ABC transporter compound knockout mice: physiological and pharmacological characterization
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Chapter 7

Impact of Abcc2 (Mrp2), Abcc3 (Mrp3) and Abcg2 (Bcrp1) on the oral pharmacokinetics of the anti-cancer drug methotrexate and its main metabolite 7-hydroxymethotrexate in mice

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Impact of Abcc2 (Mrp2), Abcc3 (Mrp3) and Abcg2 (Bcrp1) on the oral pharmacokinetics of the anti-cancer drug methotrexate and its main metabolite 7-hydroxymethotrexate in mice

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The ATP-binding cassette (ABC) transporters ABCC2 (MRP2), ABCC3 (MRP3) and ABCG2 (BCRP) are involved in the efflux of potentially toxic compounds from the body. We have shown before that ABCC2, ABCC3 and ABCG2 together influence the pharmacokinetics of the anti-cancer and anti-rheumatic drug methotrexate (MTX) and its toxic metabolite 7-hydroxymethotrexate (7OH-MTX) after iv MTX administration. We now used female Abcc2;Abcc3;Abcg2⁻/⁻ and corresponding single and double knockout mice to investigate the relative influences of these transporters on MTX and 7OH-MTX pharmacokinetics after oral MTX administration (50 mg/kg). The plasma areas under the curve (AUCplasma) of Abcc2⁻/⁻ and Abcg2⁻/⁻ mice were 1.5- and 2.0-fold increased compared to wild-type, respectively. Abcc2;Abcg2⁻/⁻ mice had a 3.3-fold increased AUCplasma, suggesting additive effects of Abcc2 and Abcg2. The AUCplasma in Abcc2;Abcc3;Abcg2⁻/⁻ mice was not different from wild-type, suggesting that the Abcc3 protein is necessary for the increased plasma concentrations in the absence of Abcc2 and/or Abcg2. Furthermore, 2 hr after administration, MTX liver levels were increased in Abcg2-deficient strains and MTX kidney levels were 2.2-fold increased compared to wild-type in Abcc2;Abcg2⁻/⁻ mice. Absence of Abcc2 and/or Abcg2 furthermore led to significantly increased liver and kidney levels of 7OH-MTX 2 hr after MTX administration. Our results suggest that combined inhibition of ABCC2 and/or ABCG2 may increase the oral availability of MTX. Furthermore, SNPs or mutations in ABCC2 and/or ABCG2 that reduce expression or activity of these proteins may be risk factors for increased MTX-related toxicity in patients treated with oral MTX.
INTRODUCTION
The ATP-binding cassette (ABC) transporters ABCC2 (MRP2), ABCC3 (MRP3) and ABCG2 (BCRP) 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 (anti-cancer) drugs (1-5). Whereas ABCC2 and ABCG2 are present at the apical membranes of cells, transporting their substrates into bile, feces and urine, ABCC3 is located basolaterally, and it generally transports its substrates into the blood circulation (1,2, Supplementary Figure 1).

ABCC2, ABCC3 and ABCG2 have broad and substantially overlapping substrate specificities (1,2,4), but their relative impact on the pharmacokinetics of shared substrates is not clear yet. We have recently generated compound knockout mice for these transporters (6,7, Vlaming et al., submitted), which together with the previously generated single knockout mice for Abcc2 (8), Abcc3 (9) and Abcg2 (10) form a complete set of mouse models that can be used to elucidate the relative and possibly overlapping effects of these proteins on the pharmacokinetics of endogenous and exogenous substrates. Using this set of mouse strains we have recently shown that Abcc2, Abcc3 and Abcg2 have profound overlapping and additive effects on the iv pharmacokinetics of the widely used anti-cancer drug methotrexate (MTX) and its main toxic metabolite 7-hydroxymethotrexate (7OH-MTX) (6,7, Vlaming et al., submitted).

In cancer treatment, most drugs are given iv due to low and/or highly variable bioavailability, which can be caused by expression of ABC transporters in the intestine (5). However, because oral administration of drugs is more patient friendly as well as more cost effective, attempts are being made to improve the oral bioavailability of several drugs by co-administration of inhibitors of ABC transporters (5,11). Since ABCC2, ABCC3 and ABCG2 are all expressed in epithelial cells of the small intestine (1,3), they may, besides affecting the iv pharmacokinetics, also influence the oral pharmacokinetics 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 to wild-type rats (12,13). In mice, the effect of Abcc2 after oral MTX has not been investigated yet. Kitamura et al. (2008) (14) did show an effect of murine Abcc3 on plasma pharmacokinetics of [3H]MTX after oral administration. Surprisingly, although the impact of Abcg2 on the oral pharmacokinetics of many drugs has been extensively studied (15,16), its effect on the disposition of MTX and 7OH-MTX after oral 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 Abcc2 and/or Abcg2 increases the plasma concentrations of MTX after oral administration, but that Abcc3 expression is necessary for this 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 of Abcc2<sup>−/−</sup> (8), Abcc3<sup>−/−</sup> (9), Abcg2<sup>−/−</sup> (10), Abcc2;Abcc3<sup>−/−</sup> (6,17), Abcc2;Abcg2<sup>−/−</sup> (7), and Abcc3;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice (Vlaming et al., submitted) has been described. All animals were of >99% FVB background and between 9-14 weeks of age. Animals were kept in a temperature-controlled environment with a 12-hour light/12-hour dark cycle. They received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*.

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

**Plasma and tissue pharmacokinetic experiments.** Before MTX administration, mice were fasted for at least 4 hrs. MTX was administered to female wild-type, Abcc2<sup>−/−</sup>, Abcc3<sup>−/−</sup>, Abcg2<sup>−/−</sup>, Abcc2;Abcc3<sup>−/−</sup>, Abcc2;Abcg2<sup>−/−</sup>, Abcc3;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice (n = 3-9) by dosing 10 µl/g body weight of 5 mg/ml MTX in 5% glucose solution by gavage into the stomach. Blood samples (~60 µl) were taken from the tail vein in heparinised Microvette® CB 300 LH capillary tubes (Sarstedt, Nümbrecht, Germany) at 7.5, 15, 30 and 60 min after administration. At 120 min, animals were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia and organs were removed. Small intestinal tissue and contents (feces) were separated. Samples were stored at -20º C until analysis.

**HPLC analysis of MTX and 7OH-MTX.** Collected organs and feces were homogenized in an ice-cold 4% BSA solution and plasma was diluted in human plasma before HPLC analysis. MTX and 7OH-MTX concentrations in the different matrices were determined as described (18).

**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 2 groups were analyzed, one-way ANOVA followed by Tukey’s multiple comparison test was
performed, as indicated. Results are presented as means ± standard deviations (SD). Differences were considered to be statistically significant when $P < 0.05$. Averaged concentrations for each time point were used to calculate the area under the plasma concentration versus time curve (AUC) from $t = 0$ to the last sampling point by the linear trapezoidal rule; standard errors (SE) were calculated by the law of propagation of errors (19). Because in many cases the plasma concentrations of MTX at 7.5 and 15 min could not be measured, for AUC calculations the concentrations at 30, 60 and 120 min after administration were used.

![Figure 1. MTX plasma concentration versus time curve after oral administration of 50 mg/kg to female wild-type, $Abcc2^-/-$, $Abcc3^-/-$, $Abcg2^-/-$, $Abcc2;Abcc3^-/-$, $Abcc2;Abcg2^-/-$, $Abcc3;Abcg2^-/-$ and $Abcc2;Abcc3;Abcg2^-/-$ mice (n = 3-9). Data are presented as means ± SD. Where no data points are given in the graph, the MTX plasma levels were below the lower limit of quantification (LLQ, 0.11 µg/ml). Additional data points (4 and 6 hrs) will be added to this graph in the near future.](image)

**RESULTS**

**Impact of Abcc2, Abcc3 and Abcg2 on oral plasma pharmacokinetics of MTX.** We have previously shown 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 iv bolus administration of MTX at 50 mg/kg (6,7,8, Vlaming et al., submitted). We now investigated the impact of these proteins on the pharmacokinetics of MTX and 7OH-MTX after oral administration of MTX at the same dose.

The plasma levels of MTX in all strains were relatively low (Figure 1): the AUCs_{oral} of the different strains over 120 min were in the order of 10-fold lower than the previously determined AUCs_{iv} (Table 1) (6,7, Vlaming et al., submitted). This suggests that at this (relatively high) dose of 50 mg/kg the oral bioavailability of MTX is quite low. However, as shown in Figure 1, both Abcc2 and especially Abcg2 did significantly affect the oral plasma pharmacokinetics of MTX. The plasma AUCs_{oral} in Abcc2^{+/+} and Abcg2^{+/+} mice were 1.5- and 2.0-fold higher than in wild-type mice, respectively (Table 1). Furthermore, whereas in most other strains MTX was undetectable at 7.5 min after administration (Figure 1), in Abcg2^{+/+} mice MTX was already clearly present in the blood. This suggests that already very early after administration Abcg2 reduces the oral uptake of MTX. The AUC_{oral} of Abcc2;Abcg2^{-/-} mice was even 3.3-fold increased compared to wild-type (Table 1), showing an additive effect of Abcg2 and Abcc2 on the oral plasma pharmacokinetics of MTX. Also in Abcc2;Abcg2^{+/+} mice MTX could already be detected at 7.5 min (Figure 1), and tended to be somewhat higher than for Abcg2^{+/+} mice (n = 3, P = 0.052), suggesting that Abcg2 is important early after administration, but Abcc2 may have a small additive effect as well.

It was shown recently by Kitamura et al. (2008) (14), that Abcc3 plays a role in [3H]MTX plasma pharmacokinetics after oral administration of [3H]MTX at 1 mg/kg (2.2 µmol/kg). After administration of MTX at 50 mg/kg a similar tendency of reduced MTX plasma concentrations was found in Abcc3^{+/+} mice (Figure 1). However, the AUC_{oral} over 120 min of Abcc3^{+/+} mice was not significantly different compared to wild-type mice (Table 1). The effect of Abcc3 on MTX pharmacokinetics became clearer when Abcc2 and/or Abcg2 were absent. Whereas in Abcc2^{+/+}, Abcg2^{+/+} and Abcc2;Abcg2^{+/+} mice the AUCs_{oral} were significantly increased compared to wild-type (see above), this was not the case in strains that additionally lacked Abcc3 (Abcc2;Abcc3^{+/+}, Abcc3;Abcg2^{+/+} and Abcc2;Abcc3;Abcg2^{+/+} mice, Table 1). This shows that, like for iv administration (6,7,Vlaming et al., submitted, Table 1), Abcc3 expression (in intestine and/or liver) was necessary for the increased MTX plasma levels in Abcc2^{+/+}, Abcg2^{+/+} and Abcc2;Abcg2^{+/+} mice after oral application of MTX.

7OH-MTX concentrations in plasma of mice after oral administration were, due to the small sample volumes, difficult to detect. Therefore, no conclusions on the effects of the different ABC transporters on 7OH-MTX plasma pharmacokinetics could be drawn.
Table 1. MTX plasma AUC<sub>0-120 min</sub> of female mice after iv (Vlaming et al., submitted) and oral administration of MTX (50 mg/kg).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Wild-type</th>
<th>Abcc&lt;sup&gt;2&lt;/sup&gt;&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc&lt;sup&gt;3&lt;/sup&gt;&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcg&lt;sup&gt;2&lt;/sup&gt;&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc&lt;sup&gt;2&lt;/sup&gt;;Abcc&lt;sup&gt;3&lt;/sup&gt;&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc&lt;sup&gt;2&lt;/sup&gt;;Abcg&lt;sup&gt;2&lt;/sup&gt;&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc&lt;sup&gt;3&lt;/sup&gt;;Abcg&lt;sup&gt;2&lt;/sup&gt;&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Abcc&lt;sup&gt;2&lt;/sup&gt;;Abcc&lt;sup&gt;3&lt;/sup&gt;;Abcg&lt;sup&gt;2&lt;/sup&gt;&lt;sup&gt;-/-&lt;/sup&gt;</th>
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<tr>
<td>AUC&lt;sub&gt;iv&lt;/sub&gt; (min·µg/ml)</td>
<td>444 ± 44</td>
<td>870 ± 103&lt;sup&gt;***&lt;/sup&gt;</td>
<td>368 ± 34</td>
<td>692 ± 56&lt;sup&gt;*&lt;/sup&gt;</td>
<td>435 ± 47</td>
<td>1446 ± 229&lt;sup&gt;**&lt;/sup&gt;</td>
<td>451 ± 26</td>
<td>603 ± 56&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;oral&lt;/sub&gt; (min·µg/ml)</td>
<td>23 ± 4</td>
<td>35 ± 6&lt;sup&gt;†&lt;/sup&gt;</td>
<td>16 ± 3</td>
<td>44 ± 10&lt;sup&gt;†&lt;/sup&gt;</td>
<td>25 ± 6</td>
<td>74 ± 11&lt;sup&gt;**&lt;/sup&gt;</td>
<td>24 ± 5</td>
<td>19 ± 2</td>
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<tr>
<td>F (oral/iv) %</td>
<td>5.0 ± 1.0</td>
<td>3.8 ± 0.7</td>
<td>4.3 ± 0.9</td>
<td>6.3 ± 1.5</td>
<td>5.7 ± 1.5</td>
<td>5.1 ± 1.1</td>
<td>5.4 ± 1.2</td>
<td>3.2 ± 0.3&lt;sup&gt;†&lt;/sup&gt;</td>
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Note: MTX plasma AUCs are presented as min·µg/ml and oral availabilities (F) are given as % of AUC<sub>iv</sub> (means ± SD, n = 3-12, *P < 0.05, ** P<0.01, *** P< 0.001 compared to wild-type mice over the same time period, Student’s t-test was used for statistical analysis). For oral administration additional data points (4 and 6 hrs) will be added in the near future.
Impact of Abcc2, Abcc3 and Abcg2 on tissue distribution of MTX and 7OH-MTX.

We further analyzed the levels of MTX and 7OH-MTX in tissues of the different strains at 120 min after oral administration of MTX (50 mg/kg). The liver levels of MTX were significantly increased compared to wild-type mice in most of the Abcg2-deficient strains (Figure 2A). In Abcg2<sup>−/−</sup> mice, liver levels were 1.7-fold increased and in Abcc2;Abcg2<sup>+/−</sup> mice they were 2.0-fold increased. This is very likely primarily a reflection of the increased plasma concentrations. In Abcc2<sup>−/−</sup> mice, however, despite increased plasma concentrations, the liver levels of MTX were not significantly different from wild-type and tended to be even somewhat lower (P = 0.07, Figure 2A), suggesting increased MTX liver elimination in Abcc2<sup>−/−</sup> mice, possibly by Abcc3, as we have previously shown after iv administration of MTX (6). In Abcc3;Abcg2<sup>−/−</sup> mice, although the plasma AUC was similar, liver levels of MTX were 1.6-fold increased compared to wild type, suggesting impaired liver elimination of MTX in Abcc3;Abcg2<sup>−/−</sup> mice (Figure 2A). A similar tendency was seen in Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice (P = 0.07, Figure 2A).

The levels of MTX in small intestinal tissue and contents were relatively high in all strains (25-55% of the dose) and not significantly different from wild type in any of the strains, due to a high variation between individual mice (Figure 2B).

In most strains the MTX kidney levels were quite low and not significantly different from wild-type at 120 min after administration (Figure 2C). In Abcc2;Abcg2<sup>−/−</sup> mice, however, these were 2.2-fold increased compared to wild-type mice (Figure 2C). In Abcc3<sup>−/−</sup> mice, in line with a tendency of decreased plasma levels, MTX kidney levels were 2.0-fold lower than in wild-type (Figure 2C). Contrary to what we previously found after iv administration of MTX (6,7), the kidney levels did not simply follow the plasma levels: whereas in Abcc2<sup>−/−</sup> and Abcg2<sup>−/−</sup> mice the MTX plasma levels were respectively, 1.6- and 2.4-fold increased at 120 min, kidney levels were not different from wild type (Figure 2C).

7OH-MTX, the main, toxic metabolite of MTX, is mainly formed in the liver (20,21). The liver levels of 7OH-MTX at 120 min are shown in Figure 3A. This shows that in Abcc2<sup>−/−</sup> and Abcg2<sup>−/−</sup> mice the liver levels of 7OH-MTX were 2.3- and 2.1-fold increased compared to wild type. Furthermore, in Abcc2;Abcc3<sup>−/−</sup>, Abcc2;Abcg2<sup>−/−</sup> and Abcc2;Abcc3;Abcg2<sup>−/−</sup> mice the 7OH-MTX liver levels were 5.6-, 8.0- and 8.9-fold increased compared to wild type, respectively (Figure 3A). In Abcc3;Abcg2<sup>−/−</sup> mice on the other hand, 7OH-MTX liver levels were only mildly increased (Figure 3A). Apparently, absence of Abcc2 in particular, combined with either Abcc3 or Abcg2 deficiency leads to increased accumulation of 7OH-MTX in the liver.
Abcc2, Abcc3 and Abcg2 influence oral MTX pharmacokinetics

Figure 2. MTX tissue distribution 2 hr after oral administration of 50 mg/kg to female wild-type, Abcc2\(^+/\), Abcc3\(^+/\), Abcg2\(^+/\), Abcc2; Abcc3\(^+/\), Abcc2; Abcg2\(^+/\), Abcc3; Abcg2\(^+/\) and Abcc2; Abcc3; Abcg2\(^+/\) mice (n = 5-9). A, MTX liver levels (as % of the dose) in the different strains. B, MTX small intestinal (SI) tissue and contents levels (as % of the dose) in the different strains (n = 5-9). C, MTX kidney levels (as % of the dose) in the different strains (n = 5-9). Data are presented as means ± SD (*, P < 0.05; **, P < 0.01; ***, P < 0.001, compared to wild type, Student’s t-test).

We have shown previously that Abcc2 is important for the biliary excretion of 7OH-MTX after iv administration of MTX, and that Abcg2 is also involved when Abcc2 is absent (6,7). After oral MTX administration this appears to be similar, as shown in Figure 3B. In nearly all Abcc2-deficient strains, despite increased liver levels (Figure 3A), levels of 7OH-MTX in the small intestinal tissue and contents were significantly decreased. In Abcc2; Abcc3\(^+/\) mice this was not the case, likely due to biliary excretion of 7OH-MTX by Abcg2 (7). Furthermore, in Abcg2\(^+/\) mice the levels of 7OH-MTX in small intestine were significantly higher than in wild-type mice, suggesting that Abcc2 expression, in combination with increased 7OH-MTX liver levels can cause 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 (Figure 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, possibly due to increased plasma levels of 7OH-MTX (not determined, see above). Furthermore, combined absence of Abcc2 and Abcg2 caused even further increased 7OH-MTX kidney levels (n = 4, P = 7*10^{-3}) (Figure 3C).

Figure 3. 7OH-MTX tissue distribution 2 hr after oral administration of MTX (50 mg/kg) to female wild-type, *Abcc2^/-*, *Abcc3^/-*, *Abcg2^/-*, *Abcc2;Abcc3^/-*, *Abcc2;Abcg2^/-*, *Abcc3;Abcg2^/-* and *Abcc2;Abcc3;Abcg2^/-* mice (n = 5-9). A, 7OH-MTX liver levels (as % of the dose) in the different strains (n = 5-9). B, 7OH-MTX small intestinal (SI) tissue and contents levels (as % of the dose) in the different strains (n = 5-9). C, 7OH-MTX kidney levels (as % of the dose) in the different strains (n = 5-9) (nd, not detectable, detection limit 24 nM). Data are presented as means ± SD (*, P < 0.05; **, P < 0.01; ***, P < 0.001, compared to wild type, Student’s t-test).
DISCUSSION
In this study we used the recently generated \textit{Abcc2;Abcc3;Abcg2}\(^{-/-}\) mice (Vlaming et al., submitted) to determine the relative effects of Abcc2, Abcc3 and Abcg2 on the oral pharmacokinetics of MTX and its main toxic metabolite 7OH-MTX. We show that Abcc2 and Abcg2 can both influence MTX plasma pharmacokinetics after oral application and that they have additive effects. Furthermore, we show that Abcc3 expression (either in the intestine or the liver) is necessary for the effects of Abcc2 and Abcg2 deletion on oral MTX plasma levels. Combined deletion of Abcc2 and Abcg2 furthermore led to increased concentrations of MTX and its toxic metabolite 7OH-MTX in liver and kidney. For MTX, this was not seen when Abcc3 was additionally deleted, illustrating the overlapping functions between the different transporters in determining the oral pharmacokinetics of MTX.

When MTX is used for cancer treatment, high doses (>15 mg/m\(^2\)) are usually given (22). Because the oral bioavailability of MTX, especially at high doses, is unpredictable and relatively poor, it is given iv (22). However, oral application of MTX would be much more favourable, as this is more patient friendly and cost-effective in general (5,11). We show here that by combined deletion of Abcg2 and Abcc2 the plasma AUC after oral administration of MTX (50 mg/kg) can be increased by more than three-fold. However, our results also show that for this effect Abcc3 protein needs to be present, suggesting that Abcc3 expression is relatively important for the oral availability of MTX, as was previously shown by Kitamura et al. (2008) (14). This suggests that specific inhibition of ABCG2 and ABCC2 (without ABCC3 inhibition), may be an effective strategy to improve the oral pharmacokinetics of MTX. Inhibitors for ABCG2 have been developed and used in clinical trials to improve the oral bioavailability of drugs (5,23,24). However, no effective, specific inhibitors for ABCC2 are known at the moment, so before practical application of this option, more research will have to be performed.

Kitamura et al. (2008) have shown that single deletion of Abcc3 in mice led to a 3-fold decreased AUC over 4 hrs after oral MTX administration at a dose of 2.2 \(\mu\)mol/kg (1 mg/kg) (14). In the here described experiment, with a much higher dose, we did not find a significant effect on the AUC of MTX after deletion of Abcc3 alone, although there was a similar tendency of a reduced AUC (Figure 1). Possibly, the effect of Abcc3 is dependent on the dose, and other transporters are more important at higher MTX doses. We have shown previously that the effect of Abcc2 on the plasma elimination of \[^{3}\text{H}]\text{MTX}\) after iv administration was dose dependent as well (8). Possibly, at a lower dose of 1 mg/kg orally, the effect of Abcg2 and Abcc2 is less pronounced, making the effect of Abcc3 more clearly visible.

Although we show here that Abcc2 and Abcg2 limit the systemic exposure of MTX after oral application, it is not clear if this is mainly caused by limiting the uptake from the intestine or by their impact on the biliary excretion of MTX (or both). The AUC\(_{\text{oral}}\) was 3.3-fold increased in \textit{Abcc2;Abcg2}\(^{-/-}\) mice compared to wild
type. However, we previously found that the AUCiv, with the same dose was 3.3-fold increased compared to wild type as well (most likely due to dramatically decreased biliary excretion of MTX) (7). It may therefore be that the increased plasma MTX levels in Abcc2;Abcg2−/− mice after oral administration are primarily caused by decreased biliary excretion of MTX. On the other hand, especially in Abcg2-deficient mice, already 7.5 min after oral administration, increased plasma levels were found (Figure 1). When Abcc2 was additionally deleted, this difference became even bigger, although this may also be caused by increased Abcc3 expression in the liver of these mice (7). Furthermore, whereas after iv administration the effect of Abcc2 on the plasma elimination was more prominent, after oral administration this was more affected by absence of Abcg2. It will be interesting to study the effects of these ABC transporters after oral MTX application in more detail in the future.

Although we did find significant effects of Abcc2 and Abcg2 on the pharmacokinetics of MTX after oral application, still in all strains analyzed we found between 25-55% of the dose present in the small intestine after 2 hrs and this was not significantly different between the different strains, possibly due to high variation between individual mice. We have shown before after iv administration that deletion of Abcc2 and/or Abcg2 led to significantly reduced levels of MTX in the intestine at 1 hr after administration, due to dramatically decreased biliary excretion (7). The fact that we don’t see this effect after oral administration suggests that biliary excretion in this set up has little influence on the intestinal MTX levels and that the effect of Abcc2 and Abcg2 on decreasing the intestinal uptake after oral administration is limited compared to the total amount of MTX in the small intestine, for example due to saturation of specific uptake transporters for MTX in the intestine.

Oral MTX administration is often used in the treatment of rheumatoid arthritis as well as psoriasis. We show here that Abcg2 and Abcc2 can influence the pharmacokinetics of MTX after oral administration. Interestingly, in a patient study with oral MTX, correlations between 3 SNPs in ABCC2 and MTX toxicity have been found recently (25). Furthermore, in a study with psoriasis patients 2 ABCG2 SNPs positively correlated with efficacy of MTX therapy (26). Our results show that deletion of Abcc2 or Abcg2, in combination with expression of Abcc3, increases MTX levels in the circulation, but also in liver and kidney. Furthermore, 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 are therefore likely caused by direct effects of reduced ABCC2 and/or ABCG2 activity. When patients are treated with oral MTX it may therefore be advisable to check for mutations in ABCC2, ABCG2 and ABCC3 in order to predict possible adverse effects.
REFERENCES


Supplementary Figure 1. Localization of ABCC2, ABCC3 and ABCG2 in intestine (A), liver (B) and kidney (C).