Abcc2 (Mrp2), Abcc3 (Mrp3), and Abcg2 (Bcrp1) are the main determinants for rapid elimination of methotrexate and its toxic metabolite 7-hydroxymethotrexate in vivo

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Abstract

The multidrug transporters ABCC2, ABCC3, and ABCG2 can eliminate potentially toxic compounds from the body and have overlapping substrate specificities. To investigate the overlapping functions of Abcc2, Abcc3, and Abcg2 in vivo, we generated and characterized Abcc3−/− Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice. We subsequently analyzed the relative impact of these transport proteins on the pharmacokinetics of the anticancer drug methotrexate (MTX) and its main, toxic, metabolite 7-hydroxymethotrexate (7OH-MTX) after i.v. administration of MTX (50 mg/kg). Whereas in single and double knockout mice, the plasma and liver concentrations of MTX and 7OH-MTX decreased rapidly after MTX administration, in the Abcc2;Abcc3;Abcg2−/− mice, they remained very high. One hour after administration, 67% of the MTX dose was still present in livers of Abcc2;Abcc3;Abcg2−/− mice as MTX or 7OH-MTX versus 7% in wild-type, showing dramatic liver accumulation of these toxic compounds when Abcc2, Abcc3, and Abcg2 were all absent. Furthermore, the urinary and fecal excretion of the nephrotoxic metabolite 7OH-MTX were increased 27- and 7-fold, respectively, in Abcc2;Abcc3;Abcg2−/− mice. Thus, Abcc2, Abcc3, and Abcg2 together mediate the rapid elimination of MTX and 7OH-MTX after i.v. administration and can to a large extent compensate for each other’s absence. This may explain why it is still comparatively safe to use a toxic drug such as MTX in the clinic, as the risk of highly increased toxicity due to dysfunctioning of ABCC2, ABCC3, or ABCG2 alone is limited. Nevertheless, cotreatment with possible inhibitors of ABCC2, ABCC3, and ABCG2 should be done with utmost caution when treating patients with methotrexate. [Mol Cancer Ther 2009;8(12):3350–9]
basolateral efflux in liver and duodenum. They found no difference between wild-type and Abcc4−/− mice in the oral plasma pharmacokinetics of [3H]MTX. Also, in ABC2-deficient rats, it was shown that the biliary excretion of MTX was only 10% of the biliary MTX excretion in wild-type rats (15). Combining these literature data, there seem to be important and overlapping functions of especially Abcc2, Abcc3, and Abcg2 in the pharmacokinetics of MTX and 7OH-MTX. Also, in humans, it was shown that specific mutations or polymorphisms in ABC2 or ABCG2 could influence the pharmacokinetics of MTX, which was in some cases associated with increased toxicity or efficacy in patients with loss-of-function mutations (16–20). No correlations between ABC3 polymorphisms and MTX pharmacokinetics or toxicity have been reported yet.

Although quite extensive in vivo studies on the influence of MTX (and 7OH-MTX) have been done already, the relative effect of the different ABC transporters is unclear. When using single (and even double) knockout mice, the effects of the deleted gene(s) might be underestimated because other transporters may compensate for their loss. For example, we did not find an effect of Abcc3 on MTX pharmacokinetics after i.v. bolus administration when we analyzed Abcc3−/− mice, but when Abcc2 was additionally deleted, a clear effect of Abcc3 expression was found (13). The same was true for the effect of Abcg2 on 7OH-MTX pharmacokinetics, which only became apparent in the absence of Abcc2 (12).

In the present study, we describe the generation and characterization of Abcc3;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice. These mice were subsequently used to further unravel the overlapping and possibly compensatory functions of Abcc2, Abcc3, and Abcg2 in determining the pharmacokinetics of MTX and 7OH-MTX in vivo. We show here that these three transporters are the main determinants of the rapid elimination of MTX and 7OH-MTX from the liver and plasma. Other ABC transporters seem less important in these processes.

### Materials and Methods

#### Animals

Mice were housed and handled according to institutional guidelines complying with Dutch legislation. The generation of Abcc2−/− (21), Abcc3−/− (22), Abcg2−/− (23), Abcc2; Abcc3−/− (13, 24), and Abcc2; Abcg2−/− mice (12) was described before. Abcc3; Abcg2−/− mice were generated by crossbreeding the two single knockout strains. Abcc2; Abcc3; Abcg2−/− mice were generated by crossbreeding Abcc2; Abcc3−/− and Abcc2; Abcg2−/− mice. All animals were of >99% FVB background and between 9 to 14 wk of age. Animals were kept in a 12-h light/12-h dark cycle. They received a standard diet (AM-II, 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. Methoxyflurane (Metofane) was from Medical Developments Australia Pty. Ltd. MRPr1 and MjL-80 were kind gifts of Dr. G.L. Scheffer (Free University Hospital, Amsterdam, the Netherlands). A polyclonal antibody against mouse Abcc2 (25) was kindly provided by Prof. Dr. J.-M. Fritschi (University of Zürich, Zürich, Switzerland). Real-time PCR primers were from Qiagen.

#### Western Analysis

Crude membrane fractions from tissues were prepared as described (23, 26). Western blotting was done as described (27). Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer. Abcc1 (Mrp1), Abcc2 (Mrp2), and Abcc4 (Mrp4) were detected with monoclonal antibodies MRPr1 (dilution, 1:1,000), a polyclonal antimurine Abcc2 antibody (dilution, 1:1,000), and MjL-80 (dilution, 1:400), respectively. Bound primary antibodies were detected by incubating the blot with horseradish peroxidase–labeled rabbit anti-rat IgG (1:1,000; DAKO) or goat anti-rabbit IgG (1:1,000; DAKO). Densitometric analysis was done using 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 (28).

#### Histologic, Clinical-Chemical, and Hematologic Analysis

Histologic analysis of tissues (n = 5, male and female), standard clinical chemistry analyses on serum (twice within a time span of 1.5 y, n = 5–6), and standard hematologic analysis of male and female mice (n = 6) were done as described (21).

#### Plasma and Tissue Pharmacokinetic Experiments

MTX was administered to female wild-type, Abcc3;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice (n = 3–13) by injecting 5 µL/g body weight of a 10 mg/mL MTX solution in saline into the tail vein. Animals were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia at 7.5, 15, 30, 60, or 120 min after MTX administration, and organs were removed at each time point.

#### Fecal and Urinary MTX Excretion Experiment

Female wild-type, Abcc3;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice (n = 5–9) were individually housed in Ruco Type M/1 stainless steel metabolic cages. They were allowed 24 h to adapt before MTX was injected at 50 mg/kg into the tail vein as described above, and feces and urine were collected for 24 h.

#### High Performance Liquid Chromatography Analysis of MTX and 7OH-MTX

Collected organs and feces were homogenized in an ice-cold 4% bovine serum albumin solution, and plasma was diluted in human plasma before high performance liquid chromatography analysis. Urine was diluted in water. MTX and 7OH-MTX concentrations in the different matrices were determined as described (29).

#### 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 statistical differences between more than two groups were
analyzed, one-way ANOVA followed by Tukey’s multiple comparison test was done, as indicated. Results are presented as means ± SDs. Differences were considered to be statistically significant when $P < 0.05$. Average 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; SEMs were calculated by the law of propagation of errors (30).

### Results

#### Macroscopic and Microscopic Analysis of Abcc3; Abcg2−/− and Abcc2; Abcc3; Abcg2−/− Mice

Abcc3; Abcg2−/− and Abcc2; Abcc3; Abcg2−/− mice were viable, fertile, and had normal life spans and body weights. Macroscopic and microscopic histologic and pathologic analysis did not reveal obvious specific aberrations in tissues of both knockout strains, including the liver. However, in Abcc2; Abcc3; Abcg2−/− mice (but not in Abcc3; Abcg2−/− mice), the liver weight was increased 41% to 67% compared with wild-type (Fig. 1A). Increased liver weight was previously shown in other Abcc2-deficient strains (Fig. 1A; refs. 12, 13, 21, 31). However, the liver weight in Abcc2; Abcc3; Abcg2−/− mice (both male and female) was also significantly higher than in most Abcc2, Abcc3, and Abcg2 single and double knockout mice (ANOVA).

#### Protein and mRNA Expression of other ABC Transporters in Tissues of Abcc3; Abcg2−/− and Abcc2; Abcc3; Abcg2−/− Mice

Protein expression of Abcc1 in liver of male wild-type, Abcc3; Abcg2−/−, and Abcc2; Abcc3; Abcg2−/− mice was low and therefore hard to quantify (data not shown). Abcc2 protein in liver of male and female Abcc3; Abcg2−/− mice was not different from wild-type mice (data not shown). Abcc2 protein in kidney of female Abcc3; Abcg2−/− mice was not different from wild-type either.

Abcc4 protein expression in kidney of female Abcc2; Abcc3; Abcg2−/− mice was increased 1.6-fold compared with wild-type mice and similar to that of Abcc2; Abcg2−/− mice (Fig. 1B). Abcc4 protein in livers of male Abcc3; Abcg2−/− and Abcc2; Abcc3; Abcg2−/− mice was low and therefore hard to quantify (data not shown). In female Abcc3; Abcg2−/− mice, liver Abcc4 protein was not different from wild-type. In female Abcc2; Abcc3; Abcg2−/− mice, Abcc4 liver expression seemed to increase about 2-fold compared with wild-type, comparable with that in Abcc2; Abcg2−/− mice (12), although this was hard to quantify due to low expression levels in all strains (data not shown). Quantitative real-time PCR analysis confirmed that in female Abcc2; Abcg2−/− and Abcc2; Abcc3; Abcg2−/− mice, Abcc4 mRNA expression in liver was significantly (14- to 19-fold) increased compared with wild-type mice (Fig. 1C). Abcc4 mRNA levels in Abcc3; Abcg2−/− mice were not different from wild-type mice (Fig. 1C).

#### Expression Levels of Genes Involved in Methotrexate Disposition

Because we aimed to analyze the pharmacokinetics of MTX using Abcc3; Abcg2−/− and Abcc2; Abcc3; Abcg2−/− mice, we determined mRNA expression levels of other genes that may be involved in MTX metabolism and transport in livers of female knockout mice. We found that, similar to Abcc2−/− and Abcc2; Abcg2−/− mice (12), mRNA levels of the enzyme aldehyde oxidase 1, which is implicated in the conversion of MTX to 7OH-MTX (32), was increased 2.5-fold compared with wild-type mice ($n = 3$, $P = 0.008$; data not shown). Aldehyde oxidase 3 mRNA expression in liver was not significantly different from wild-type (data not shown).

### Serum Clinical Chemistry and Hematologic Analysis of Abcc3; Abcg2−/− and Abcc2; Abcc3; Abcg2−/− Mice

Table 1 shows clinical chemistry parameters of Abcc3; Abcg2−/− and Abcc2; Abcc3; Abcg2−/− mice that were different from wild-type mice. The results of all measured parameters are shown in Supplementary Table S1. Conjugated bilirubin levels were below the detection limit of the analyzer (2 μmol/L). In the Abcc2; Abcc3; Abcg2−/− mice, the serum total bilirubin concentrations were mildly increased compared with wild-type, as was previously shown for Abcg2−/− and Abcc2; Abcg2−/− mice (12, 23). In contrast to what was previously shown for Abcc2−/− and Abcc2; Abcg2−/− mice (12, 21, 33), we did not detect increased conjugated bilirubin levels in Abcc2; Abcc3; Abcg2−/− mice. This is in line with the earlier observation that Abcc3 is responsible for transport of conjugated bilirubin from liver to blood when Abcc2 is absent (13). Besides bilirubin, triglyceride serum concentrations were also increased ~2-fold in Abcc2; Abcc3; Abcg2−/− mice (but not in Abcc3; Abcg2−/− mice; Table 1). Furthermore, total protein levels were mildly increased in serum of Abcc2; Abcc3; Abcg2−/− mice (Table 1). In male (but not female) Abcc2; Abcc3; Abcg2−/− mice, alanine aminotransferase, creatinine, and urea serum concentrations were also mildly but significantly increased (Table 1).

Hematologic analysis of Abcc3; Abcg2−/− mice did not show consistent differences with wild-type mice (data not shown). Female Abcc2; Abcc3; Abcg2−/− mice had, similar to other Abcc2-deficient mice (12, 21), mildly reduced hemoglobin levels compared with wild-type (7.2 ± 0.2 mmol/L versus 7.8 ± 0.3 mmol/L, $n = 6$, $P = 2.3 \times 10^{-3}$). In males, this was not found.

#### Effect of Abcc2, Abcc3, and Abcg2 on MTX and 7OH-MTX Pharmacokinetics

We have previously shown that Abcc2 and Abcg2 are the main determinants for the biliary excretion of MTX and 7OH-MTX after i.v. administration of MTX, (50 mg/kg; ref. 12). Furthermore, when Abcc2 is absent, (upregulated) Abcc3 in the liver causes increased efflux of MTX and 7OH-MTX from liver to blood, leading to increased urinary excretion of these compounds (13). To further investigate the functional overlap and importance of these ABC transporters, we administered MTX i.v. at a dose of 50 mg/kg to wild-type, Abcc3; Abcg2−/−, and Abcc2; Abcc3; Abcg2−/− mice

Mol Cancer Ther 2009;8(12). December 2009
and analyzed the pharmacokinetics of MTX and 7OH-MTX. The results for all compound and single Abcc2, Abcc3, and Abcg2 knockout mice (12, 13) are given in Supplementary Tables S2 (for MTX) and S3 (for 7OH-MTX). Figure 2 shows the pharmacokinetics of MTX and 7OH-MTX in wild-type, Abcc2;Abcc3;Abcg2−/− mice (Fig. 2A). Whereas in all other strains, the MTX plasma levels rapidly decreased 30 minutes after administration, the plasma levels of 7OH-MTX increased rapidly, probably due to reduced biliary excretion of MTX, 7OH-MTX, or both (12). In Abcc2;Abcc3;Abcg2−/− mice, however, this increase was less pronounced, showing that part of the increased plasma 7OH-MTX concentrations in Abcc2;Abcg2−/− mice was dependent on the presence of (overexpressed) Abcc3 in these strains, leading to increased sinusoidal efflux (13).

**Figure 1.** Physiologic characterization of Abcc3;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice. A, liver weight (as percentage of body weight) of male (left) and female (right) wild-type, Abcc2−/+ , Abcc2;Abcg2−/−, Abcc2;Abcc3−/−, Abcc2;Abcg2−/−, and Abcc2;Abcc3;Abcg2−/− mice (columns, means (n = 3–12); bars, SD; **, P < 0.01; ***, P < 0.001, relative to wild-type, ANOVA). B, protein levels of Abcc4 (molecular weight, 150 kDa) in crude membrane fractions of kidney samples from two independent female wild-type, Abcc2;Abcc3;Abcg2−/−, and (for comparison) Abcc2;Abcg2−/− mice (dilution experiment). Densitometric analysis of the blots showed that on average Abcc4 expression was increased 1.6-fold in Abcc2;Abcc3;Abcg2−/− mice compared with wild-type. The lane with the positive control (Sf-9 vesicles containing ABCC4; ref. 40), is indicated with “+”. Underglycosylation of ABCC4 in the Sf-9 cells causes the faster migration compared with the murine Abcc4. The amount of protein loaded in each lane (in micrograms) is noted above the respective lanes. C, mRNA expression of Abcc4 in liver of female wild-type, Abcc2;Abcg2−/−, Abcc3;Abcg2−/−, and Abcc2;Abcc3;Abcg2−/− mice. Results are expressed as the fold change in liver of knockout mice compared with wild-type mice. Data were normalized against the endogenous control glyceraldehyde-3-phosphate dehydrogenase (n = 3; ***, P < 0.05; ns, not significant; ANOVA, statistical significance was determined by comparison of NCi values of different strains). Columns, means; bars, SD.

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hepatic Abcc3 (Fig. 2C, see also below). It should be noted that in the Abcc2;Abcc3;Abcg2−/− mice, substantial amounts of 7OH-MTX still reached the circulation, leading to increased plasma levels and a 9.6-fold increased AUCplasma compared with wild-type mice over the first 120 minutes (Supplementary Table S3; Fig. 2C). This indicates that in the Abcc2;Abcc3;Abcg2−/− mice, substantial amounts of 7OH-MTX can still leave the liver over the basolateral membrane, even without Abcc3.

Analysis of hepatic 7OH-MTX concentrations clearly showed the importance of Abcc2, Abcc3, and Abcg2 for the elimination of 7OH-MTX from the liver. Liver levels of 7OH-MTX were dramatically (up to 90-fold at t = 120 minutes) increased in the Abcc2;Abcc3;Abcg2−/− mice (Fig. 2D), also when compared with all double knockout strains, including Abcc2;Abcc3−/− mice (Supplementary Table S3). At 60 minutes, the cumulative MTX and 7OH-MTX levels in wild-type as well as single and double knockout strains did not exceed 22% of the given dose (ranging from 7.0 ± 0.9% of the dose in wild-type to 22.0 ± 7.2% of the dose in Abcc2;Abcc3−/− mice). However, in Abcc2;Abcc3;Abcg2−/− mice, the combined MTX and 7OH-MTX liver levels amounted to 67.1 ± 3.5% of the dose (Supplementary Fig. S1), illustrating a drastically reduced liver elimination of both compounds in these mice. In the triple knockout mice, accumulated 7OH-MTX was slowly released from the liver, primarily toward plasma, resulting in relatively high 7OH-MTX plasma levels over a prolonged time frame (Fig. 2C and D).

MTX levels in the kidney in general reflected the plasma MTX levels, leading to 7- and 2-fold increased kidney levels in Abcc2;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice, respectively, at 60 minutes after administration (Supplementary Table S2; Fig. 3A; ref. 12). The 7OH-MTX kidney levels also followed the plasma levels, resulting in substantial kidney accumulation in both Abcc2;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice at 60 minutes after administration (Fig. 3C).

We have shown before that the presence of MTX and 7OH-MTX in the small intestine after i.v. administration (up to about 50% of the dose in 60 minutes) is mainly due to their biliary excretion by Abcc2 and Abcg2, whereas direct excretion across the intestinal wall is relatively low (12, 13). Figure 3B shows that in Abcc2;Abcc3;Abcg2−/− mice, the excretion of MTX into the small intestine was clearly reduced to only 15% of wild-type levels at 60 minutes after administration (Supplementary Table S2). Despite markedly increased hepatic levels in the triple knockout mice (Fig. 2B), relatively little MTX was excreted into the small intestine, once more showing that Abcc2 and Abcg2 are the predominant factors involved in the hepatobiliary excretion of MTX into the small intestine. The somewhat higher small intestinal MTX levels in Abcc2;Abcc3;Abcg2−/− compared with Abcc2;Abcg2−/− mice (P = 4.8 × 10−3 by Student's t test at 60 minutes) probably reflected the higher liver MTX concentrations (Fig. 3B). Clearly, also in the absence of Abcc2 and Abcg2, there is some remaining biliary excretion of MTX, as we still found low levels of MTX in the small intestine of Abcc2;Abcg2−/− and Abcc2;Abcc3;Abcg2−/− mice.

Figure 3D shows that, despite the increased liver levels of 7OH-MTX in the Abcc2;Abcc3;Abcg2−/− mice, the excretion into the small intestine was significantly lower than in wild-type mice. This confirms that Abcc2 and Abcg2 are the main transporters responsible for (biliary) excretion of 7OH-MTX into the small intestine. However, like for MTX (see above), the small intestinal levels of 7OH-MTX were significantly higher at 60 minutes after administration in Abcc2;

### Table 1. Clinical chemical analysis of serum from wild-type, Abcc3;Abcg2−/−, and Abcc2;Abcc3;Abcg2−/− mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sex</th>
<th>Wild-type</th>
<th>Abcc3;Abcg2−/−</th>
<th>Abcc2;Abcc3;Abcg2−/−</th>
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<tbody>
<tr>
<td>Total Bilirubin (μmol/L)</td>
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<td>2.2 ± 0.4</td>
<td>2.7 ± 0.8</td>
<td>3.7 ± 0.5*</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>3.0 ± 1.0</td>
<td>3.7 ± 1.9</td>
<td>4.2 ± 1.3*</td>
</tr>
<tr>
<td>ALAT (U/l)</td>
<td>Male</td>
<td>34 ± 6</td>
<td>36 ± 7</td>
<td>55 ± 14*</td>
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<tr>
<td></td>
<td>Female</td>
<td>27 ± 8</td>
<td>22 ± 9</td>
<td>33 ± 6</td>
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<tr>
<td>Creatinine (μmol/L)</td>
<td>Male</td>
<td>11 ± 2</td>
<td>19 ± 4</td>
<td>25 ± 4*</td>
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<td>Urea (mmol/L)</td>
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<td>13 ± 2</td>
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</tr>
<tr>
<td>Total protein (g/l)</td>
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<td>48 ± 2</td>
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<td>Triglyceride (mmol/L)</td>
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<td>2.0 ± 0.7</td>
<td>2.0 ± 0.6</td>
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NOTE: Results are presented as means ± SD (n = 5–6).

Abbreviation: ALAT, alanine aminotransferase.

*P < 0.001 compared with wild-type mice (Student's t test was used for statistical analysis).

†P < 0.01 compared with wild-type mice (Student's t test was used for statistical analysis).

‡P < 0.05 compared with wild-type mice (Student's t test was used for statistical analysis).

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Abcc3;Abcg2<sup>−/−</sup> compared with Abcc2;Abcg2<sup>−/−</sup> mice (P < 0.01, ANOVA), suggesting that the increased liver and plasma concentrations in these mice led to somewhat increased (hepatobiliary and/or direct) excretion into the small intestine of 7OH-MTX, not mediated by Abcc2 or Abcg2.

**Fecal and Urinary Excretion of MTX and 7OH-MTX**

The cumulative excretion of MTX and 7OH-MTX over 24 hours into urine and feces in wild-type, Abcc3;Abcg2<sup>−/−</sup>, and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice is shown in Fig. 4. Despite the delayed hepatic elimination of MTX and 7OH-MTX early after administration (see above), especially in the triple knockout mice, virtually all of the administered MTX was excreted as MTX or 7OH-MTX within 24 hours (Fig. 4C and D). The total urinary excretion of MTX was not different from wild-type in the compound knockout strains, showing that even if Abcc2, Abcc3, and Abcg2 are absent, MTX can still leave the liver and be excreted via the kidney. The fecal excretion of MTX was not significantly different from wild-type in the Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice either. Interestingly, in Abcc3;Abcg2<sup>−/−</sup> mice, the fecal excretion of MTX was significantly increased compared with wild-type (Fig. 4A).

The urinary excretion of 7OH-MTX in wild-type and Abcc3;Abcg2<sup>−/−</sup> mice was low, in line with the relatively high plasma and kidney 7OH-MTX concentrations in these strains. The fecal excretion of 7OH-MTX was, like that of MTX, somewhat increased in Abcc3;Abcg2<sup>−/−</sup> mice. In contrast, in Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice, both the urinary and fecal excretion of 7OH-MTX were markedly increased (27.0- and 7.3-fold, respectively) compared with wild-type (Fig. 4B). In fact, in Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice, the total amount of 7OH-MTX recovered in urine and feces represented ~43% of the total MTX dose, as opposed to <8% in wild-type and double knockout strains (Fig. 4C and D).

**Discussion**

To investigate the possibly overlapping functions of Abcc2, Abcc3, and Abcg2, we generated and characterized Abcc3;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice. Despite the absence of up to three ABC transporters with overlapping or complementary functions in the protection from toxic compounds (1–4), both strains are viable, fertile, and have a normal life span. They also do not display obvious physiologic or pathologic aberrations under standard housing conditions, except for an increased liver size and some mild changes in serum clinical chemistry.
We therefore consider these double and triple knockout mice quite suitable for pharmacokinetic, toxicologic, and physiologic research on the functions of Abcc2, Abcc3, and Abcg2. We used the mice to evaluate the overlapping or complementary functions of Abcc2, Abcc3, and Abcg2 in the pharmacokinetics of the anticancer and antirheumatic drug MTX and its main, toxic, metabolite 7OH-MTX in vivo.

We show here that Abcc2, Abcc3, and Abcg2 together are the primary ABC transporters responsible for the fast elimination of both MTX and 7OH-MTX from the liver and body, and that they can largely compensate for the loss of each other. Absence of all three ABC transporters leads to a dramatic accumulation of MTX and 7OH-MTX in the liver, as well as prolonged systemic exposure to both compounds after MTX administration.

In Abcc2;Abcc3;Abcg2−/− mice, some changes in clinical chemistry parameters of serum were found (Table 1). However, although the differences were consistent in two separate measurements, they were relatively small, and extensive pathologic analysis of liver and kidney of these mice did not reveal any signs of pathologic lesions. Furthermore, the mice had normal life spans. This suggests that there are no serious effects on the health of Abcc2;Abcc3;Abcg2−/− mice, showing that at least in the protective environment of our animal facility, Abcc2, Abcc3, and Abcg2 do not have crucial overlapping physiologic functions.

We found that after i.v. administration of MTX, Abcc2, Abcc3, and Abcg2 play important and clearly overlapping or complementary roles in the elimination of MTX and 7OH-MTX from the liver. Whereas in single and double knockout mice there was only relatively mild accumulation of both compounds in the liver (Supplementary Tables S2 and S3), in the Abcc2;Abcc3;Abcg2−/− mice this was much more dramatic, especially for 7OH-MTX, and it lasted longer as well (Supplementary Fig. S1; Fig. 2B and D). Furthermore, whereas in all other strains the plasma levels of MTX and 7OH-MTX at 120 minutes after i.v. administration of MTX were quite low and comparable with wild-type (Supplementary Tables S2 and S3), in Abcc2;Abcc3;Abcg2−/− mice, they were still relatively high, suggesting prolonged systemic exposure to these toxic compounds in the combined absence of these transporters. In Abcc2;Abcc3;Abcg2−/− mice, the total amount of 7OH-MTX recovered in urine and feces represented ~43% of the total MTX dose, as opposed to <8% in wild-type and double knockout strains (Fig. 4C and D). It is likely that the absence of Abcc2, Abcc3, and Abcg2 leads
to increased retention of MTX in the liver and subsequent extensive 7OH-MTX formation and accumulation in the liver. This is subsequently eliminated over a relatively prolonged period via both urine and feces. Combined, these results show that Abcc2, Abcc3, and Abcg2 can to a large extent compensate for absence of one another in the liver. Other ABC transporters, which have been shown to transport MTX in vitro, such as Abcc1, Abcc4, Abcc5, and P-gp (10, 11, 34, 35), do not seem to play a significant role in the rapid elimination of MTX and 7OH-MTX in vivo, at least not after bolus i.v. administration of MTX at 50 mg/kg. Abcc2, Abcc3, and Abcg2 clearly are important for the disposal of MTX and 7OH-MTX early after administration. However, also in the absence of all three proteins, virtually all of the administered MTX was excreted as MTX or 7OH-MTX within the first 24 hours after administration (Fig. 4). This shows that other (low capacity) elimination systems are apparently still able to eliminate these compounds from the body. Which mechanisms are responsible for this is not clear and may be investigated in the future.

The combined absence of Abcc2, Abcc3, and Abcg2 led to trapping of MTX in the liver due to reduced biliary (via Abcc2 and Abcg2) and sinusoidal (via Abcc3) elimination of MTX. This in turn led to the increased hepatic formation of the toxic MTX metabolite 7OH-MTX (Fig. 2B and D), showing that Abcc2, Abcc3, and Abcg2 are very important for limiting the hepatic formation of this toxic metabolite by keeping liver MTX levels low. Moreover, 7OH-MTX is also efficiently transported out of the liver for fecal or urinary excretion by Abcc2, Abcc3, and Abcg2. Together, these processes result in a reduced exposure of the body to the highly toxic 7OH-MTX. It is therefore possible that decreased activity and/or expression of ABCC2, ABCC3, and/or ABCG2 may lead to accumulation of MTX and 7OH-MTX in patients treated with MTX. This may cause increased risk of hepatotoxicity when patients with polymorphisms or mutations in one or more of these genes are treated with MTX, as was previously shown for MTX-treated African-American patients carrying an ABCC2 mutation (19). However, as it may be unlikely for patients to have polymorphisms or mutations in all three transporters, the risk of increased MTX-related hepatotoxicity could be relatively small.

In fact, it is worth noting that the functional overlap or complementarity between no less than three different proteins that we observed here guarantees a considerable robustness in the protection from MTX, a xenobiotic toxin. This makes sense from both biological and clinical-therapeutic perspectives. Although individual deficiencies in Abcc2, Abcc3, or Abcg2 can affect the clearance and systemic exposure of MTX to a certain extent, from our study, it is clear that the consequences would be much more severe in the absence of functions of all these proteins. Thus, overall protection against MTX toxicity is not entirely dependent on the function of one single gene and protein, but it is determined by at least three different genes. This means that the risk of increased toxicity due to genetic polymorphisms or coincidental inhibition affecting activity of a single gene or protein is much reduced. This is clearly advantageous in the natural protection from xenobiotic toxins. It also means that it is comparatively safe to use a drug such as MTX in the clinic, as the risk of unpredictable toxicity due to dysfunctioning of one detoxifying protein is relatively limited. Clearly, drugs of which the toxicity is critically dependent on the function of just one detoxifying protein bear considerably higher risks, and they should probably be avoided in clinical practice. Nevertheless, as many drugs (and food components) may be substrates or inhibitors of these three ABC transporters, coadministration of MTX with drugs that
are also ABC2, ABC3, and ABCG2 substrates should be done with caution. Furthermore, many mutations and polymorphisms of ABC2 and ABCG2 that can influence drug pharmacokinetics exist (17, 36). Especially in patients with impaired function of ABC2 and/or ABCG2, the effect of ABC3 on MTX and 7OH-MTX pharmacokinetics may be more important than previously anticipated.

Interestingly, in Abcc3;Abcg2<sup>−/−</sup> mice, the fecal excretion of MTX and 7OH-MTX was significantly higher than in wild-type mice. In the mildly increased liver accumulation of MTX and 7OH-MTX early after administration in these mice due to combined absence of Abcc3 and Abcg2 in the liver (Supplementary Table S3) may have caused increased excretion of these compounds into the intestine over 24 hours, most likely via Abcc2 (13). In addition, absence of Abcc3 in the intestinal wall of these mice may lead to reduced reabsorption of MTX and perhaps 7OH-MTX from the intestine (13). As a consequence, enterocytes of Abcc3;Abcg2<sup>−/−</sup> mice may become exposed to higher levels of MTX and these mice could therefore be more prone to gastrointestinal toxicity. As this could mean that patients with decreased ABC3 expression or activity in the intestine may be more susceptible to gastrointestinal toxicity (a common side effect of MTX treatment (37), it would be interesting to test this hypothesis in more detail in the future.

Kidney failure is another common toxicity in patients treated with high doses of MTX (38). This is thought to be primarily due to the formation of crystalline deposits of MTX and especially of the poorly water-soluble metabolite 7OH-MTX in renal tubules (37). Because in the Abcc2<sup>−/−</sup>;Abcc3<sup>−/−</sup>;Abcg2<sup>−/−</sup> mice the formation of 7OH-MTX and, as a result, the urinary excretion of 7OH-MTX was dramatically increased compared with wild-type mice (and the other (compound) knockout strains), this suggests that ABCC2, ABCC3, and ABCG2 together are very important in the protection of the kidneys from 7OH-MTX exposure and that they may prevent MTX- and 7OH-MTX-related toxicities in patients.

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 may therefore be lower as well. Whether this is indeed the case should be investigated in future studies.

The data presented here illustrate the dramatic changes in the pharmacokinetics of both MTX and 7OH-MTX when two or more ABC transporters are absent. In the future, these results may be helpful in predicting responses to MTX treatment in patients based on individual expression or activity data of ABCC2, ABCC3, and/or ABCG2. Furthermore, the Abcc2<sup>−/−</sup>;Abcc3<sup>−/−</sup>;Abcg2<sup>−/−</sup> mice we generated will be excellent tools to determine the relative and overlapping effects of these ABC transporters on the pharmacokinetics of a wide range of existing and newly developed drugs.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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Abcc2 (Mrp2), Abcc3 (Mrp3), and Abcg2 (Bcrp1) are the main determinants for rapid elimination of methotrexate and its toxic metabolite 7-hydroxymethotrexate in vivo

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