of male and female mice (n = 6; twice within a time span of 1.5 y) were done as described (12).

Bile and plasma collection for bilirubin analysis. Gall bladder cannulations in male wild-type, Abkg2-/-, Abcc2-/-, and Abcc2;Abkg2-/- mice (n = 5–6), and bile and urine collection were done as described (16, 25). Heparin plasma was collected from male mice (n = 4–6) by cardiac puncture under methoxylfluorane anesthesia. To each plasma sample, 10 μL of 100 mg/mL ascorbic acid solution in water was added. All samples were frozen immediately and stored at -80°C.

Bilirubin and bile component analysis. Concentrations of bilirubin monoglucuronides, bilirubin diglucuronides, and unconjugated bilirubin in bile (first 15-min fraction, 100× diluted in water) and in collected urine and plasma were determined as described (26).

Pharmacokinetic experiments. MTX was given to female wild-type, Abkg2-, Abcc2-, and Abcc2;Abkg2- mice (n = 3–13) by injecting 5 μL/g of body weight of a 10 mg/mL MTX solution in saline into the tail vein. The animals were killed by terminal bleeding through cardiac puncture after methoxylfluorane anesthesia at 7.5, 15, 30, 60, or 120 min after MTX administration, and the organs were removed.

Biliary MTX excretion experiment. Gall bladder cannulations in female wild-type, Abkg2-, Abcc2-, and Abcc2;Abkg2- mice (n = 3–5) were done as described (25). After successful cannulation, 50 mg/kg MTX was given i.v. as described above. Bile was collected in 15-min fractions for 60 min. The mice were killed by cardiac puncture, and blood and organs were collected. Small intestinal tissue and contents were separated.

Fecal and urinary MTX excretion experiment. Female wild-type, Abkg2-, Abcc2-, and Abcc2;Abkg2- mice (n = 5–9) were individually housed in Rucio Type M1 stainless steel metabolic cages. They were allowed 24 h to adapt before 50 mg/kg MTX was injected into the tail vein as described above. Feces and urine were collected for 24 h.

High-performance liquid chromatography analysis of MTX and 7OH-MTX. Collected organs were homogenized in an ice-cold 4% bovine serum albumin solution, and plasma and bile were diluted in

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**Results**

Macroscopic and microscopic analyses of Abcc2;Abcg2-/- mice. Abcc2;Abcg2-/- mice were viable, fertile, and had normal life spans and body weights. Macroscopic and microscopic histologic and pathologic analyses did not reveal obvious specific aberrations in tissues of the Abcc2;Abcg2-/- mice, including the liver. Similar to what was previously shown for Abcc2-/- mice (12), the liver weight of Abcc2;Abcg2-/- mice was significantly (26%-36%) increased compared with wild-type (Fig. 1A) but not significantly different from that of Abcc2-/- mice (ANOVA), suggesting that the increase in liver weight is primarily caused by Abcc2 absence.

Protein expression of other ABC transporters in tissues of Abcc2;Abcg2-/- mice. The absence of both Abcg2 and Abcc2 in tissues of the double knockout mice may lead to compensatory increases in expression of other ABC transporters. Therefore, we analyzed the expression of Abcc1 (Mrp1), Abcc3 (Mrp3), Abcc4 (Mrp4), Abcc5 (Mrp5), and Abcb11 (Bsep) in tissues of Abcc2;Abcg2-/- mice (>99% FVB background). Abcc3 protein levels in the liver [but not kidney (not shown)] of male (not shown) and female (Fig. 1B) Abcc2;Abcg2-/- mice were ~1.7-fold increased compared with wild-type. Abcc4 protein in female liver and kidney (Fig. 1C and D) and male kidney (not shown) of Abcc2;Abcg2-/- mice was ~1.5-fold increased. Abcc4 in male liver was very low in both wild-type and Abcc2;Abcg2-/- mice (not shown). Abcc1 in male liver, and Abcc5 in male and female liver were quite low and not different between wild-type and Abcc2;Abcg2-/- mice (not shown). Abcb11 expression was not increased in Abcc2;Abcg2-/- mice and, possibly, even mildly decreased compared with wild-type (not shown). Combined, the expression patterns of Abcc3 and Abcc4 in the liver and kidney of Abcc2;Abcg2-/- mice were similar to what was found for Abcc2-/- mice of >99% FVB background (12), suggesting that these inductions are mainly the result of the absence of Abcc2.

Expression levels of genes involved in MTX disposition. Because we wanted to analyze the pharmacokinetics of MTX in Abcc2;Abcg2-/- mice, we determined the mRNA expression levels of genes that may be involved in MTX metabolism and transport in the livers of female knockout mice. We found that mRNA levels of aldehyde oxidase 1 and 3, which are implicated in the conversion of MTX to 7OH-MTX (29) were 3-fold to 8-fold increased in Abcc2;Abcg2-/- mice (>99% FVB background) of male and female knockout mice. We analyzed the expression of Abcc1 (Mrp1), Abcc3 (Mrp3), Abcc4 (Mrp4), Abcc5 (Mrp5), and Abcb11 (Bsep) in tissues of Abcc2;Abcg2-/- mice (>99% FVB background) of male and female knockout mice. We found that mRNA levels of aldehyde oxidase 1 and 3, which are implicated in the conversion of MTX to 7OH-MTX (29) were 3-fold to 8-fold increased in the livers of Abcc2-/+ and Abcc2;Abcg2-/- mice (Supplementary Fig. S1A and B). The mRNA levels of the uptake transporter Slco1a4 were not different from wild-type in the knockout strains (Supplementary Fig. S1D). The mRNA of Slco1b2 was ~2-fold increased in Abcc2-/- and Abcc2;Abcg2-/- mice (Supplementary Fig. S1D).

Plasma clinical chemistry and hematologic analysis of Abcc2;Abcg2-/- mice. Except for bilirubin (see below), the standard plasma clinical chemistry variables were not significantly different between any of the strains analyzed (not shown). Hematologic analysis of male and female Abcc2;Abcg2-/- mice showed a tendency for reduced hemoglobin levels, as was previously seen in Abcc2-/- mice with a mixed genetic background (not shown; ref. 12). Other measured human plasma before high-performance liquid chromatography analysis. Urine was diluted in water, and feces were homogenized in ice-cold 4% bovine serum albumin solution. MTX and 7OH-MTX concentrations in the different matrices were determined as described (27).

**Statistical analysis.** Unless otherwise indicated, the two-sided unpaired Student’s t test was used to assess the statistical significance of differences between wild-type and knockout mice. When differences between >2 groups were analyzed, one-way ANOVA followed by Tukey’s multiple comparison test was done as indicated. The results are presented as means ± SD. Differences were considered statistically significant when P < 0.05. The 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; SEs were calculated by the law of propagation of errors (28).
variables did not consistently show differences with wild-type mice (not shown).

Bilirubin in plasma, bile, and urine of Abcc2;Abcg2-/- mice. We have previously shown that Abcc2-/- mice had mildly increased plasma levels of conjugated bilirubin due to reduced biliary excretion of this compound (12). In Abcg2-/- mice, on the other hand, the plasma levels of unconjugated bilirubin were increased (9). Clinical chemistry (not shown) and subsequent high-performance liquid chromatography analysis (Fig. 2A) of plasma confirmed these findings. In Abcc2;Abcg2-/- mice, bilirubin monoglucuronide, diglucuronide, and unconjugated bilirubin concentrations were all increased, leading to 7-fold higher total bilirubin plasma levels in Abcc2;Abcg2-/- mice compared with wild-type.

Because Abcg2 and Abcc2 both excrete their substrates into bile, we analyzed the bile flow and biliary bilirubin output in the different strains. As in Abcc2-/- mice (12), the cumulative bile flow over 1 hour in Abcc2;Abcg2-/- mice was significantly reduced to 54% of wild-type bile flow and was not significantly different from Abcc2-/- mice (ANOVA; Fig. 2B). The total biliary bilirubin output in Abcc2-/- mice was comparable with wild-type (Fig. 2C). As shown before (11, 12), the total biliary bilirubin excretion of Abcc2-/- mice was significantly reduced due to a decreased bilirubin monoglucuronide output. This was also seen in Abcc2;Abcg2-/- mice. Biliary bilirubin diglucuronide excretion in wild-type mice was very low and, in Abcc2-/- mice, this was not detected at all. As shown before (16), the biliary excretion of bilirubin diglucuronide was significantly increased in Abcc2-/- mice. This was similar for Abcc2;Abcg2-/- mice and is apparently related to Abcc2 absence. The biliary excretion of unconjugated bilirubin was modest in wild-type and Abcc2-/- mice, and not significantly different (Fig. 2C). In Abcc2-/- and Abcc2;Abcg2-/- mice, however, the biliary unconjugated bilirubin excretion was significantly reduced.

In urine collected from the gall bladder-cannulated mice, bilirubin monoglucuronide was detected only in Abcc2-/- and Abcc2;Abcg2-/- mice (Fig. 2D), reflecting the increased bilirubin monoglucuronide plasma levels in these strains (Fig. 2A). Bilirubin diglucuronides or unconjugated bilirubin were not detected in the urine of any of the strains.

Functionally overlapping roles of Abcg2 and Abcc2 in the elimination of MTX. It was shown previously with the use of Abcg2-/- and Abcc2-/- mice that both ABC transporters play a significant role in plasma elimination of [1H]MTX after i.v. administration (12, 16, 30). However, whereas transport of MTX by murine Abcg2 in vitro has been shown before (19), direct transport by murine Abcc2 has not. We therefore investigated the transport of [1H]MTX using Sf9 vesicles expressing murine Abcc2 (24). We found that, like human ABCC2, murine Abcc2 actively transports MTX (Supplementary Fig. S2).

To investigate the relative roles of Abcc2 and Abcg2 in MTX pharmacokinetics, we gave MTX i.v. (50 mg/kg) to the different strains, and measured the plasma and tissue concentrations over 120 min. The plasma AUCs for Abcg2-/- and Abcc2-/- mice were 1.6-fold and 2.0-fold increased compared with wild-type, respectively (Fig. 3A and Supplementary Table S1). In Abcc2;Abcg2-/- mice, the plasma AUC was 3.3-fold increased, suggesting additive effects of Abcg2 and Abcc2 on MTX plasma elimination.

MTX liver concentrations were, like plasma, highest (44%-54% of the dose) early after the administration and decreased quite rapidly thereafter (Fig. 3B and Supplementary Table S1). As shown before for Abcc2-/- mice (16) and despite increased Slco1b2 expression (Supplementary Fig. S1D), the liver-to-plasma ratios over 120 minutes (Fig. 3C) were significantly reduced in Abcc2-/- and Abcc2;Abcg2-/- mice. The liver-to-plasma ratios of Abcg2-/- mice over 120 minutes were not significantly
different from wild-type. This suggests a more effective liver elimination of MTX towards plasma (over the sinusoidal membrane of hepatocytes), most likely via (up-regulated) Abcc3 (16). This may contribute to the increased plasma concentrations of MTX in both Abcc2−/− and Abcc2;Abcg2−/− mice compared with wild-type. Note that Abcc3 was not up-regulated in single Abcg2−/− mice (not shown), consistent with liver-to-plasma MTX ratios being similar to wild-type values.

The small intestinal tissue and content levels of MTX between 7.5 and 60 minutes (Fig. 3D and Supplementary Table S1) were significantly reduced compared with wild-type in Abcg2−/−, Abcc2−/−, and Abcc2;Abcg2−/− mice at every measured time point. Whereas in Abcg2−/− and Abcc2−/− mice still substantial MTX excretion into the small intestine was observed, in Abcc2;Abcg2−/− mice this was virtually absent and significantly lower than in Abcg2−/− and Abcc2−/− mice 60 minutes after administration ($P < 0.01$; ANOVA). This shows that Abcg2 and Abcc2 together dictate the excretion of MTX into the small intestine and that each partly compensates for the absence of the other.

To investigate the functions of Abcg2 and Abcc2 in more detail, we measured their influence in biliary excretion and/or direct intestinal MTX excretion with gall bladder cannulation experiments. Figure 4A shows that Abcc2 and Abcg2 both influence the biliary excretion of MTX and that each can partly compensate for loss of the other. In Abcc2;Abcg2−/− mice, the biliary excretion of MTX was dramatically reduced to only 2% ± 1% of the dose versus 45% ± 20% in wild-type ($P = 0.011$), showing that Abcg2 and especially Abcc2 are the main determinants for biliary excretion of MTX.

As shown before (16), the MTX levels in the small intestinal tissue and contents of the gall bladder–cannulated mice were low (<3% of the dose) compared with the biliary MTX levels, suggesting that direct intestinal excretion is not important in MTX elimination. Surprisingly, whereas the plasma concentrations after this experiment (Supplementary Fig. S3A) were not significantly different from wild-type in any of the knockout strains (ANOVA), ~3-fold increased levels of MTX in the small intestinal contents of Abcc2;Abcg2−/− mice compared with the other mouse strains were found ($P < 0.05$; ANOVA; Fig. 4B), suggesting the presence of an active direct intestinal MTX excretion mechanism in Abcc2;Abcg2−/− mice, which is not present/active in the other strains.

Figure 4C shows the role of Abcg2 and Abcc2 in the urinary and fecal excretion of MTX the first 24 hours after i.v. administration (50 mg/kg). As described before (16), MTX urinary excretion in Abcc2−/− mice was significantly increased compared with wild-type mice, thereby reflecting increased
plasma and kidney levels. In Abcg2<sup>−/−</sup> and Abcc2;Abcg2<sup>−/−</sup> mice, however, despite 1.6-fold and 3.3-fold increased plasma AUCs and increased kidney concentrations of MTX (Fig. 3A and Supplementary Table S1), the urinary excretion of MTX was not different from wild-type mice. This suggests that Abcg2 in the kidney is (at least partly) responsible for the increased urinary excretion of MTX in Abcg2<sup>−/−</sup> mice. Fecal excretion of MTX over 24 hours was not significantly different from wild-type mice in any of the knockout strains.

**Functionally overlapping roles of Abcg2 and Abcc2 in the elimination of 7OH-MTX.** ABCG2 and ABCC2 can transport 7OH-MTX, the main toxic metabolite of MTX, in vivo (16). The Abcg2<sup>−/−</sup> mice have a 6.2-fold increased 7OH-MTX plasma AUC compared with wild-type (Fig. 5A and Supplementary Table S2). In Abcc2<sup>−/−</sup> mice, on the other hand, the 7OH-MTX plasma AUC was not significantly different from wild-type, which would suggest that Abcg2 is not important for 7OH-MTX elimination in vivo. However, in Abcc2;Abcg2<sup>−/−</sup> mice, the 7OH-MTX plasma AUC was 12.4-fold higher than in wild-type mice and 2.0-fold higher compared with Abcg2<sup>−/−</sup> mice (P < 0.03; Student’s t test). This shows that when Abcc2 is absent, Abcg2 does influence the elimination of 7OH-MTX from the circulation.

Already 7.5 minutes after MTX administration, the 7OH-MTX liver levels (Fig. 5B) were relatively high in all strains and significantly higher in Abcc2<sup>−/−</sup> and Abcc2;Abcg2<sup>−/−</sup> mice compared with wild-type, suggesting a rapid 7OH-MTX formation in the liver, which may even have been increased compared with wild-type in Abcc2-deficient mice due to the overexpression of aldehyde oxidase 1 and 3 in the livers of these mice (Supplementary Fig. S1). Whereas in Abcc2<sup>−/−</sup> mice, the 7OH-MTX liver levels decreased rapidly after 30 minutes (Fig. 5B), the liver levels in Abcc2;Abcg2<sup>−/−</sup> mice stayed relatively high up to 60 minutes after administration, suggesting initially delayed elimination of 7OH-MTX from the liver in Abcc2; Abcg2<sup>−/−</sup> mice.

There was little difference between Abcg2<sup>−/−</sup> and wild-type mice in 7OH-MTX small intestinal tissue and contents levels (Fig. 5C), suggesting only a minor role for Abcg2 in the elimination of 7OH-MTX via the small intestine. As shown before (16), in Abcc2<sup>−/−</sup> mice the small intestinal 7OH-MTX levels were markedly lower compared with wild-type at every time point (Fig. 5C). In Abcc2;Abcg2<sup>−/−</sup> mice, the small intestinal levels of 7OH-MTX were even more reduced to only 11% of wild-type levels at 60 minutes (P = 3.1*10<sup>−5</sup>), suggesting that when Abcc2 is absent, Abcg2 is involved in the excretion of 7OH-MTX into the small intestine, most likely via biliary excretion (see below).

7OH-MTX-related toxicity in patients is suggested to mainly occur in the kidney, leading to renal failure (31, 32). Therefore, we analyzed the kidney levels of 7OH-MTX after i.v. administration of MTX (Fig. 5D). These, in general, followed the plasma levels of 7OH-MTX. Thus, in Abcc2<sup>−/−</sup> and in Abcc2;Abcg2<sup>−/−</sup> mice, they were significantly increased, up to 11-fold and 35-fold compared with wild-type mice at 60 minutes.

The effect of Abcg2 and Abcc2 on the biliary excretion of 7OH-MTX after i.v. injection of MTX is shown in Fig. 6A. Clearly, Abcc2 is the main determinant for the biliary excretion of 7OH-MTX, but in the absence of Abcc2, Abcg2 can (partly) take over its function. In Abcc2<sup>−/−</sup> and in Abcc2;Abcg2<sup>−/−</sup> mice, despite increased liver levels (Fig. 5B), the biliary 7OH-MTX output was decreased to only 5% of wild-type output (0.12% ± 0.04% versus 2.51% ± 1.53% of the dose; P = 0.04), illustrating the important role of both transporters in the biliary excretion of 7OH-MTX. The levels of 7OH-MTX in the small intestinal tissue
and contents 60 minutes after MTX administration in the gall bladder cannulation experiment were negligible (not shown), indicating that direct intestinal excretion is not important for 7OH-MTX elimination.

The urinary excretion of 7OH-MTX during the first 24 hours after i.v. administration of MTX (Fig. 6B) was quite low and not significantly different from wild-type in Abcg2−/− mice. As shown before (16), the urinary excretion of 7OH-MTX was significantly increased in Abcc2−/− mice. Although in Abcc2;Abcg2−/− mice, the urinary excretion of 7OH-MTX was significantly higher compared with wild-type, it was lower than in Abcc2−/− mice (n = 5-9; P < 0.001; ANOVA). Because the AUCplasma of 7OH-MTX in Abcc2;Abcg2−/− mice was 2-fold higher than in Abcc2−/− mice (Fig. 5A), this suggests that Abcg2 is (at least partly) responsible for the urinary excretion of 7OH-MTX in Abcc2−/− mice. The fecal excretion of 7OH-MTX was very low in all strains (<3% of the dose) and difficult to determine due to endogenous background peaks (not shown).

Discussion

To investigate the possibly overlapping roles of Abcg2 and Abcc2 in the elimination of shared endogenous and exogenous substrates in vivo, we generated Abcc2;Abcg2−/− mice that are viable and fertile. The spontaneous phenotypes of the double knockout mice seem to be a combination of what we observed previously for Abcg2−/− and Abcc2−/− mice (9, 12), suggesting separate roles of Abcg2 and Abcc2 in most physiologic functions. We show here that Abcg2 and Abcc2 play functionally overlapping roles in the elimination of the anticancer drug MTX and its toxic metabolite 7OH-MTX. We conclude that Abcc2;Abcg2−/− mice are quite healthy overall and can be used to study overlapping Abcg2 and Abcc2 functions in vivo.

It was shown previously that in Abcg2−/− mice, unconjugated bilirubin plasma levels were mildly increased (9), whereas in Abcc2−/− mice, conjugated bilirubin plasma levels were increased (11, 12). This was confirmed in the present study with the use of high-performance liquid chromatography. In Abcc2;Abcg2−/− mice, a combination of both was observed: 7-fold increased plasma concentrations of total bilirubin due to significantly increased unconjugated as well as conjugated bilirubin. This is probably the consequence of reduced biliary excretion of conjugated and unconjugated bilirubin in Abcc2;Abcg2−/− mice. Combined, this suggests that Abcc2 is mainly involved in the biliary excretion of bilirubin monoglucurononides, whereas Abcg2 excretes unconjugated bilirubin. However, if Abcg2 transports unconjugated bilirubin, it clearly has a low efficiency because the amount excreted into bile is very small, also in wild-type mice. Even in Gunn rats, which have elevated serum unconjugated bilirubin due to the absence of the conjugating enzyme UGT1A1, very little is excreted into bile (33). An alternative explanation for the observed changes is decreased unconjugated bilirubin uptake into the liver of Abcg2-deficient mice. An uptake system for unconjugated bilirubin in mice has, however, not been unambiguously identified yet (34), making this difficult to investigate. Note that in Abcc2;Abcg2−/− mice, still substantial biliary output of conjugated bilirubin is present, suggesting that an alternative canalicular transport mechanism transports bilirubin into bile. Nevertheless, the increased bilirubin plasma levels in Abcc2;Abcg2−/− mice are still relatively low compared with normal bilirubin plasma levels in humans (5-30 μmol/L). It is therefore unlikely that plasma bilirubin levels would markedly influence drug pharmacokinetics in these mice.

We have studied the overlapping roles of Abcc2 and Abcg2 in plasma and tissue pharmacokinetics of MTX and 7OH-MTX.
Analysis of the biliary excretion of MTX and 7OH-MTX revealed excretion of MTX and 7OH-MTX in the strains analyzed. 6.2-fold in fractionsover 1h (gallbladder^cannulatedmice of the various strains. Bile was collected in 15-min A, 7OH-MTX biliary excretion in the first 60 min after i.v. administration of MTX to mice of the various strains (n = 6-9; * P < 0.05; ***, P < 0.001; ANOVA). Data, means ± SD.

Fig. 6. Biliary, urinary, and fecal excretion of 7OH-MTX after i.v. administration of 50 mg/kg MTX to female wild-type, Abcg2^−/−, Abcc2^−/−, and Abcc2;Abcg2^−/− mice. A, 7OH-MTX biliary excretion in the first 60 min after i.v. administration of MTX to gall bladder – cannulated mice of the various strains. Bile was collected in 15-min fractions over 1h (n = 3-5; *, P < 0.05; Student’s t test). B, 7OH-MTX plasma and liver concentrations of the cannulated mice at t = 60 min are depicted in Supplementary Fig. 3. B, 7OH-MTX urinary excretion in the first 24 h after i.v. administration of MTX to mice of the various strains (n = 6-9; *, P < 0.05; ***, P < 0.001; ANOVA).

The MTX plasma pharmacokinetics in the different strains revealed additive effects of Abcg2 and Abcc2. Because MTX-related toxicity and outcome in patients after high-dose MTX therapy seem to correlate with MTX serum levels (35–37), differences in expression or activity of ABCG2 and/or ABCC2 may have profound effects on MTX-induced toxicity in patients. Because many known ABCC2 and ABCG2 polymorphisms drastically reduce or even abrogate the function of these proteins (2, 6, 7), this could be an important contributory factor to variable MTX efficacy and toxicity in patients. Interestingly, in psoriasis patients treated with MTX, single nucleotide polymorphisms in ABCG2 were associated with a good response to therapy (38).

For the plasma pharmacokinetics of 7OH-MTX after MTX administration, we found Abcc2 to be the main determinant. Nevertheless, when Abcc2 is absent, Abcg2 still plays a significant role. This was best illustrated by the 12.4-fold increased 7OH-MTX plasma AUC in Abcc2^−/−;Abcg2^−/− mice versus 6.2-fold in Abcc2^−/− mice.

Supplementary Table S3 provides an overview of the overall excretion of MTX and 7OH-MTX in the strains analyzed. Analysis of the biliary excretion of MTX and 7OH-MTX revealed that Abcg2 and Abcc2 together dictate these processes. Abcc2 was the most important transporter in the biliary excretion of both compounds after i.v. MTX administration. Although mRNA levels of aldehyde oxidase were increased in both Abcc2-deficient strains (Supplementary Fig. S1), the hepatic MTX levels in these strains were not significantly different from wild-type levels. The reduced biliary excretion of MTX must therefore primarily be caused by reduced Abcc2;Abcg2-mediated transport of MTX into bile and not by increased conversion of MTX to 7OH-MTX.

It is interesting to note that males have higher hepatic Abcg2 levels than females (39). Because our experiments were done in female mice, the effect of Abcg2 in male liver may be underestimated here. Still, a clear effect of Abcg2 on biliary excretion in female Abcc2^−/− mice could be shown, both for MTX and 7OH-MTX. This was reflected by drastically decreased levels of both compounds in the small intestine and its contents in Abcc2;Abcg2^−/− mice. Interestingly, Rau et al. (2006; ref. 40) found an association between a frequent ABCG2 polymorphism and MTX pharmacokinetics, but only in females. This suggests that because Abcg2 apparently can (partly) compensate for Abcc2 absence, in males ABCG2 expression is sufficient for effective compensatory MTX excretion, whereas in females it is not.

MTX-related toxicity in humans is often seen in bone marrow and the gastrointestinal tract, although this is generally rendered manageable by coadministration of leucovorin. Because Abcc2 and Abcg2 are primarily responsible for the biliary excretion of MTX and 7OH-MTX, reduced expression or activity of ABCG2 and/or ABCC2 in patients may cause reduced biliary excretion of MTX and 7OH-MTX. This could lead to reduced exposure of the intestine to these compounds after i.v. administration and, therefore, to reduced gastrointestinal toxicity.

Besides bone marrow and gastrointestinal toxicity, high-dose MTX treatment in patients may result in renal failure (41). This is thought to be caused by the precipitation of MTX and especially 7OH-MTX, which is highly insoluble, in kidney tubules (31, 32). As shown before (16), the urinary output of MTX and 7OH-MTX in Abcc2^−/− mice was significantly increased compared with wild-type in the first 24 hours after MTX administration. Although in Abcc2^−/−;Abcg2^−/− mice the plasma AUCs for MTX and 7OH-MTX were even higher than in Abcc2^−/− mice, the urinary excretion of both compounds was reduced compared with Abcc2^−/− mice and was comparable with wild-type. This suggests that in Abcc2^−/− mice the increased urinary excretion of MTX and 7OH-MTX is mediated through Abcg2 in the kidney. Furthermore, whereas the Abcg2^−/− mice had (like the Abcc2^−/− mice) increased plasma MTX levels, they did not show increased urinary MTX excretion. This suggests that even in the wild-type situation, Abcg2 may be involved in urinary MTX excretion. These findings are in line with the previously described effect of Abcg2 on the urinary excretion of another Abcg2 substrate, E3040S (42).

Given the increased kidney levels and urinary output of MTX and 7OH-MTX in Abcc2-deficient mice, reduced ABCG2 expression/activity may be a risk factor for kidney toxicity, which is primarily due to the precipitation of these compounds in urine (31, 32). Interestingly, renal failure after high-dose MTX treatment in a patient with an ABCC2 mutation has been reported (43). In contrast, because Abcg2 seems to mediate
the excretion of MTX and especially 7OH-MTX into urine, one could speculate that decreased activity of ABCG2 may decrease the risk of nephrotoxicity after MTX treatment.

Because the Abcc4 protein was 1.5-fold increased in the kidneys of Abcc2-/- and Abcc2;Abcg2-/- mice (Fig. 1D; ref. 12), we cannot exclude a minor contribution of Abcc4 overexpression on the increased urinary excretion of MTX and/or 7OH-MTX in Abcc2-deficient mice. However, this does not explain the differences in urinary excretion between Abcc2-/- and Abcc2; Abcg2-/- mice, as both strains had increased kidney Abcc4. If any, the effects of increased Abcc4 on the urinary MTX and 7OH-MTX excretion are very small compared with the effect of Abcg2.

It should be noted that the i.v. bolus injection of 50 mg/kg MTX given here is comparable with a dose of 154 mg/m2 in man (12, 44), which is relatively low for high-dose cancer treatment, during which doses of up to 12 g/m2 are given (29). Whether the effects of Abcc2 and/or Abcg2 found here are also valid for higher doses or longer infusion times remains to be elucidated. Interestingly, associations between ABCC2 and ABCG2 polymorphisms/mutations and MTX pharmacokinetics or toxicities in patients were found previously after high-dose (1-5 g/m2) MTX infusions (40, 43, 45), suggesting that our results are also relevant for higher doses and longer infusions.

Of course, as always, extrapolating data from mice to humans should be done with caution. For example, in humans, the biliary excretion of MTX seems somewhat less important (up to 20% of the dose is excreted into bile, although this was measured in very few patients; refs. 46, 47), whereas the urinary excretion of MTX seems somewhat more important compared with mice (46). Furthermore, other MTX/7OH-MTX transporters in human liver or kidney may be more significant in humans than in mice.

Overall, our data show that Abcg2 and Abcc2 are important transporters for plasma elimination as well as biliary and urinary excretion of MTX and its toxic metabolite 7OH-MTX. When one is absent, the other can (at least partly) compensate for its loss. These findings provide direct explanations for previously found correlations between ABCB2 and ABCG2 polymorphisms/mutations and MTX pharmacokinetics or toxicity in patients (38, 40, 43, 48), and show the potential value of Abcc2;Abcg2-/- mice for studying the pharmacokinetics of endogenous and exogenous compounds in vivo.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Functionally Overlapping Roles of Abcg2 (Bcrp1) and Abcc2 (Mrp2) in the Elimination of Methotrexate and Its Main Toxic Metabolite 7-Hydroxymethotrexate In vivo

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