Impact of Abcc2 [Multidrug Resistance-Associated Protein (Mrp) 2], Abcc3 (Mrp3), and Abcg2 (Breast Cancer Resistance Protein) on the Oral Pharmacokinetics of Methotrexate and Its Main Metabolite 7-Hydroxymethotrexate

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ABSTRACT:
The ATP-binding cassette (ABC) transporters ABCB2 [multidrug resistance-associated protein (MRP) 2], ABCB3 (MRP3), and ABCG2 [breast cancer resistance protein] are involved in the efflux of potentially toxic compounds from the body. We have shown before that ABCB2, ABCB3, and ABCG2 together influence the pharmacokinetics of the anticancer and antirheumatic drug methotrexate (MTX) and its toxic metabolite 7-hydroxymethotrexate (7OH-MTX) after intravenous MTX administration. We now have used Abcc2;Abcc3;Abcg2−/− mice and corresponding single and double knockout mice to investigate the relative impact of these transporters on MTX and 7OH-MTX pharmacokinetics after oral MTX administration (50 mg/kg). The plasma areas under the curve (AUC plasma) in Abcg2−/− and Abcc2;Abcg2−/− mice were 1.7- and 3.0-fold higher than those in wild-type mice, respectively, suggesting additive effects of Abcc2 and Abcg2 on oral MTX pharmacokinetics. However, the AUC plasma in Abcc2;Abcc3;Abcg2−/− mice was not different from that in wild-type mice, indicating that Abcc3 protein is necessary for increased MTX plasma concentrations in the absence of Abcc2 and/or Abcg2. Furthermore, 2 h after administration, MTX liver levels were increased in Abcg2-deficient strains and MTX kidney levels were 2.2-fold increased in Abcc2;Abcg2−/− mice compared with those in wild-type mice. The absence of Abcc2 and/or Abcg2 also led to significantly increased liver and kidney levels of 7OH-MTX. Our results suggest that inhibition of ABCG2 and/or ABC2, genetic polymorphisms or mutations reducing expression or activity of these proteins may increase the oral availability of MTX. Such conditions may also present risk factors for increased MTX-related toxicity in patients treated with oral MTX.

Introduction
The ATP-binding cassette (ABC) transporters ABCB2 (multidrug resistance-associated protein 2), ABCB3 (multidrug resistance-associated protein 3), and ABCG2 (breast cancer resistance protein) are membrane proteins that are involved in the efflux of potentially toxic endogenous and exogenous substrates from cells. They are expressed in epithelial cells of excretory organs, such as liver, kidney, and small intestine, and can influence the pharmacokinetics of a wide range of (anticancer) drugs (Borst and Oude Elferink, 2002; Schinkel and Jonker, 2003; Borst et al., 2006; Breedveld et al., 2006; Kruh et al., 2007). Whereas ABCB2 and ABCG2 are expressed at the apical membranes of cells, transporting their substrates into bile, feces, and urine, ABCB3 is expressed basolaterally, especially in hepatocytes and enterocytes, and it generally transports its substrates into the blood circulation (Borst and Oude Elferink, 2002; Borst et al., 2006).

ABC2, ABC3, and ABCG2 have broad and substantially overlapping substrate specificities (Borst and Oude Elferink, 2002; Borst et al., 2006; Kruh et al., 2007), but their relative impact on the pharmacokinetics of shared substrates is not clear yet. We have recently generated compound knockout mice for these transporters (Vlaming et al., 2008, 2009a,b), which, together with the previously generated single knockout mice for Abcc2 (Vlaming et al., 2006), Abcc3 (Zelcer et al., 2006), and Abcg2 (Jonker et al., 2002), form a complete set of mouse models that can be used to elucidate the relative and possibly overlapping effects of these proteins on the

ABBREVIATIONS: ABC, ATP-binding cassette; MTX, methotrexate; 7OH-MTX, 7-hydroxymethotrexate; AUC, area under the plasma concentration versus time curve.
pharmacokinetics of endogenous and exogenous substrates. Using this set of mouse strains, we have recently shown that Abcc2, Abcc3, and Abcg2 have profoundly overlapping and additive effects on the intravascular pharmacokinetics of the widely used anticancer drug methotrexate (MTX) and its major toxic metabolite 7-hydroxymethotrexate (7OH-MTX), presumably primarily through their activity in the liver (Vlamming et al., 2008, 2009a,b).

In cancer treatment, most drugs are given intravenously because of low and/or highly variable bioavailability, which can be caused, among other reasons, by expression of ABC transporters in the intestine (Breedveld et al., 2006). However, because oral administration of drugs is more patient-friendly and more cost-effective, attempts are being made to improve the oral bioavailability of several drugs by coadministration of ABC transporter inhibitors (Kuppens et al., 2005; Breedveld et al., 2006). Because Abcc2, Abcc3, and Abcg2 are all expressed in epithelial cells of the small intestine (Borst and Oude Elferink, 2002; Schinkel and Jonker, 2003), they may, besides affecting the intravascular pharmacokinetics, also influence the oral uptake of MTX (and 7OH-MTX). It was shown previously in Abcc2-deficient rats that after oral administration of MTX the plasma concentrations were significantly increased compared with those in wild-type rats (Chen et al., 2003; Naba et al., 2004). In mice, the effect of Abcc2 after oral MTX has not been investigated yet. Kitamura et al. (2008) found a positive effect of murine Abcc3 on plasma pharmacokinetics of [3H]MTX after oral administration. It was surprising that although the impact of Abcg2 on the oral pharmacokinetics of many drugs has been extensively studied (van Herwaarden and Schinkel, 2006; Murakami and Takano, 2008), its effect on the disposition of MTX and 7OH-MTX after oral MTX administration has not been investigated yet.

In the present study, we have used the recently generated Abcc2; Abcc3; Abcg2 (−/−) mice, as well as the corresponding single and double knockout mice, to investigate the relative effect of Abcc2, Abcc3, and Abcg2 on the oral pharmacokinetics of MTX and its metabolite 7OH-MTX. We show here that deletion of Abcg2 increases the plasma concentrations of MTX after oral administration and that additional deletion of Abcc2 leads to an even more pronounced increase. Of interest, Abcc3 expression is necessary for this enhancing effect. Furthermore, Abcc2, Abcc3, and Abcg2 clearly influence the tissue concentrations of MTX and 7OH-MTX, also after oral MTX application.

Materials and Methods

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. The generation and basic characterization of Abcc2(−/−) (Vlamming et al., 2006), Abcc3(−/−) (Zelcer et al., 2006), Abcg2(−/−) (Jonker et al., 2002), Abcc2;Abcc3(−/−) (van de Wetering et al., 2007; Vlamming et al., 2008), Abcc2;Abcg2(−/−) (Vlamming et al., 2009a), and Abcc3;Abcg2(−/−) and Abcc2;Abcc3;Abcg2(−/−) mice (Vlamming et al., 2009b) was described previously. All animals were of >99% FVB background and between 9 and 14 weeks of age. Animals were kept in a temperature-controlled environment with a 12:12 light:dark cycle. They received a standard diet (AM-H; Hope Farms, Woerden, The Netherlands) and acidified water ad libitum.

Chemicals. MTX (Emthexate PF; 25 mg/ml) was from Pharmachemie (Haarlem, The Netherlands), 7OH-MTX was from Toronto Research Chemicals, Inc. (North York, ON, Canada), and methoxyflurane (Metofane) was from Medical Developments Australia Pty. Ltd. (Springvale, VIC, Australia).

Plasma and Tissue Pharmacokinetic Experiments. Before MTX administration, mice were fasted for at least 4 h. MTX was administered to female wild-type. Abcc2(−/−), Abcc3(−/−), Abcg2(−/−), Abcc2;Abcc3(−/−), Abcc2;Abcg2(−/−), Abcc3;Abcg2(−/−) and Abcc2;Abcc3;Abcg2(−/−) mice (n = 2–14, as detailed in the figure legends) by dosing 10 μl/g b.wt. MTX (5 mg/ml in 5% glucose solution) by gavage into the stomach. Blood samples (~60 μl) were taken from the tail vein in heparinized Microvette CB 300 LH capillary tubes (Sarstedt, Nümbrecht, Germany) at 7.5, 15, 30, 60, 120, and 240 min after administration. At 120 or 360 min, animals were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia, and organs were removed from the mice that were killed after 120 min. Small intestinal tissue and contents (feces) were separated. Samples were stored at −20°C until analysis.

High-Performance Liquid Chromatography Analysis of MTX and 7OH-MTX. 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. MTX and 7OH-MTX concentrations in the different matrices were determined as described previously (van Tellingen et al., 1989).

Statistical Analysis. The two-sided unpaired Student’s t test was used to assess the statistical significance of differences between wild-type and knockout mice. Results are presented as means ± S.D. Differences were considered to be statistically significant when P < 0.05. Averaged concentrations for each time point were used to calculate the AUC from t = 0 to the last sampling point by the linear trapezoidal rule; S.E.s were calculated by the law of propagation of errors (Bardelmeijer et al., 2000).

Results

Impact of Abcc2, Abcc3, and Abcg2 on Oral Plasma Pharmacokinetics of MTX. We have shown previously that the ABC transporters Abcc2, Abcc3, and Abcg2 have a profound impact on the plasma pharmacokinetics of MTX and its toxic metabolite 7OH-MTX after intravenous bolus administration of 50 mg/kg MTX (Vlamming et al., 2006, 2008, 2009a,b). We now investigated the impact of these proteins on the pharmacokinetics of MTX and 7OH-MTX after oral administration of the same dose of MTX to single, double, and triple knockout mice for these transporters.

The plasma levels of MTX in all strains were relatively low (Fig. 1): the oral AUCs of the different strains over 6 h were on the order of 10-fold lower than the previously determined intravenous AUCs over 2 h (Table 1) (Vlamming et al., 2008, 2009a,b). This result indicates that at this relatively high dose of 50 mg/kg, the oral bioavailability of MTX is quite low (9–16%). As shown in Fig. 1, Abcg2 significantly affected the oral plasma pharmacokinetics of MTX. The plasma AUCoral in Abcg2(−/−) mice was 1.7-fold higher than that in the wild type (Table 1). Although the AUCoral in Abcc2(−/−) mice over 6 h was not significantly different from that in wild-type mice, the plasma levels of MTX at 60 and 120 min after administration were 1.5-fold higher than those in wild-type mice (n = 5–14, P < 0.05). This finding suggests that Abcc2 alone has some impact on the plasma concentrations of MTX after oral administration. Indeed, the AUCoral of Abcc2;Abcg2(−/−) mice was 3.0-fold increased compared with that in wild-type mice (Table 1), which was significantly higher than that in Abcg2(−/−) mice. This result indicates an additive effect of Abcg2 and Abcc2 on the oral plasma pharmacokinetics of MTX.

It was shown recently by Kitamura et al. (2008) that Abcc3 plays a role in MTX plasma pharmacokinetics after oral administration of 1 mg/kg (2.2 μmol/kg) MTX, with a 3.4-fold reduced AUCoral in Abcc3(−/−) compared with that in wild-type mice. After administration of 50 mg/kg MTX we found a modest tendency for reduced MTX plasma concentrations in Abcc3(−/−) mice (Fig. 1; Table 1), but the AUCoral over 6 h was not significantly different from that in wild-type mice (Table 1). The impact of Abcc3 on oral MTX pharmacokinetics became clearer when Abcg2, Abcc2, or both were absent: whereas in Abcg2(−/−) and Abcc2;Abcg2(−/−) mice, the oral AUCs were markedly increased compared with those in wild-type mice (see above) and increased plasma levels were measured for Abcc2(−/−) mice (Fig. 1), this was no longer the case in strains that also lacked
of MTX were significantly increased in most of the Abcg2-deficient strains compared with those in wild-type mice, but not in the strains that lacked Abcc2 but did have Abcg2 expression (Fig. 2A). In Abcg2(−/−) mice, liver levels were 1.7-fold increased and in Abcc2; Abcg2(−/−) mice they were 2.0-fold increased. These increases in MTX liver levels are probably caused in part by the increased plasma concentrations in these strains, as can be seen from the liver/plasma ratios, which are comparable to or even lower than those in wild-type mice (Fig. 2B). Note that this process may be partly counteracted by the up-regulation of hepatic Abcc3 that occurs in all Abcc2-deficient strains (Vlaming et al., 2008, 2009a,b), which probably explains why the liver/plasma ratios seen in Abcg2(−/−) mice are reduced in Abcc2;Abcg2(−/−) mice, but again increased in Abcc2;Abcc3;Abcg2(−/−) mice (Fig. 2B). A tendency for increased liver accumulation was seen in Abcc2;Abcc3;Abcg2(−/−) mice (0.59 ± 0.08% of the dose; P = 0.07) (Fig. 2A), but this was not significant. It is striking that, despite plasma levels similar to those in wild-type mice and the complete absence of three MTX-clearing transporters from the liver, there was not a much more marked accumulation of MTX in the Abcc2;Abcc3;Abcg2(−/−) liver. This result is different from the situation after intravenous MTX administration (Vlaming et al., 2009b).

### TABLE 1

MTX plasma AUCs of female mice after intravenous (0–120 min) and oral (0–6 h) administration of 50 mg/kg MTX

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wild-Type</th>
<th>Abcc2(−/−)</th>
<th>Abcc3(−/−)</th>
<th>Abcg2(−/−)</th>
<th>Abcc2;Abcc3(−/−)</th>
<th>Abcc2;Abcg2(−/−)</th>
<th>Abcc3;Abcg2(−/−)</th>
<th>Abcc2;Abcc3;Abcg2(−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{oral}</td>
<td>62 ± 13</td>
<td>85 ± 14</td>
<td>48 ± 13</td>
<td>107 ± 14 ∗</td>
<td>43 ± 9</td>
<td>184 ± 31 ∗</td>
<td>60 ± 11</td>
<td>53 ± 5</td>
</tr>
<tr>
<td>AUC_{oral}</td>
<td>1.0</td>
<td>1.4</td>
<td>0.8</td>
<td>1.7</td>
<td>0.7</td>
<td>3.0</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>AUC_{oral}</td>
<td>444 ± 44</td>
<td>870 ± 103 ∗</td>
<td>368 ± 34</td>
<td>692 ± 56 ∗</td>
<td>435 ± 47</td>
<td>1446 ± 229 ∗</td>
<td>451 ± 26</td>
<td>603 ± 56 ∗</td>
</tr>
<tr>
<td>AUC_{oral}</td>
<td>1.0</td>
<td>2.0</td>
<td>0.8</td>
<td>1.6</td>
<td>1.0</td>
<td>3.3</td>
<td>1.0</td>
<td>1.4</td>
</tr>
<tr>
<td>AUC_{oral}</td>
<td>14.0 ± 3.2</td>
<td>9.8 ± 2.0</td>
<td>13.1 ± 3.7</td>
<td>15.6 ± 2.4</td>
<td>9.8 ± 2.4</td>
<td>12.8 ± 2.9</td>
<td>13.4 ± 2.6</td>
<td>8.9 ± 1.2 ∗</td>
</tr>
</tbody>
</table>

* P < 0.05, compared with wild-type mice. Student’s t test was used for statistical analysis.
** P < 0.01.
*** P < 0.001.

Fold difference represents fold difference compared with wild-type mice with the same route of administration.
The levels of MTX in small intestinal contents and tissue were relatively high in all knockout strains (25–55% of the dose) but not significantly different from those in wild-type mice, in part because of high interindividual variation (Fig. 2C). Probably there is substantial variation in the overall uptake of MTX from the intestine among individual mice.

In most strains, the MTX kidney levels were relatively low (<0.15% of the dose) and not significantly different from wild-type mice at 120 min after administration (Fig. 2D). In Abcc2; Abcg2(-/-) mice, however, they were 2.2-fold increased compared with levels in wild-type mice, presumably reflecting the 3- to 4-fold higher plasma concentrations in this strain. In Abcc3(-/-) mice, MTX kidney levels were 2.0-fold lower than those in wild-type mice. This result may reflect the 1.5-fold decreased plasma levels of MTX at 120 min in Abcc3(-/-) mice. In contrast to what we previously found after intravenous administration of MTX (Vlaming et al., 2008, 2009a), the kidney levels did not always simply follow the plasma levels: whereas in Abcc2(-/-) and Abcg2(-/-) mice, the MTX plasma levels were 1.5- and 2.4-fold increased at 120 min, respectively (Fig. 1), kidney levels were not different from those in wild-type mice (Fig. 2D).

7OH-MTX, the main and toxic metabolite of MTX, is primarily formed in the liver (Bremnes et al., 1989; Chládek et al., 1997). The liver levels of 7OH-MTX at 120 min after MTX administration are shown in Fig. 3A. In Abcc2(-/-) and Abcg2(-/-) mice, the liver levels of 7OH-MTX were 2.3- and 2.1-fold increased compared with wild-type levels (P < 0.01). Furthermore, in Abcc2; Abcg2(-/-), Abcc2; Abcc3(-/-), and Abcc2; Abcc3; Abcg2(-/-) mice, the 7OH-MTX liver levels were 8.0-, 5.6-, and 8.9-fold increased compared with those in wild-type mice, respectively (P < 1 × 10^{-4}). In Abcc3; Abcg2(-/-) mice, on the other hand, 7OH-MTX liver levels were only mildly increased (1.5-fold, P = 0.045). It is apparent that absence of Abcc2 in particular, combined with either Abcc3 or Abcg2 deficiency, leads to increased accumulation of 7OH-MTX in the liver.

We have shown previously that Abcc2 is important for the biliary excretion of 7OH-MTX after intravenous administration of MTX and that Abcg2 is also involved when Abcc2 is absent (Vlaming et al., 2008, 2009a). After oral MTX administration, this process appears to be similar, as shown in Fig. 3B (note that there is little or no direct intestinal excretion of 7OH-MTX (Vlaming et al., 2009a), so most intestinal 7OH-MTX must derive from biliary excretion). In nearly all Abcc2-deficient strains, despite increased liver levels (Fig. 3A), levels of 7OH-MTX in the small intestinal tissue and contents were significantly decreased. In Abcc2; Abcc3(-/-) mice this was not the case, probably because of biliary excretion of 7OH-MTX by Abcg2 (Vlaming et al., 2009a), enhanced by the increased hepatic 7OH-MTX concentration. Furthermore, in Abcg2(-/-) mice, the levels of 7OH-MTX in small intestine were significantly higher than those in wild-type mice, suggesting that Abcc2, in combination with the increased 7OH-MTX liver levels, mediates the increased biliary excretion of 7OH-MTX in these mice.

Kidney levels of 7OH-MTX were very low (<0.025% of the dose) in all strains and undetectable in all Abcc2-proficient mice (Fig. 3C). In all Abcc2-deficient strains, 7OH-MTX was detected, suggesting that absence of Abcc2 leads to increased exposure of the kidney to 7OH-MTX, probably due to increased plasma levels of 7OH-MTX (which could not be determined reliably, see above). Furthermore, combined absence of Abcc2 and Abcg2 caused an even further (5-fold) increase in 7OH-MTX kidney levels in Abcc2; Abcg2(-/-) mice compared with those in Abcc2(-/-) mice (P = 7 × 10^{-3}) (Fig. 3C).

**Discussion**

In this study, we used the recently generated Abcc2; Abcc3; Abcg2(-/-) mice (Vlaming et al., 2009b) to study the relative effects of Abcc2, Abcc3, and Abcg2 on the oral pharmacokinetics of MTX and its main toxic metabolite 7OH-MTX. We show that Abcg2 in particular and to a lesser extent Abcc2 can reduce MTX plasma levels after oral application and that these transporters have clear additive effects. Furthermore, we show that Abcc3 expression is in each case

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**Fig. 2.** MTX tissue distribution 2 h after oral administration of 50 mg/kg MTX to female wild-type (n = 6–7/tissue), Abcc2(-/-) (n = 4–5/tissue), Abcc3(-/-) (n = 4–5/tissue), Abcc2; Abcc3(-/-) (n = 5), Abcc2; Abcg2(-/-) (n = 5), Abcc3; Abcg2(-/-) (n = 5), and Abcc2; Abcc3; Abcg2(-/-) (n = 4–5/tissue) mice. A, MTX liver levels (percentage of dose) in the different strains. B, MTX liver concentration versus plasma concentration ratios in the different strains. C, MTX small intestinal tissue (SI) and contents (SIC) levels (percentage of dose) in the different strains. D, MTX kidney levels (percentage of dose) in the different strains. Data are presented as means ± S.D. (*, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with wild-type mice, Student’s t test).
necessary for the effects of Abcc2 and/or Abcg2 deletion on oral MTX plasma levels. Combined deletion of Abcc2 and Abcg2 further led to increased concentrations of MTX and its toxic metabolite 7OH-MTX in liver and kidney.

When MTX is used for cancer treatment, high doses (> 5 mg/m²) are usually given (Gorlick and Bertino, 1999). Because the oral bioavailability of MTX, especially at high doses, is unpredictable and relatively poor, it is given intravenously for cancer treatment (Gorlick and Bertino, 1999). However, oral administration of MTX would be much more desirable because this route is, in general, more patient-friendly and cost-effective (Kuppens et al., 2005; Breedveld et al., 2006). In this study, we therefore used a moderately high oral dosage of MTX (50 mg/kg), which also allows direct comparison with the extensive intravenous data at 50 mg/kg MTX we previously obtained (Vlaming et al., 2009a). Thus, it can be concluded that the oral AUCs of MTX demonstrate quite similar shifts between the different mouse strains (Vlaming et al., 2009a). Therefore, a more pronounced effect on the oral AUC compared with the intravenous AUC would have been expected. We have shown before that Abcc2 in particular, and, when Abcc2 is absent, Abcg2 also can mediate very substantial (virtually all) biliary excretion of MTX, up to 50% of the dose in wild-type mice. We have shown before (Vlaming et al., 2009a,b) that substantial effects of Abcg2 and Abcc2 on the plasma pharmacokinetics of MTX after oral application, in all strains analyzed we found between 25 and 55% of the dose present in the small intestine after 2 h, and this result was not significantly different among the different strains. We have shown before that with intravenous administration the deletion of Abcc2 and/or Abcg2 led to markedly reduced levels of MTX in the intestine at 1 h after administration (down from nearly 50% of the dose in wild-type mice) because of dramatically decreased biliary excretion. The fact that we did not find this effect after oral administration suggests that biliary excretion in this setup has little impact on the intestinal MTX levels, probably because of the large amount of residual MTX that has not yet been available to our knowledge.

The primary mechanism by which Abcc2 and Abcg2 reduce oral MTX plasma levels could be either by reducing intestinal uptake of MTX or by mediating hepatobiliary (and perhaps some renal) MTX excretion. However, the observation that the intravenous and oral AUCs of MTX demonstrate quite similar shifts between the different mouse strains (Table 1) suggests that there is not a big impact of these transporters on intestinal MTX uptake at the MTX dose used; otherwise, a more pronounced effect on the oral AUC compared with the intravenous AUC would have been expected. We have shown before that Abcc2 in particular, and, when Abcc2 is absent, Abcg2 also can mediate very substantial (virtually all) biliary excretion of MTX, up to 50% of an intravenous dose in 1 h (Vlaming et al., 2009a). Thus, it seems more likely that the hepatic function of Abcc2 and Abcg2 is most important in reducing the oral AUC of MTX at 50 mg/kg.

Although we did find substantial effects of Abcg2 and Abcc2 on the plasma pharmacokinetics of MTX after oral application, in all strains analyzed we found between 25 and 55% of the dose present in the small intestine after 2 h, and this result was not significantly different among the different strains. We have shown before that with intravenous administration the deletion of Abcc2 and/or Abcg2 led to markedly reduced levels of MTX in the intestine at 1 h after administration (down from nearly 50% of the dose in wild-type mice) because of dramatically decreased biliary excretion. The fact that we did not find this effect after oral administration suggests that biliary excretion in this setup has little impact on the intestinal MTX levels, probably because of the large amount of residual MTX that has not yet been available to our knowledge.
absorbed from the intestine after oral administration and the much lower overall hepatic MTX uptake and hence hepatobiliary excretion upon oral as opposed to intravenous MTX administration. The increases in AUCoral in Abcc2(−/−), Abcg2(−/−), and Abcc2; Abcg2(−/−) mice are completely abrogated as soon as Abcc3 is also deleted (Fig. 1; Table 1). In principle, one can envision two mechanisms by which Abcc3 could have such a strong impact on the MTX plasma levels (Kitamura et al., 2008): it might enhance the uptake of oral MTX across the intestinal wall because of its localization in the basolateral membrane of enterocytes or it could mediate the basolateral (back)flux of MTX from the liver, thus reducing biliary clearance and increasing plasma levels of MTX. Both mechanisms may, of course, also act simultaneously.

Our data suggest that the first mechanism does not play a substantial role at the MTX dose tested (50 mg/kg). One reason is that when the oral and intravenous plasma AUCs of all the strains tested are compared (Table 1), there is a remarkable similarity between the shifts in AUC among the strains, resulting in at best modest changes in the oral bioavailability (AUCoral/AUCiv.). This finding argues against an overruling impact of any of the transporters on primary intestinal uptake. Furthermore, there is little effect of the single Abcc3 knockout on the AUCoral of MTX (Table 1), arguing against an important role of Abcc3 in uptake from the intestine. This finding seems to contrast with the results of Kitamura et al. (2008), who observed a 3.4-fold decreased MTX oral AUC in Abcc3(−/−) mice. These authors provided evidence that the decreased plasma AUC was partly due to reduced basolateral efflux from the liver (and hence increased biliary clearance) and partly due to reduced intestinal uptake of MTX. However, their experiment was done at a low MTX dose of 1 mg/kg (2.2 μmol/kg). Subsequent experiments in everted intestinal sacs showed that Abcc3 could indeed increase mucosal to serosal MTX uptake but only at low MTX doses. Higher MTX dosages resulted in saturation of the overall uptake process, with similar MTX uptake rates in wild-type and Abcc3(−/−) intestinal sacs. Because we used a 50-fold higher oral dosage of MTX than Kitamura et al. (2008), we have probably saturated the possible contribution of Abcc3 to intestinal uptake of MTX, and other (lower affinity) net MTX absorptive processes may have taken over.

In summary, at the 50 mg/kg MTX dosage, it thus seems more likely that Abcc3 in the liver is important for revealing the impact of reduced hepatobiliary excretion of MTX by Abcc2 and Abcg2 deficiency on plasma MTX levels. Abcc3 probably allows rapid backflux of MTX from liver to plasma when it is not efficiently cleared by the bile canalicular transporters Abcg2 and Abcc2 (Vlaming et al., 2009a), thus raising the plasma MTX levels.

Although Abcg2, Abcc2, and Abcc3 appear to be the main determinants for MTX elimination, expression of other (compensatory) mechanisms such as transporters or metabolizing enzymes may be altered because of genetic deletion of ABC transporters. We have shown before that Abcc1 and Abcc5, which also transport MTX, are not increased in the liver of the knockout strains investigated here. On the other hand, levels of Abcc4 were modestly increased in the livers of all Abcc2-deficient strains (Vlaming et al., 2008, 2009a,b). In the present study we found that, despite these increased Abcc4 liver protein levels in Abcc2-deficient strains, decreased liver/plasma ratios (probably due to rapid backflux from liver to plasma, as described above) only occurred when Abcc3 was present. It therefore seems likely that Abcc3 is the main determining factor in this process and not Abcc4.

We note that also other mechanisms can mediate MTX and 7OH-MTX elimination, because even in Abcc2;Abcc3;Abcg2(−/−) mice the MTX levels in plasma and liver were comparable to those in wild-type mice. Indeed, other elimination processes such as metabolism (and possibly biliary and sinusoidal efflux) play a role in the elimination of MTX from the liver when Abcc2, Abcc3, and Abcg2 are absent. This is shown by the increased liver levels of 7OH-MTX in Abcc2-deficient strains (Fig. 3A), which is probably partly due to increased expression of aldehyde oxidase 1 in the liver of these strains (Vlaming et al., 2009a,b). Besides this process, other (possibly yet unknown) changes in these knockout strains may also affect MTX pharmacokinetics. These processes may have a higher affinity but lower capacity for MTX elimination compared with Abcc2, Abcg2, and Abcc3, explaining the larger effects seen after intravenous compared with oral MTX administration (Vlaming et al., 2009b).

Oral MTX administration is often used in the treatment of rheumatoid arthritis as well as psoriasis, and if taken long term it can easily result in toxicity. The dose must therefore be carefully titrated. Of interest, in a recent patient study with oral MTX, correlations between three single nucleotide polymorphisms in ABCC2 and MTX toxicity have been found (Ranganathan et al., 2008). Furthermore, in a study of patients with psoriasis, two ABCG2 single nucleotide polymorphisms positively correlated with the efficacy of MTX therapy (Warren et al., 2008). Our results show that deletion of Abcc2 and Abcc2 increases oral MTX levels in the circulation but also in liver and kidney. Furthermore, the absence of Abcc2 and/or Abcg2 leads to increased exposure of liver and kidney to the toxic metabolite 7OH-MTX. The effects found in patients with polymorphisms in these genes may therefore be expected to be caused by direct effects of reduced activity of ABCC2 and/or ABCG2. When patients are treated with oral MTX it may therefore be advisable to check for mutations in ABCC2, ABCG2, and perhaps ABCB3 to predict and circumvent possible adverse effects.

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Authorship Contributions

Participated in research design: Vlaming, Pala, van Tellingen, and Schinkel.
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Performed data analysis: Vlaming, van Esch, Pala, van Tellingen, and Schinkel.
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