Abstract

Purpose: ATP-binding cassette sub-family C member 2 [ABCC2; multidrug resistance-associated protein 2 (MRP2)] and ABCC3 (MRP3) mediate the elimination of toxic compounds, such as drugs and carcinogens, and have a large overlap in substrate specificity. We investigated the roles of Abcc2 and Abcc3 in the elimination of the anticancer drug methotrexate (MTX) and its toxic metabolite 7-hydroxymethotrexate (7OH-MTX) in vivo.

Experimental Design: Abcc2;Abcc3-/- mice were generated, characterized, and used to investigate possibly overlapping or complementary roles of Abcc2 and Abcc3 in the elimination of MTX and 7OH-MTX after i.v. administration of 50 mg/kg MTX.

Results: Abcc2;Abcc3-/- mice were viable and fertile. In Abcc2;/- mice, the plasma area under the curve (AUC) for MTX was 2.0-fold increased compared with wild type, leading to 1.6-fold increased urinary excretion, which was not seen in Abcc2;Abcc3-/- mice. Biliary excretion of MTX was 3.7-fold reduced in Abcc2;/- but unchanged in Abcc2;Abcc3-/- mice. The plasma AUCs of 7OH-MTX were 6.0-fold and 4.3-fold increased in Abcc2;/- and Abcc2;Abcc3-/- mice, respectively, leading to increased urinary excretion. The biliary excretion of 7OH-MTX was 5.8-fold reduced in Abcc2;/- but unchanged in Abcc2;Abcc3-/- mice. 7OH-MTX accumulated substantially in the liver of Abcc2;/- and especially Abcc2;Abcc3-/- mice.

Conclusions: Abcc2 is important for (biliary) excretion of MTX and its toxic metabolite 7OH-MTX. When Abcc2 is absent, Abcc3 transports MTX and 7OH-MTX back from the liver into the circulation, leading to increased plasma levels and urinary excretion. Variation in Abcc2 and/or Abcc3 activity may therefore have profound effects on the elimination and severity of toxicity of MTX and 7OH-MTX after MTX treatment of patients.

Impact of Abcc2 (Mrp2) and Abcc3 (Mrp3) on the In vivo Elimination of Methotrexate and its Main Toxic Metabolite 7-hydroxymethotrexate

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The multidrug resistance proteins (MRP) ATP-binding cassette sub-family C member 2 (ABCC2; MRP2) and ABCC3 (MRP3) are members of the ATP-binding cassette (ABC) transporter super-family. ABCC2 is present in the apical membranes of hepatocytes and the epithelial cells of the small intestine and kidney, and is involved in the elimination of both endogenous and exogenous compounds from the body (1). ABCC3 is also found in the liver, kidney, and small intestine as well as in the adrenal glands and pancreas. In contrast to ABCC2, ABCC3 localizes to the basolateral membrane of polarized cells (2). Both transporters are expressed in various tumors and can transport a range of (anticancer) drugs (1, 2). There is a large overlap in the substrate specificity of ABCC2 and ABCC3. They can, for example, both transport bilirubin glucuronides (3-5) and, in patients with the Dubin-Johnson syndrome, who have functionally deficient ABCC2 (6, 7), both plasma bilinubin glucuronide levels and ABCC3 protein levels are increased. It has been speculated that these two findings are related: ABCC3 up-regulation in the absence of ABCC2 would allow increased basolateral efflux of bilirubin glucuronides from the liver (7). Because Abcc2 and Abcc3 are both involved in the elimination of xenobiotics, the absence of each or both of them can have a profound effect on the pharmacokinetics of drugs or their glucuronide conjugates, as has been shown with the use of single knockout mice for Abcc2 and Abcc3 (8) as well as with the recently generated Abcc2;Abcc3-/- mice (9).

Methotrexate (MTX), a widely used anticancer and antirheumatic drug, is a substrate for both ABCC2 and ABCC3 in vitro (2). Abcc2-deficient mice and rats are hampered in the (biliary) elimination of [3H]MTX (10, 11) whereas the effect of Abcc3 on the pharmacokinetics of MTX in vivo thus far has not been
Translational Relevance

Methotrexate (MTX) treatment, commonly used in the treatment of cancer, can lead to severe and even lethal toxicity, the severity of which seems to be associated with MTX serum levels. The toxic metabolite 7-hydroxymethotrexate is found in the urine of patients after treatment with MTX. It has a very low aqueous solubility and is therefore associated with kidney failure in patients. Using Abcc2-/-, Abcc3-/-, and Abcc2;Abcc3-/- mice, we show that Abcc2 has a major impact on the pharmacokinetics of MTX and especially its toxic metabolite 7-hydroxymethotrexate. In the absence of Abcc2, Abcc3 transports these toxic compounds across the sinusoidal membrane of the hepatocyte into the circulation, thereby providing an alternative route of elimination via the urine. This leads to increased plasma and kidney levels, and urinary excretion of both compounds in Abcc2-deficient mice. Our results suggest that Dubin-Johnson patients, who are deficient in functional ABCC2 (and have increased expression of ABCC3), have an increased risk of developing toxicity upon MTX treatment. In addition, patients with (heterozygous) polymorphisms in Abcc2 or Abcc3 resulting in altered Abcc2 or Abcc3 activity might also 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. Abcc2-/- (10) and Abcc3-/- (5) mice have been described. Abcc2;Abcc3-/- mice were generated by crossbreeding. All animals were of 99% FVB background and between 9 and 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 (Emthexate PF, 25 mg/mL) was from Pharmachemie, and 7OH-MTX was from Toronto Research Chemicals Inc.; Ketamine was from Parke-Davis, xylazine was from Sigma Chemical Co., and methoxyflurane (Metofane) was from Medical Developments Australia Pty. Ltd.; MRPr1, M1-I-80, and M1-II-54 were the kind gifts of Dr. George L. Scheffer (Free University Hospital, Amsterdam, The Netherlands); K12 was kindly provided by Dr. Bruno Siesler (University Hospital, University of Zürich, Zürich, Switzerland). BXP-53 was described before (21).

Western analysis. Crude membrane fractions from tissues were prepared as described (21, 22). Western blotting was done as described (23). Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer (not shown). Abcc1 (Mrp1), Abcc4 (Mrp4), Abcc5 (Mrp5), Abcg2 (breast cancer resistance protein 1), and Abcb11 ( bile salt export pump) were detected with the antibodies MRPr1 (dilution 1:1,000), M1-I-80 (dilution 1:400), M1-II-54 (dilution 1:1), BXP-53 (dilution 1:400), and K12 (dilution 1:2,000), respectively. Bound primary antibodies were detected by incubating the blot with horseradish peroxidase–labeled rabbit anti-rat IgG (1:1,000; DAKO;
Abcc1, Abcc4, Abcc5, and Abcg2) or horseradish peroxidase–labeled goat anti-rabbit IgG (1:2,000; DAKO; Abcb11).

Histologic, clinical-chemical, and hematologic analysis. Histologic analysis of mouse tissues, standard clinical chemistry analyses on serum, and standard hematologic analysis (twice within a time span of 1.5 y) were done as described (10).

Bileflowmeasurements and analysis. Gall bladder cannulations and collection of bile in male wild-type, Abcc2-/-, Abcc3-/-, and Abcc2;Abcc3-/- mice (n = 5 for each group) were done as described (10;24). Concentrations of bilirubin monoglucuronides, bilirubin diglucuronides, and unconjugated bilirubin in bile and urine were determined as described (25). Bile salts, choline-containing phospholipids, and cholesterol were determined enzymatically as described (26).

Plasmaticandtissuemodells. MTX was administered to female mice by injecting 5 mL/g body weight of 10 mg/mL MTX in 0.9% NaCl solution into the tail vein. The animals were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia, and organs were removed. Intestinal contents (feces) and tissue were separated.

Biliary excretion ofMTX and 7OH-MTX. Gall bladder cannulations in female mice were done as described (24). After cannulation, 50 mg/kg MTX was administered i.v. as described above. Bile was collected in 15-min fractions for 60 min. Subsequently, the mice were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia, and organs were removed. Intestinal contents (feces) and tissue were separated.

Fecal and urinary excretion ofMTX and 7OH-MTX. Female mice were individually housed in Ruco Type M/1 stainless-steel metabolic cages and 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 over 0 to 24 and 24 to 48 h. Forty-eight hours after injection, the mice were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia. Organs were removed, and intestinal contents (feces) and tissue were separated.

High-performance liquid chromatography analysis ofMTX and 7OH-MTX. The collected organs were homogenized in an ice-cold 4% bovine serum albumin solution before high-performance liquid chromatography analysis. MTX and 7OH-MTX concentrations in plasma, urine, and tissue homogenates 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 two sets of data. When more than two groups were compared, one-way ANOVA followed by Tukey’s multiple
Comparison test was used, as indicated in text and/or figure legends. Results are presented as the means F SD. Differences were considered 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; SEs were calculated by the law of propagation of errors (28). The results of AUC measurements are presented as means ± SD.

Results

Macroscopic and microscopic analysis of Abcc2;Abcc3−/− mice. Abcc2;Abcc3−/− mice were viable, fertile, and had normal life spans, body weights, and anatomy. Adult Abcc2;Abcc3−/− mice had a 36% to 49% increased liver weight compared with wild-type mice (Fig. 1A). The liver weight in male Abcc2;Abcc3−/− mice was also significantly higher than in the Abcc2−/− mice (Fig. 1A), which by themselves already had ~27% increased liver weight (Fig. 1A; ref. 10). Similarly increased liver weight was seen in Abcc2−/− and Abcc2;Abcc3−/− mice in C57BL/6 background (29). Despite the markedly increased liver size, detailed microscopic analysis of liver sections did not reveal obvious pathologic changes.

Protein levels of other ABC multidrug transporter proteins in tissues of Abcc2;Abcc3−/− mice. We checked the protein levels of various ABC transporters in the liver, kidney, and/or small intestine of male and female Abcc2;Abcc3−/− mice using immunoblot analysis. Abcc4 expression in livers (Fig. 1B) and kidneys (Fig. 1C) of female but not male (not shown) Abcc2;Abcc3−/− mice was about 2-fold increased compared to wild-type livers. Levels of Abcc4 in the small intestine were very low, and no differences were seen between wild-type and Abcc2;Abcc3−/− mice (not shown). Abcg2 protein levels in male and female liver, kidney, and small intestine of Abcc2;Abcc3−/− mice were not different from wild-type (not shown). Abcc1 and Abcc5 expression in the liver of male and female Abcc2;Abcc3−/− mice were not different from wild-type (not shown). Abc11 and Abcc2;Abcc3−/− mice were not different from those found in wild-type either (not shown) nor was Abcb11 expression in the liver of male Abcc2−/− and Abcc2;Abcc3−/− mice (not shown).

Plasma clinical chemistry and hematologic analysis of Abcc2;Abcc3−/− mice. It has been hypothesized (7, 10, 30) that Abcc3
expression (most likely in liver) could be related to increased conjugated bilirubin levels observed in the circulation of Abcc2−/− mice, possibly as a compensation for the reduced biliary excretion of bilirubin glucuronides via Abcc2. Analysis of plasma from wild-type, Abcc2−/−, Abcc3−/−, and Abcc2;Abcc3−/− mice showed that Abcc3 is indeed necessary for the increased plasma bilirubin glucuronide levels seen in Abcc2−/− mice: whereas in Abcc2−/− mouse plasma levels of total and conjugated bilirubin were markedly increased as compared with wild-type controls, in Abcc2;Abcc3−/− mice these plasma levels returned to wild-type values. Abcc3−/− mice had plasma levels of total and conjugated bilirubin similar to those found in wild-type mice as well (Fig. 2A and B).

All other clinical-chemical parameters measured showed no significant differences between wild-type and knockout strains (not shown). Also, hematologic analysis did not yield any consistent differences between Abcc2;Abcc3−/− and wild-type mice.

**Biliary and urinary excretion and composition in Abcc2;Abcc3−/− mice.** We analyzed the bile and urine composition of wild-type, Abcc2+/−, Abcc3+/−, and Abcc2;Abcc3+/− mice after ligation of the common bile duct and gall bladder cannulation. We previously found that Abcc2−/− mice have a significantly reduced bile flow (Fig. 3A; ref. 10). Bile flow in the Abcc2−/− mouse was significantly reduced as well, to about 76% of wild-type bile flow (Fig. 3A). Surprisingly, this was still significantly higher than in Abcc2−/− mice (P < 0.01; ANOVA) although this was not the case after correction for differences in liver weight (ANOVA; not shown).

The biliary excretion of total bilirubin in Abcc2−/−;Abcc3−/− mice was significantly reduced compared with wild-type mice (Fig. 3B). The reduced total biliary bilirubin excretion in Abcc2−/−;Abcc3−/− mice was caused by a reduction in bilirubin monoglucuronide output, which was also reduced in Abcc2−/− mice (Fig. 3B). Interestingly, the (much lower) biliary bilirubin diglucuronide output in both strains lacking Abcc2 was increased compared with wild-type and Abcc3−/− mice (Fig. 3B). The (modest) output of unconjugated bilirubin in the bile of Abcc2−/−; Abcc3−/−, and Abcc2;Abcc3−/− mice was not significantly different from wild-type mice (Fig. 3B).

Analysis of the urine of the cannulated mice showed that bilirubin monoglucuronide was found in the urine of Abcc2−/− but not of Abcc2−/−;Abcc3−/− mice, consistent with the increased bilirubin glucuronide levels in the plasma of Abcc2−/− mice (Figs. 2B and 3C).

The biliary output of cholesterol and bile acids was not significantly different in any of the knockout strains (not shown). The biliary phospholipid output was significantly increased in Abcc2−/− and Abcc2−/−;Abcc3−/− mice compared with wild-type mice (Supplementary Fig. S1A). However, after correction for liver weight, no significant differences were seen in any of the strains (Supplementary Fig. S1B).

**Influence of Abcc2 and Abcc3 on MTX pharmacokinetics in vivo.** To investigate the relative roles of Abcc2 and Abcc3 in the pharmacokinetics of MTX, we administered MTX i.v. to female wild-type, Abcc2−/−; Abcc3−/−, and Abcc2;Abcc3−/− mice at a dose of 50 mg/kg (comparable with 154 mg/m² in man; ref. 31) and measured MTX levels in plasma and a set of organs at different time points. The results for plasma are shown in Fig. 4A and Supplementary Table S1. Compared with wild-type mice, the MTX plasma AUC between 7.5 and 120 minutes was 2-fold increased in Abcc2−/− mice (870 ± 103 min·μg/mL versus 444 ± 44 min·μg/mL for wild type; P = 6.2 × 10−4). In contrast, in the Abcc2−/−; Abcc3−/− mice, the MTX plasma AUC was not significantly different from wild type (435 ± 47 min·μg/mL; P = 0.76) and 2-fold lower than in single Abcc2−/− mice (P = 2.2 × 10−12). In Abcc3−/− mice, the MTX plasma AUC was not significantly different from wild type either although there was a tendency of a reduced AUCplasma in these mice (368 ± 34 min·μg/mL; P = 0.084). This suggests that, in the presence of Abcc2, there is only a minor influence of Abcc3 on the pharmacokinetics of MTX. However, the role of Abcc3 becomes important when Abcc2 is absent.

We also measured liver levels of MTX between 7.5 and 120 minutes after administration (Fig. 4B and Supplementary Table S1). Already 7.5 minutes after administration, livers contained high amounts of MTX (44-55% of the dose), which decreased thereafter. Interestingly, the liver-to-plasma ratios of MTX over 120 minutes (Fig. 4C and Supplementary Table S1) were lower in Abcc2−/− mice than in wild-type mice whereas Abcc3−/− mice tended to have increased MTX liver-to-plasma ratios. In Abcc2−/−; Abcc3−/− mice, the liver-to-plasma ratios were
similar to those found in wild type. These results, combined with the MTX plasma AUCs, suggest that Abcc2;Abcc3−/− mice had reduced sinusoidal elimination of MTX from the liver compared with Abcc2−/− mice.

We next determined the MTX levels in small intestinal tissue and contents of the different strains (Fig. 4D and Supplementary Table S1). MTX levels in all strains steadily increased up to 60 minutes after administration. At all time points, MTX levels in the small intestine of Abcc2−/− mice were significantly lower compared with wild-type mice (Supplementary Table S1), indicating an important role for Abcc2 in the elimination of MTX, most likely via hepatobiliary excretion (see also below). At 60 minutes after administration, MTX levels in Abcc2;Abcc3−/− mice were significantly reduced as well compared with wild-type and Abcc3−/− mice (P < 0.01; ANOVA). However, MTX levels in Abcc2;Abcc3−/− mice were significantly higher than in Abcc2−/− mice at this time point (P < 0.01; ANOVA). Collectively, these data suggest that apical elimination mechanisms different from Abcc2 still mediate substantial hepatobiliary excretion of MTX in the Abcc2−/− and Abcc2;Abcc3−/− mice. Tendencies of increased liver MTX concentrations in the Abcc2;Abcc3−/− mice (Fig. 4B) and higher MTX hepatobiliary excretion compared with Abcc2−/− mice (see below) likely explain the higher small intestinal values in the Abcc2;Abcc3−/− mice.

Kidney toxicity is a clinically relevant dose-limiting factor for (high-dose) MTX treatment (32). Compared with wild-type mice, kidney levels of MTX were significantly increased in Abcc2−/− mice 15 to 60 minutes after MTX administration whereas levels in kidneys of Abcc2;Abcc3−/− mice were not (Supplementary Table S1). MTX kidney levels seemed to correlate with MTX plasma levels in all strains.

Subsequent gall bladder cannulation experiments revealed that excretion of MTX into bile the first 60 minutes after i.v. MTX administration (50 mg/kg) was reduced in Abcc2−/− mice to about 27% of that found in wild-type mice. Surprisingly, in Abcc2;Abcc3−/− mice, the biliary excretion of MTX over the first 60 minutes did not differ from that found in wild-type or Abcc3−/− mice (ANOVA; Fig. 5A). MTX levels in small intestinal contents and tissue after gall bladder cannulation were very low (<1% of the dose) in all strains and did not differ between the strains (not shown), indicating that direct intestinal excretion is a negligible route for the elimination of MTX in mice.

Clearly, Abcc2−/− mice excreted less MTX into bile, resulting in increased MTX plasma levels. The fact that in Abcc2;Abcc3−/− mice MTX plasma levels were not increased illustrates that in the absence of Abcc2, Abcc3 substantially transports MTX across the sinusoidal membrane of the hepatocyte towards the circulation, thereby possibly facilitating the elimination of MTX via the urine. To investigate this, we determined the urinary and
fetal excretion of MTX (50 mg/kg) in the various strains after i.v. administration (Fig. 5B). Indeed, Abcc2<sup>-/-</sup> mice displayed increased elimination of unchanged MTX via the urine whereas urinary excretion of MTX in Abcc2;Abcc3<sup>+/+</sup> mice was reduced to rates found in wild-type and Abcc3<sup>-/-</sup> mice. Despite the markedly reduced biliary excretion of MTX in Abcc3<sup>-/-</sup> mice, the fecal excretion of MTX did not differ from wild type in any of the strains (Fig. 5B). Compared with the high amount of MTX excreted into bile (Fig. 5A) and small intestine (Fig. 4D) in the 1st hour after administration, the amount of MTX found in feces is relatively low. This indicates that MTX is substantially reabsorbed from the intestine in all mouse strains tested.

**Influence of Abcc2 and Abcc3 on 7OH-MTX pharmacokinetics in vivo.** In patients receiving high-dose MTX, a significant amount of the toxic metabolite 7OH-MTX, which is primarily formed in the liver (18–20), is detected in the urine (33). The effects of Abcc2 and Abcc3 on the pharmacokinetics of 7OH-MTX after i.v. administration of 50 mg/kg MTX are shown in Fig. 6 and Supplementary Table S2. The plasma AUCs for 7OH-MTX in wild-type, Abcc2<sup>-/-</sup>, Abcc3<sup>+/+</sup>, and Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice (Fig. 6A) suggest a very important role for Abcc2 in the elimination of this toxic metabolite. Whereas the plasma AUCs for 7OH-MTX in wild-type and Abcc3<sup>-/-</sup> mice were quite low and not significantly different from each other (wild type: 17 ± 3 min-μg/ml; Abcc3<sup>-/-</sup>: 12 ± 1 min-μg/ml; P = 0.084), the plasma AUC for 7OH-MTX in Abcc3<sup>-/-</sup> mice was 6-fold increased compared with wild-type mice (106 ± 11 min-μg/ml; P = 3.7 × 10<sup>-5</sup>). At early time points (0-30 minutes), plasma levels of 7OH-MTX in Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice were not different from wild-type and Abcc3<sup>-/-</sup> mice and clearly lower than in Abcc3<sup>-/-</sup> mice. This suggests that in the absence of Abcc2, Abcc3 efficiently transports 7OH-MTX from liver towards the circulation. Interestingly, at later time points after MTX administration (>30 minutes), plasma 7OH-MTX levels were substantially increased in Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice, leading to a 4.3-fold higher plasma AUC compared with wild-type mice (73 ± 20 min-μg/mL, P = 7.1 × 10<sup>-3</sup>). The concomitant liver levels of 7OH-MTX in Abcc2<sup>-/-</sup> mice were markedly higher than in wild-type mice at all time points tested, and the 7OH-MTX levels in livers of Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice were even more increased, especially between 30 and 120 minutes after administration of MTX (Fig. 6B and Supplementary Table S2). This suggests an important role for Abcc2 in clearing the liver from the toxic metabolite 7OH-MTX. Abcc3 only plays a role in the absence of Abcc2 because the 7OH-MTX levels in liver of Abcc3<sup>-/-</sup> mice were not different from those found in wild-type mice. Kidney levels of 7OH-MTX in Abcc2<sup>-/-</sup> (0-60 minutes) and Abcc2;Abcc3<sup>-/-</sup> (15-60 minutes) mice were 3-fold to 11-fold and 4-fold to 7-fold increased compared with wild type, respectively, correlating with the increased 7OH-MTX plasma levels (Supplementary Table S2).

Gall bladder cannulation experiments furthermore showed that the cumulative biliary excretion of 7OH-MTX in Abcc2<sup>-/-</sup> mice in the first 60 minutes after MTX administration was reduced to only 17% of the biliary 7OH-MTX output in wild-type mice (Fig. 6C). In contrast, the biliary excretion of 7OH-MTX was not significantly different from wild type in Abcc3<sup>-/-</sup> and Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice (Fig. 6C).

Whether Abcc2 and Abcc3 affect urinary and fecal excretion of 7OH-MTX in the first 24 hours after i.v. administration of MTX (50 mg/kg) was subsequently investigated (Fig. 6D). Compared with control mice, urinary excretion of 7OH-MTX was markedly higher in both Abcc2<sup>-/-</sup> and Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice, consistent with the increased 7OH-MTX plasma levels in these strains (Fig. 6D). Urinary 7OH-MTX output in Abcc3<sup>-/-</sup> mice was not significantly different from wild-type output (Fig. 6D). The fecal output of 7OH-MTX was similar to wild type in both Abcc2<sup>-/-</sup> and Abcc3<sup>-/-</sup> mice but 2-fold increased in Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice (Fig. 6D).

**Discussion**

Abcc2 and Abcc3 have overlapping substrate specificities, and Abcc3 protein expression is often increased when Abcc2 is absent (7, 10). Using Abcc2<sup>-/-</sup> and the here described Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice, we show that in the absence of Abcc2, Abcc3 transports bilirubin glucuronide across the sinusoidal membrane, resulting in increased plasma levels and urinary excretion. Similar results were obtained for the pharmacokinetics of the anticancer and antirheumatic drug MTX: Abcc2 transports MTX into bile whereas in the absence of Abcc2, Abcc3 transports MTX back into the circulation, allowing increased urinary excretion. Using Abcc2<sup>-/-</sup> and Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice, we additionally show that Abcc2 is important for the biliary excretion of 7OH-MTX, the main (toxic) metabolite of MTX. In the absence of Abcc2, Abcc3 markedly influences the sinusoidal elimination of 7OH-MTX from the liver, and the absence of both ABC transporters leads to significant liver accumulation of 7OH-MTX. The absence of Abcc2 leads to a profound increase in plasma and kidney levels of MTX and especially 7OH-MTX, which is partly dependent on the presence of Abcc3. Analysis of Abcc3<sup>-/-</sup> mice alone would have suggested little impact of Abcc3 on either MTX or 7OH-MTX pharmacokinetics. Our findings suggest that differences in Abcc2 and (when Abcc2 is absent or reduced) Abcc3 activity could profoundly influence plasma and tissue levels as well as MTX-related and 7OH-MTX-related toxicity in patients who are treated with MTX.

It is quite surprising that, in the absence of both Abcc2 and Abcc3, the health of the mice does not seem to be affected. This may be caused by the fact that the mice live in a relatively protected environment and therefore may not be confronted with many toxic compounds during their life. Other elimination mechanisms possibly (partly) compensate for the absence of these ABC transporters; for example, Abcc4, which is 2-fold increased in liver and kidney of Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice, drug treatment can clearly lead to substantial accumulation of potentially toxic metabolites in liver and plasma, as we show here for 7OH-MTX after MTX treatment and as was previously shown for morphine-3-glucuronide upon morphine treatment of Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice (9).

Analysis of the plasma and urine of the Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice (Figs. 2 and 3C) showed that Abcc3 is necessary for the increased bilirubin glucuronide levels in blood and urine of Abcc2<sup>-/-</sup> mice. This indicates that Abcc3 functions as a backup pathway for Abcc2 when toxic compounds in the liver cannot be excreted into the bile, Abcc3 transports them into the blood and the compounds are alternatively excreted via the urine. Surprisingly, in Abcc2<sup>-/-</sup>;Abcc3<sup>-/-</sup> mice, both biliary and urinary excretion of bilirubin are relatively low, raising the question of
how the bilirubin formed is handled in these mice. Abcc2; Abcc3−/− mice do not display obvious pathologies, suggesting that alternative bilirubin elimination pathways are involved. Bilirubin accumulating in the liver might, for example, be degraded by cytochrome P450 1A enzymes (34), but (possibly unknown) other mechanisms could also play a role.

Using Abcc2−/− mice, we confirmed that the absence of Abcc2 causes a reduction in bile flow (10, 35). Surprisingly, the bile flow (when corrected for body weight) of Abcc2;Abcc3−/− mice was significantly higher than that of Abcc2−/− mice (albeit still lower than wild-type bile flow). The reason for this is unknown. Possibly, the accumulation of Abcc2 and Abcc3 substrates in liver can induce compensatory mechanisms that increase the bile flow, perhaps via other ABC transporters, such as Abcg2 or Abcb11, although protein expression of these two transporters was not increased in the liver of male Abcc2;Abcc3−/− mice.

Using gallbladder cannulation experiments, we show that murine Abcc2 plays an important role in the biliary excretion of MTX, as was shown previously for rat Abcc2 (11). The results for rats and mice were similar although, in rats, the effect of Abcc2 was even more pronounced than in mice. This suggests that additional transport mechanisms (for example, Abcg2) are more important for biliary excretion of MTX in mice compared with rats. Another explanation for this difference could be that, in Abcc2-deficient rats, Abcc3 liver protein expression is much more increased than in Abcc2−/− mice (~6-fold in rats versus ~2-fold in mice; refs. 10, 36). Strongly induced Abcc3 in liver of Abcc2-deficient rats may lead to an overestimation of the effect of Abcc2 on biliary MTX excretion.

In the absence of Abcc2, Abcc3 is clearly involved in elimination of MTX from the liver. Increased plasma and decreased liver levels of MTX in the Abcc2−/− mice were reflected by increased urinary excretion of MTX, which was not observed in Abcc2;Abcc3−/− mice. Thus, hepatic Abcc3 expression is necessary for the increased urinary MTX excretion in the Abcc2−/− mice. The Abcc3-mediated (back) transport of MTX from the liver into the circulation leads to a reduced volume of distribution of MTX in Abcc2−/− mice due to the up-regulation of Abcc3. Interestingly, although hepatic Abcc3 transports MTX towards the circulation, MTX is still quite rapidly taken up by the liver, leading to the accumulation of >44% of the dose 7.5 minutes after administration in all strains. At later time points, other transport mechanisms than Abcc2 and Abcc3 (possibly Abcg2 and/or Abcc4), passive diffusion, and/or metabolism, also seem to play a role in liver elimination of MTX: in Abcc2;Abcc3−/− mice 120 minutes after i.v. administration, only 1% of the dose was left in the liver.

The effect of other (apical) transporters was also suggested by the gall bladder cannulation experiments because, in the absence of Abcc2, there was still significant transport of MTX into the bile. When Abcc2 and Abcc3 were both absent, the biliary excretion of MTX was markedly higher than in the absence of Abcc2 alone. Combined, these results suggest the presence of one or more alternative canalicular transporters that become increasingly important in biliary excretion of MTX as a consequence of (mildly) increasing liver concentrations in Abcc2;Abcc3−/− mice. An obvious candidate would be Abcg2, which has been shown to influence MTX pharmacokinetics in mice (37), but other apical transport proteins could also be involved.

The severities of MTX-related toxicities in humans seem to correlate with MTX serum levels (12, 16) and might, based on our results, be related to the expression levels and/or activity of Abcc2 and Abcc3. High-dose MTX therapy additionally leads to persistent renal dysfunction in nearly 30% of all patients (32). Renal failure is thought to be caused by crystalline deposits of MTX and especially 7OH-MTX (12). In rats, 7OH-MTX is much more toxic than MTX (38) and, after high-dose MTX treatment (>50 mg/kg), a significant amount of 7OH-MTX is detected in patient urine (33). This suggests that MTX-related toxicity may correlate with 7OH-MTX plasma and tissue levels. Insight into in vivo mechanisms that affect plasma levels of 7OH-MTX is therefore important. Recently, it was shown in vitro that ABCG2 and ABCC2 can transport 7OH-MTX (17), and we now show in mice that hepatic Abcc2 and Abcc3 are involved in the excretion of 7OH-MTX from the liver by transporting it into bile and circulation, respectively. Especially the absence of Abcc2 affects plasma and kidney levels of 7OH-MTX. Abcc3 provides an alternative (sinusoidal) route of 7OH-MTX elimination from the liver when Abcc2 is absent, and combined absence leads to substantial accumulation of 7OH-MTX in liver and, probably secondary to that, also in plasma. Abcc3 is not exclusively responsible for transport of 7OH-MTX into the circulation and subsequent urinary excretion. Especially at somewhat later time points (>30 minutes) after administration, another mechanism seems to be relevant as well, as was seen in the Abcc2;Abcc3−/− mice. This mechanism becomes apparent when liver concentrations of 7OH-MTX are high (Fig. 6B). A possible candidate for this would be Abcc4, which is expressed at the basolateral membrane of hepatocytes and the apical membrane in renal cells and is ~2-fold up-regulated in the liver of female Abcc2−/− and Abcc2;Abcc3−/− mice.

In Abcc2−/− and especially Abcc2;Abcc3−/− mice, the total amount of excreted 7OH-MTX (via urine and feces) is higher than in the other strains (Fig. 6D). It could be that MTX accumulation in the liver of these strains causes more 7OH-MTX formation. Another possible explanation is increased liver expression of the enzyme aldehyde oxidase, which is responsible for 7OH-MTX formation (18–20). Indeed, preliminary microarray analysis showed that mRNA expression of aldehyde oxidase in Abcc2−/− and Abcc2;Abcc3−/− mice is 1.4-fold to 2.4-fold increased. The 7OH-MTX formed is subsequently (slowly) eliminated from the liver and body, leading to a high 7OH-MTX exposure in Abcc2-deficient mice. It would be interesting to know whether the increased expression of aldehyde oxidase is also seen in patients with ABC2C2 mutations because this could make them more vulnerable to 7OH-MTX–related toxicity.

In this study, we administered an i.v. bolus injection of 50 mg/kg MTX to mice, which is comparable with a dose of 154 mg/m^2 in man (31). It cannot be excluded that the effects of Abcc2 and/or Abcc3 found in this study might differ when higher doses or different routes of administration (e.g., oral or constant infusion) are used. However, because associations between ABC2C2 mutations and MTX-related toxicity in patients have been found previously after high-dose (3–5 g/m^2) MTX infusions as well (13, 15), it is likely that, at least, the impact of ABC2C2 is similar even at very high doses and longer infusion times.

For practical reasons, this study was done in female mice. In females, the liver expression of Abcg2/ABCG2 protein is lower than in males (39), and the effect of Abcg2 on MTX and 7OH-MTX pharmacokinetics therefore may be lower as well. The
observed impact of Abcc2 (and Abcc3) on MTX and 7OH-MTX pharmacokinetics may thus be bigger in females. Interestingly, Rau et al. (2006); (15) found an association between an ABCC2 mutation and MTX-related toxicity in female but not in male patients. The fact that Abcc4 protein in liver is increased in female Abcc2;Abcc3+/- mice may additionally lead to gender-specific differences in MTX pharmacokinetics.

Our data show that Abcc3 can often compensate for Abcc2 when this protein is absent or nonfunctional, transporting shared substrates like bilirubin glucuronides, MTX, and 7OH-MTX from the liver back into the circulation, thereby leading to increased elimination via the urine. Our results show a direct effect of Abcc2 absence (and concomitant Abcc3 up-regulation) on the pharmacokinetics of MTX and 7OH-MTX and are in line with previously reported associations between ABCC2 mutations in patients and increased toxicity after MTX treatment (13–15). Clearly, Abcc2;Abcc3+/- mice provide useful tools to investigate the overlapping and compensatory roles of the ABC transporters Abcc2 and Abcc3 in the physiology and pharmacology of shared substrates in vivo.

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

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References
Impact of Abcc2 (Mrp2) and Abcc3 (Mrp3) on the In vivo Elimination of Methotrexate and its Main Toxic Metabolite 7-hydroxymethotrexate

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