DMD Fast Forward. Published on May 12, 2011 as doi:10.1124/dmd.111.038794

DMD # 38794

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

Maria L.H. Vlaming[1], Anita van Esch, Evita van de Steeg[1], Zeliha Pala, Els Wagenaar, Olaf van Tellingen, Alfred H. Schinkel

Authors' Affiliations: Divisions of Molecular Biology (M.L.H.V., A.v.E., E.v.d.S., E.W., A.H.S.) and Clinical Chemistry (O.v.T.), The Netherlands Cancer Institute, Amsterdam, The Netherlands; and Department of Pharmacology, Faculty of Pharmacy, Istanbul University, Istanbul, Turkey (Z.P.).

DMD Fast Forward. Published on May 12, 2011 as DOI: 10.1124/dmd.111.038794
This article has not been copyedited and formatted. The final version may differ from this version.

DMD # 38794

Running title: Abcc2, Abcc3 and Abcg2 affect oral pharmacokinetics of MTX

Corresponding author:

Dr. Alfred H. Schinkel

Division of Molecular Biology

The Netherlands Cancer Institute

Plesmanlaan 121

1066 CX Amsterdam

The Netherlands

Phone: +31 20 512 2046

Fax: +31 20 669 1383

E-mail: a.schinkel@nki.nl

Number of text pages (including references, figure legends and table): 26

Number of tables: 1

Number of figures: 3

Number of references: 28

Number of words in the *Abstract*: 238

Number of words in the *Introduction*: 581

Number of words in the *Discussion*: 1566

List of abbreviations:

7OH-MTX, 7-hydroxymethotrexate

ABC, ATP-binding cassette

AUC, area under the plasma concentration versus time curve

BCRP, breast cancer resistance protein

MTX, methotrexate

MRP, multidrug resistance-associated protein

DMD Fast Forward. Published on May 12, 2011 as DOI: 10.1124/dmd.111.038794
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

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 i.v. MTX administration. We now used *Abcc2;Abcc3;Abcg2*$^{-/-}$ 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 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 compared to wild-type in *Abcc2;Abcg2*$^{-/-}$ mice. 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 ABCC2, or 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 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 (Borst and Oude Elferink, 2002; Schinkel and Jonker, 2003; Borst, 2006; Breedveld, 2006; Kruh, 2007). Whereas ABCC2 and ABCG2 are expressed at the apical membranes of cells, transporting their substrates into bile, feces and urine, ABCC3 is expressed basolaterally, especially in hepatocytes and enterocytes, and it generally transports its substrates into the blood circulation (Borst and Oude Elferink, 2002; Borst, 2006).

ABCC2, ABCC3 and ABCG2 have broad and substantially overlapping substrate specificities (Borst and Oude Elferink, 2002; Borst, 2006; Kruh, 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, 2008; Vlaming, 2009a; Vlaming, 2009b), which, together with the previously generated single knockout mice for Abcc2 (Vlaming, 2006), Abcc3 (Zelcer, 2006), and Abcg2 (Jonker, 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 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 i.v. pharmacokinetics of the widely used anti-cancer drug methotrexate (MTX) and its main toxic metabolite 7-hydroxymethotrexate (7OH-MTX), presumably primarily through their activity in the liver (Vlaming, 2008; Vlaming, 2009a; Vlaming, 2009b).

In cancer treatment most drugs are given i.v. due to low and/or highly variable bioavailability which can be caused, amongst others, by expression of ABC transporters in the intestine (Breedveld, 2006). 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 ABC transporter inhibitors (Breedveld, 2006; Kuppens, 2005). Since 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 i.v. 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 to wild-type rats (Chen, 2003; Naba, 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 [^{3}H]MTX after oral administration. Surprisingly, although the impact of Abcg2 on the oral pharmacokinetics of many drugs has been extensively studied (van Herwaarden and Schinkel, 2006; Murakami, 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. Interestingly, 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.

METHODS

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. The generation and basic characterization of *Abcc2*$^{-/-}$ (Vlaming, 2006), *Abcc3*$^{-/-}$ (Zelcer, 2006), *Abcg2*$^{-/-}$ (Jonker, 2003), *Abcc2;Abcc3*$^{-/-}$ (van de Wetering, 2007; Vlaming, 2008), *Abcc2;Abcg2*$^{-/-}$ (Vlaming, 2009a), and *Abcc3;Abcg2*$^{-/-}$ and *Abcc2;Abcc3;Abcg2*$^{-/-}$ mice (Vlaming, 2009b) 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 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 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, 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 -20º 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 (van Tellingen, 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, 2000).

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 i.v. bolus administration of 50 mg/kg MTX (Vlaming, 2006; Vlaming, 2008; Vlaming 2009a; Vlaming 2009b). 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 (Figure 1): the oral AUCs of the different strains over 6 h were in the order of 10-fold lower than the previously determined i.v. AUCs over 2 h (Table 1) (Vlaming, 2008; Vlaming 2009a; Vlaming 2009b). This indicates that at this relatively high dose of 50 mg/kg the oral bioavailability of MTX is quite low (9-16%). As shown in Figure 1, Abcg2 significantly affected the oral plasma pharmacokinetics of MTX. The plasma AUC_{oral} in *Abcg2*$^{-/-}$ mice was 1.7-fold higher than in wild type (Table 1). Although the AUC_{oral} in *Abcc2*$^{-/-}$ mice over 6 h was not significantly different from wild-type, the plasma levels of MTX at 60 and 120 min after administration were 1.5-fold higher than in wild-type mice ($n = 5\text{-}14$, $P < 0.05$). This suggests that also Abcc2 alone has some impact on the plasma concentrations of MTX after oral administration. Indeed, the AUC_{oral} of *Abcc2;Abcg2*$^{-/-}$ mice was 3.0-fold increased compared to wild-type mice (Table 1), significantly higher than that in *Abcg2*$^{-/-}$ mice. This 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 AUC_{oral} in *Abcc3*$^{-/-}$ compared to wild-type mice. After administration of 50 mg/kg MTX we found a modest tendency of reduced MTX plasma concentrations in *Abcc3*$^{-/-}$

DMD Fast Forward. Published on May 12, 2011 as DOI: 10.1124/dmd.111.038794
This article has not been copyedited and formatted. The final version may differ from this version.

mice (Figure 1, Table 1), but the AUC_{oral} over 6 h was not significantly different compared to 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 to wild-type (see above), and increased plasma levels were measured for *Abcc2*$^{-/-}$ mice (Figure 1), this was no longer the case in strains that additionally lacked Abcc3. *Abcc2;Abcc3*$^{-/-}$, *Abcc3;Abcg2*$^{-/-}$, and *Abcc2;Abcc3;Abcg2*$^{-/-}$ mice displayed, respectively, 2.0-, 1.8-, and 3.5-fold decreases in oral AUC compared to the corresponding strains with Abcc3 expression (Figure 1, Table 1). This shows that, like for i.v. administration (Vlaming, 2008; Vlaming, 2009b; Table 1), Abcc3 expression (in liver and/or intestine) is necessary for the increased MTX plasma levels in *Abcc2*$^{-/-}$, *Abcg2*$^{-/-}$ and *Abcc2;Abcg2*$^{-/-}$ mice after oral application of MTX.

7OH-MTX in plasma of mice after oral MTX administration was, due to the small sample volumes and low concentrations, difficult to detect, precluding conclusions on the effects of the different ABC transporters on 7OH-MTX plasma pharmacokinetics.

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 MTX administration, when plasma MTX concentrations were close to the C_{max} in all strains. In the interpretation of these results it is useful to keep in mind that the absence of Abcc2 consistently causes upregulation of Abcc3 expression in the liver of the various mouse strains (Vlaming, 2008; Vlaming 2009a; Vlaming 2009b). Absolute levels of hepatic MTX were quite low at 120 min (< 1% of the dose). The liver levels of MTX were significantly increased in most of the Abcg2-deficient strains compared to wild-type mice, but not in the strains that lacked Abcc2 but did have Abcg2 expression (Figure 2A). In *Abcg2*$^{-/-}$ mice, liver levels were 1.7-fold increased and in *Abcc2;Abcg2*$^{-/-}$ mice they were 2.0-fold

DMD Fast Forward. Published on May 12, 2011 as DOI: 10.1124/dmd.111.038794
This article has not been copyedited and formatted. The final version may differ from this version.

increased. These increases in MTX liver levels are likely 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 (Figure 2B). Note that this process may be partly counteracted by the upregulation of hepatic Abcc3 that occurs in all Abcc2-deficient strains (Vlaming, 2008; Vlaming, 2009a; Vlaming, 2009b). This probably explains why the liver/plasma ratios seen in *Abcg2*$^{-/-}$ mice are reduced in *Abcc2;Abcg2*$^{-/-}$ mice, but again increased in *Abcc2;Abcc3;Abcg2*$^{-/-}$ (Fig. 2B). A tendency of increased liver accumulation was seen in *Abcc2;Abcc3;Abcg2*$^{-/-}$ mice (0.59 ± 0.08% of the dose, P = 0.07, Figure 2A), but this was not significant. It is striking that, in spite of similar plasma levels as 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 is different from the situation after i.v. MTX administration (Vlaming, 2009b).

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 due to a high interindividual variation (Figure 2C). Probably there is substantial variation in overall uptake of MTX from the intestine between individual mice.

In most strains the MTX kidney levels were relatively low (< 0.15% of the dose) and not significantly different from wild-type levels at 120 min after administration (Figure 2D). In *Abcc2;Abcg2*$^{-/-}$ mice, however, they were 2.2-fold increased compared to wild-type mice, presumably reflecting the 3-4-fold higher plasma concentrations in this strain. In *Abcc3*$^{-/-}$ mice, MTX kidney levels were 2.0-fold lower than in wild-type. This 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 i.v. administration of MTX (Vlaming, 2008; Vlaming 2009a; Vlaming, 2009b), 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 (Figure 1), kidney levels were not different from those in wild-type mice (Figure 2D).

7OH-MTX, the main and toxic metabolite of MTX, is primarily formed in the liver (Bremnes, 1989; Chladek, 1997). The liver levels of 7OH-MTX at 120 min after MTX administration are shown in Figure 3A. This shows that in *Abcc2*$^{-/-}$ and *Abcg2*$^{-/-}$ mice the liver levels of 7OH-MTX were 2.3- and 2.1-fold increased compared to 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 to 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, $P = 0.045$). Apparently, 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 i.v. administration of MTX, and that Abcg2 is also involved when Abcc2 is absent (Vlaming, 2008; Vlaming, 2009a). After oral MTX administration this appears to be similar, as shown in Figure 3B (note that there is little or no direct intestinal excretion of 7OH-MTX (Vlaming, 2009a), so most intestinal 7OH-MTX must derive from biliary excretion). 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 (Vlaming, 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 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.

DMD Fast Forward. Published on May 12, 2011 as DOI: 10.1124/dmd.111.038794
This article has not been copyedited and formatted. The final version may differ from this version.

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, 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 to *Abcc2*$^{-/-}$ mice ($P = 7*10^{-3}$) (Figure 3C).

DISCUSSION

In this study we used the recently generated *Abcc2;Abcc3;Abcg2*$^{-/-}$ mice (Vlaming, 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 especially Abcg2, 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 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 (> 15 mg/m^2) 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 i.v. for cancer treatment (Gorlick and Bertino, 1999). However, oral administration of MTX would be much more desirable, as this is in general more patient-friendly and cost-effective (Kuppens, 2005; Breedveld, 2006). In this study we therefore used a moderately high oral dosage of MTX (50 mg/kg), that also allows a direct comparison with the extensive i.v. data at 50 mg/kg MTX we previously obtained in our mouse strains (Vlaming, 2008; Vlaming, 2009a; Vlaming, 2009b).

We show here that by combined deletion of Abcg2 and Abcc2 the plasma AUC_{oral} of MTX can be increased up to three-fold. Also Abcg2 deficiency alone, and perhaps to a lesser extent Abcc2 deficiency alone, can increase the oral MTX plasma AUC. This could mean that known polymorphisms and mutations that substantially reduce ABCG2 and ABCC2 activity in humans might affect the oral availability and hence efficacy and toxicity of oral MTX treatment (Ranganathan, 2008; Warren, 2008; Ieiri, 2009). Possibly one could also consider specific inhibition of ABCG2 and ABCC2 (without ABCC3 inhibition) 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 (Kruijtzer, 2002a; Kruijtzer, 2002b; Breedveld, 2006), but effective, specific inhibitors for ABCC2 are not yet 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 i.v. 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 the intestinal MTX uptake at the MTX dose used - otherwise a more pronounced effect on the oral AUC as compared to the i.v. AUC would have been expected. We have shown before that especially Abcc2 and, when Abcc2 is absent, also Abcg2 can mediate very substantial (virtually all) biliary excretion of MTX, up to 50% of an i.v. dose in 1 hour (Vlaming, 2009a). It thus 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, still in all strains analyzed we found between 25-55% of the dose present in the small intestine after 2 h and this was not significantly different between the different strains. We have shown before that upon i.v. 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), due to dramatically decreased biliary excretion. The fact that we don't find this effect after oral administration suggests that biliary excretion in this set-up has little impact on the intestinal MTX levels. This is probably due to the large amount of residual MTX that has not yet been absorbed from the intestine after the oral administration, and the much lower overall hepatic MTX uptake and hence hepatobiliary excretion upon oral as opposed to i.v. MTX administration.

The increases in AUC_{oral} in *Abcc2*$^{-/-}$, *Abcg2*$^{-/-}$ and *Abcc2;Abcg2*$^{-/-}$ mice are completely abrogated as soon as *Abcc3* is also deleted (Figure 1, Table 1). In principle one can envisage two mechanisms by which Abcc3 could have such a strong impact on the MTX plasma levels (Kitamura, 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 doesn't play a substantial role at the MTX dose tested (50 mg/kg). For one, when comparing the oral and intravenous plasma AUCs of all the strains tested (Table 1), there is a remarkable similarity between the shifts in AUC between the strains, resulting in at best modest changes in the oral bioavailability ($AUC_{oral}/AUC_{i.v.}$). This 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 AUC_{oral} 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. The 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 dosages. Higher MTX dosages resulted in saturation of the overall uptake process, with similar MTX uptake rates in wild-type and *Abcc3*$^{-/-}$ intestinal sacs. As 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 exretion of MTX by Abcc2- and Abcg2-deficiency on plasma MTX levels. Abcc3 probably allows rapid back-flux of MTX from liver to plasma when it is not efficiently cleared by the bile canalicular transporters Abcg2 and Abcc2 (Vlaming, 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 due to 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, 2008; Vlaming 2009a; Vlaming 2009b). In the present study we found that, despite these increased Abcc4 liver protein levels in Abcc2-deficient strains, decreased liver-plasma ratios (likely due to rapid back-flux 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, since even in *Abcc2;Abcc3;Abcg2*$^{-/-}$ mice the MTX levels in plasma and liver were quite 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 (Figure 3A), which is likely partly due to increased expression of aldehyde oxidase 1 in the liver of these strains (Vlaming, 2009a;

Vlaming, 2009b). Besides this process, also other (possibly yet unknown) changes in these knockout strains may affect MTX pharmacokinetics. These processes may have a higher affinity, but lower capacity for MTX elimination compared to Abcc2, Abcg2 and Abcc3, explaining the larger effects seen after i.v. compared to oral MTX administration (Vlaming, 2009b).

Oral MTX administration is often used in the treatment of rheumatoid arthritis as well as psoriasis, and taken long-term it can easily result in toxicity. The dose must therefore be carefully titrated. Interestingly, in a recent patient study with oral MTX, correlations between 3 single nucleotide polymorphisms in ABCC2 and MTX toxicity have been found (Ranganathan, 2008). Furthermore, in a study with psoriasis patients two ABCG2 single nucleotide polymorphisms positively correlated with efficacy of MTX therapy (Warren, 2008). Our results show that deletion of Abcg2 and Abcc2 increases oral 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 with polymorphisms in these genes may therefore 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 ABCC3 in order to predict and circumvent possible adverse effects.

ACKNOWLEDGEMENTS

We thank our colleagues for critical reading of the manuscript.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Vlaming, Pala, van Tellingen, and Schinkel.

Conducted experiments: Vlaming, van Esch, van de Steeg, Pala, and Wagenaar.

Performed data analysis: Vlaming, van Esch, Pala, van Tellingen, and Schinkel.

Wrote or contributed to the writing of the manuscript: Vlaming and Schinkel.

REFERENCES

Bardelmeijer HA, Beijnen JH, Brouwer KR, Rosing H, Nooijen WJ, Schellens, JHM, and van Tellingen O (2000) Increased oral bioavailability of paclitaxel by GF120918 in mice through selective modulation of P-glycoprotein. *Clin Cancer Res* **6**: 4416-4421.

Borst P, and Oude Elferink RP (2002) Mammalian ABC transporters in health and disease. *Annu Rev Biochem* **71**: 537-592.

Borst P, Zelcer N, and van de Wetering K (2006) MRP2 and 3 in health and disease. *Cancer Lett* **234**: 51-61.

Breedveld P, Beijnen JH, and Schellens JHM (2006) Use of P-glycoprotein and BCRP inhibitors to improve oral bioavailability and CNS penetration of anticancer drugs. *Trends Pharmacol Sci* **27**: 17-24.

Bremnes RM, Slordal L, Wist E, and Aarbakke J (1989) Formation and elimination of 7-hydroxymethotrexate in the rat in vivo after methotrexate administration. *Cancer Res* **49**: 2460-2464.

Chen C, Scott D, Hanson E, Franco J, Berryman E, Volberg M, and Liu X (2003) Impact of Mrp2 on the biliary excretion and intestinal absorption of furosemide, probenecid, and methotrexate using Eisai hyperbilirubinemic rats. *Pharm Res* **20**: 31-37.

Chladek J, Martinkova J, and Sispera L (1997) An in vitro study on methotrexate hydroxylation in rat and human liver. *Physiol Res* **46**: 371-379.

Gorlick R, and Bertino JR (1999) Clinical Pharmacology and Resistance to Dihydrofolate Reductase Inhibitors, in *Antifolate Drugs in Cancer Therapy* (Jackman AN ed) pp. 37-57 Humana Press, Totowa, New Jersey.

van Herwaarden AE, and Schinkel AH (2006) The function of breast cancer resistance protein in epithelial barriers, stem cells and milk secretion of drugs and xenotoxins. *Trends Pharmacol Sci* **27**: 10-16.

Ieiri I, Higuch S, Sugiyama Y (2009) Genetic polymorphisms of uptake (OATP1B1, 1B3) and efflux (MRP2, BCRP) transporters: implications for inter-individual differences in the pharmacokinetics and pharmacodynamics of statins and other clinically relevant drugs. *Expert Opin Drug Metab Toxicol* **5**: 703-729.

Jonker JW, Buitelaar M, Wagenaar E, van der Valk MA, Scheffer GL, Scheper RJ, Plosch T, Kuipers F, Oude Elferink RPJ, Rosing H, Beijnen JH, and Schinkel AH (2002) The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc Natl Acad Sci USA* **99**: 15649-15654.

Kitamura Y, Hirouchi M, Kusuhara H, Schuetz JD, and Sugiyama Y (2008) Increasing systemic exposure of methotrexate by active efflux mediated by multidrug resistance-associated protein 3 (mrp3/abcc3). *J Pharmacol Exp Ther* **327**: 465-473.

Kruh GD, Belinsky MG, Gallo JM, and Lee K (2007) Physiological and pharmacological functions of Mrp2, Mrp3 and Mrp4 as determined from recent studies on gene-disrupted mice. *Cancer Metastasis Rev* **26**: 5-14.

Kruijtzer CM, Beijnen JH, Rosing H, Ten Bokkel Huinink WW, Schot M, Jewell RC, Paul EM, and Schellens JHM (2002a) Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. *J Clin Oncol* **20**: 2943-2950.

Kruijtzer CM, Beijnen JH, and Schellens JHM (2002b) Improvement of oral drug treatment by temporary inhibition of drug transporters and/or cytochrome P450 in the gastrointestinal tract and liver: an overview. *Oncologist* **7**: 516-530.

Kuppens IE, Breedveld P, Beijnen JH, and Schellens JH (2005) Modulation of oral drug bioavailability: from preclinical mechanism to therapeutic application. *Cancer Invest* **23**: 443-464.

Murakami T, and Takano M (2008) Intestinal efflux transporters and drug absorption. *Expert Opin Drug Metab Toxicol* **4**: 923-939.

Naba H, Kuwayama C, Kakinuma C, Ohnishi S, and Ogihara T (2004) Eisai hyperbilirubinemic rat (EHBR) as an animal model affording high drug-exposure in toxicity studies on organic anions. *Drug Metab Pharmacokinet* **19**: 339-351.

Ranganathan P, Culverhouse R, Marsh S, Mody A, Scott-Horton TJ, Brasington R, Joseph A, Reddy V, Eisen S, and McLeod HL (2008) Methotrexate (MTX) pathway gene polymorphisms and their effects on MTX toxicity in Caucasian and African American patients with rheumatoid arthritis. *J Rheumatol* **35**: 572-579.

Schinkel AH, and Jonker JW (2003) Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* **55**: 3-29.

van Tellingen O, van der Woude HR, Beijnen JH, van Beers CJ, and Nooyen WJ (1989) Stable and sensitive method for the simultaneous determination of N5-methyltetrahydrofolate, leucovorin, methotrexate and 7-hydroxymethotrexate in biological fluids. *J Chromatogr* **488**: 379-388.

Vlaming MLH, Mohrmann K, Wagenaar E, de Waart DR, Oude Elferink RPJ, Lagas JS, van Tellingen O, Vainchtein LD, Rosing H, Beijnen JH, Schellens JHM, and Schinkel AH (2006) Carcinogen and anticancer drug transport by Mrp2 in vivo: studies using Mrp2 (Abcc2) knockout mice. *J Pharmacol Exp Ther* **318**: 319-327.

Vlaming MLH, Pala Z, van Esch A, Wagenaar E, van Tellingen O, de Waart DR, Oude Elferink RPJ, van de Wetering K., and Schinkel AH (2008) Impact of Abcc2 (Mrp2) and Abcc3 (Mrp3) on the in vivo elimination of methotrexate and its main toxic metabolite 7-hydroxymethotrexate. *Clin Cancer Res* **14**: 8152-8160.

Vlaming MLH, Pala Z, van Esch A, Wagenaar E, de Waart DR, van de Wetering K, van der Kruijssen CMM, Oude Elferink RPJ, van Tellingen O, and Schinkel AH (2009a) Functionally overlapping roles of Abcg2 (Bcrp1) and Abcc2 (Mrp2) in the elimination of methotrexate and its main toxic metabolite 7-hydroxymethotrexate in vivo. *Clin Cancer Res* **15**: 3084-3093.

Vlaming MLH, van Esch A, Pala Z, Wagenaar E, van de Wetering K, van Tellingen O, and Schinkel AH (2009b) Abcc2 (Mrp2), Abcc3 (Mrp3) and Abcg2 (Bcrp1) are the main determinants for rapid elimination of methotrexate and its toxic metabolite 7-hydroxymethotrexate in vivo. *Mol Cancer Ther* **8**: 3350-3359.

Warren RB, Smith RL, Campalani E, Eyre S, Smith CH, Barker JN, Worthington J, and Griffiths CE (2008) Genetic variation in efflux transporters influences outcome to methotrexate therapy in patients with psoriasis. *J Invest Dermatol* **128**: 1925-1929.

van de Wetering K, Zelcer N, Kuil A, Feddema W, Hillebrand M, Vlaming MLH, Schinkel AH, Beijnen JH, and Borst P (2007) Multidrug resistance proteins 2 and 3 provide alternative routes for hepatic excretion of morphine-glucuronides. *Mol Pharmacol* **72**: 387-394.

Zelcer N, van de Wetering K, de Waart DR, Scheffer GL, Marschall HU, Wielinga PR, Kuil A, Kunne C, Smith A, van der Valk M, Wijnholds J, Oude Elferink RPJ, and Borst P (2006) Mice lacking Mrp3 (Abcc3) have normal bile salt transport, but altered hepatic transport of endogenous glucuronides. *J Hepatol* **44**: 768-775.

FOOTNOTES

This work was supported by the Dutch Cancer Society [grant NKI 2003-2940]; and the Scientific and Technological Research Council of Turkey [grant TÜBITAK 2214].

A preliminary version of this study was previously presented in the PhD thesis entitled "ABC transporter compound knockout mice: physiological and pharmacological characterization" by Marijn Vlaming (University of Amsterdam, 2009), and was published online at http://dare.uva.nl/document/135716.

Reprint requests to: Dr. Alfred H. Schinkel, Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands. E-mail: a.schinkel@nki.nl.

[1]Current address: TNO, Zeist, The Netherlands.

LEGENDS FOR FIGURES

Figure 1.

MTX plasma concentration versus time curve after oral administration of 50 mg/kg MTX to female wild-type (n = 5-14 per time point), *Abcc2*$^{-/-}$ (n = 3-9 per time point), *Abcc3*$^{-/-}$ (n = 2-10 per time point), *Abcg2*$^{-/-}$ (n = 4-10 per time point), *Abcc2;Abcc3*$^{-/-}$ (n = 4-10 per time point), *Abcc2;Abcg2*$^{-/-}$ (n = 3-12 per time point), *Abcc3;Abcg2*$^{-/-}$ (n = 3-6 per time point, at 4 hr after administration no *Abcc3;Abcg2*$^{-/-}$ mice were analyzed) and *Abcc2;Abcc3;Abcg2*$^{-/-}$ mice (n = 3-8 per time point). Data are presented as means ± S.D.

Figure 2.

MTX tissue distribution 2 h after oral administration of 50 mg/kg MTX to female wild-type (n = 6-7 per tissue), *Abcc2*$^{-/-}$ (n = 4-5 per tissue), *Abcc3*$^{-/-}$ (n = 4-5 per tissue), *Abcg2*$^{-/-}$ (n = 4-5 per tissue), *Abcc2;Abcc3*$^{-/-}$ (n = 5), *Abcc2;Abcg2*$^{-/-}$ (n = 5), *Abcc3;Abcg2*$^{-/-}$ (n = 5) and *Abcc2;Abcc3;Abcg2*$^{-/-}$ (n = 4-5 per tissue) mice. A, MTX liver levels (% of dose) in the different strains. B, MTX liver concentration vs. plasma concentration ratios in the different strains. C, MTX small intestinal tissue (SI) and contents (SIC) levels (% of dose) in the different strains. C, MTX kidney levels (% of dose) in the different strains. Data are presented as means ± S.D. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared to wild type, Student's t-test).

Figure 3.

7OH-MTX tissue distribution 2 h after oral administration of 50 mg/kg MTX to female wild-type (n = 7), *Abcc2*$^{-/-}$ (n = 4-5 per tissue), *Abcc3*$^{-/-}$ (n = 5), *Abcg2*$^{-/-}$ (n = 4-5 per tissue), *Abcc2;Abcc3*$^{-/-}$ (n = 4-5 per tissue), *Abcc2;Abcg2*$^{-/-}$ (n = 4), *Abcc3;Abcg2*$^{-/-}$ (n = 5) and *Abcc2;Abcc3;Abcg2*$^{-/-}$ (n = 5) mice. A, 7OH-MTX liver levels (% of MTX dose) in the

different strains. B, 7OH-MTX small intestinal (SI) tissue and contents levels (% of MTX dose) in the different strains. C, 7OH-MTX kidney levels (% of MTX dose) in the different strains (nd, not detectable, below $2.5*10^{-3}$% of dose, the detection limit is indicated by the dashed line). Data are presented as means ± S.D. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared to wild type, Student's t-test).

Table 1. MTX plasma AUCs of female mice after iv (0-120 min) (Vlaming, 2009b) and oral (0-6 hr) administration of 50 mg/kg MTX.

	Strain							
	Wild-type	*Abcc2*$^{-/-}$	*Abcc3*$^{-/-}$	*Abcg2*$^{-/-}$	*Abcc2;Abcc3*$^{-/-}$	*Abcc2;Abcg2*$^{-/-}$	*Abcc3;Abcg2*$^{-/-}$	*Abcc2;Abcc3;Abcg2*$^{-/-}$
$AUC_{oral\ 0\text{-}6\ hr}$ (min·µg/ml)	62 ± 13	85 ± 14	48 ± 13	107 ± 14*	43 ± 9	184 ± 31**	60 ± 11	53 ± 5
Fold difference[¶]	1.0	1.4	0.8	1.7	0.7	3.0	1.0	0.9
$AUC_{iv\ 0\text{-}120\ min}$ (min·µg/ml)	444 ± 44	870 ± 103***	368 ± 34	692 ± 56*	435 ± 47	1446 ± 229**	451 ± 26	603 ± 56*
Fold difference[¶]	1.0	2.0	0.8	1.6	1.0	3.3	1.0	1.4
F (oral/iv) %	14.0 ± 3.2	9.8 ± 2.0	13.1 ± 3.7	15.6 ± 2.4	9.8 ± 2.4	12.8 ± 2.9	13.4 ± 2.6	8.9 ± 1.2*

Note: MTX plasma AUCs are presented as min·µg/ml and oral availabilities (F) are given as % of $AUC_{i.v.}$ (means ± SD, n = 2-14, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to wild-type mice, Student's t-test was used for statistical analysis). [¶]Fold difference: fold difference compared to wild-type mice with the same route of administration.

Figure 1

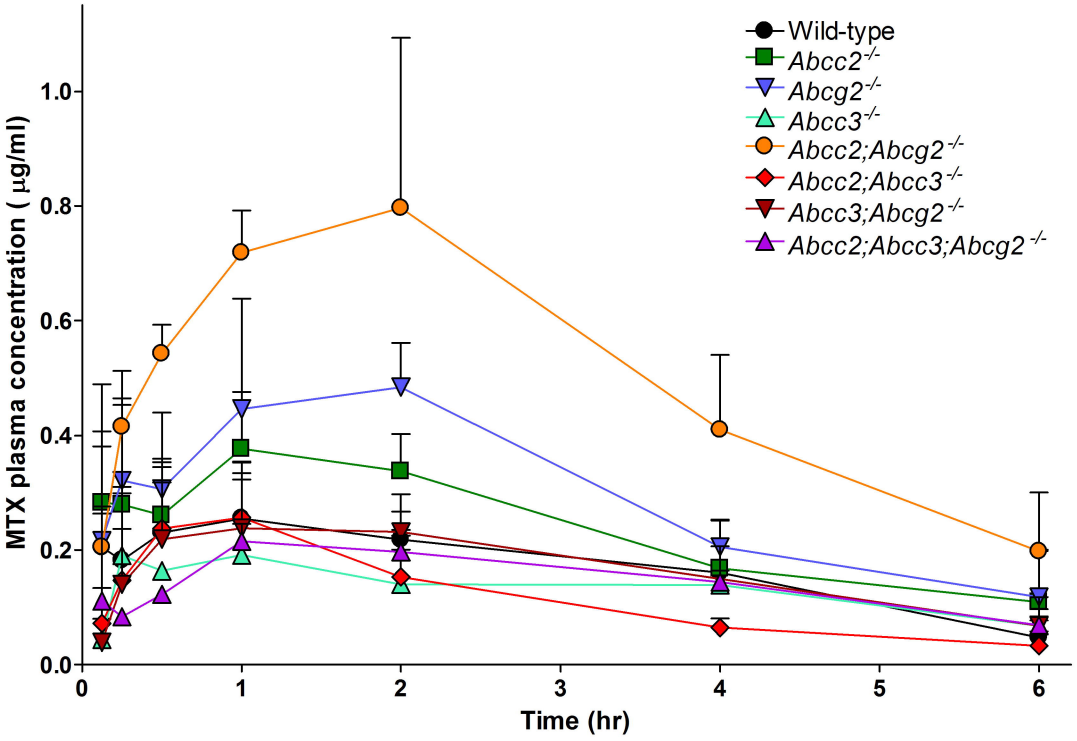

Wild-type
Abcc2-/-
Abcg2-/-
Abcc3-/-
Abcc2;Abcg2-/-
Abcc2;Abcc3-/-
Abcc3;Abcg2-/-
Abcc2;Abcc3;Abcg2-/-
MTX plasma concentration (μg/ml)
0.0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5
6
Time (hr)

Figure 2

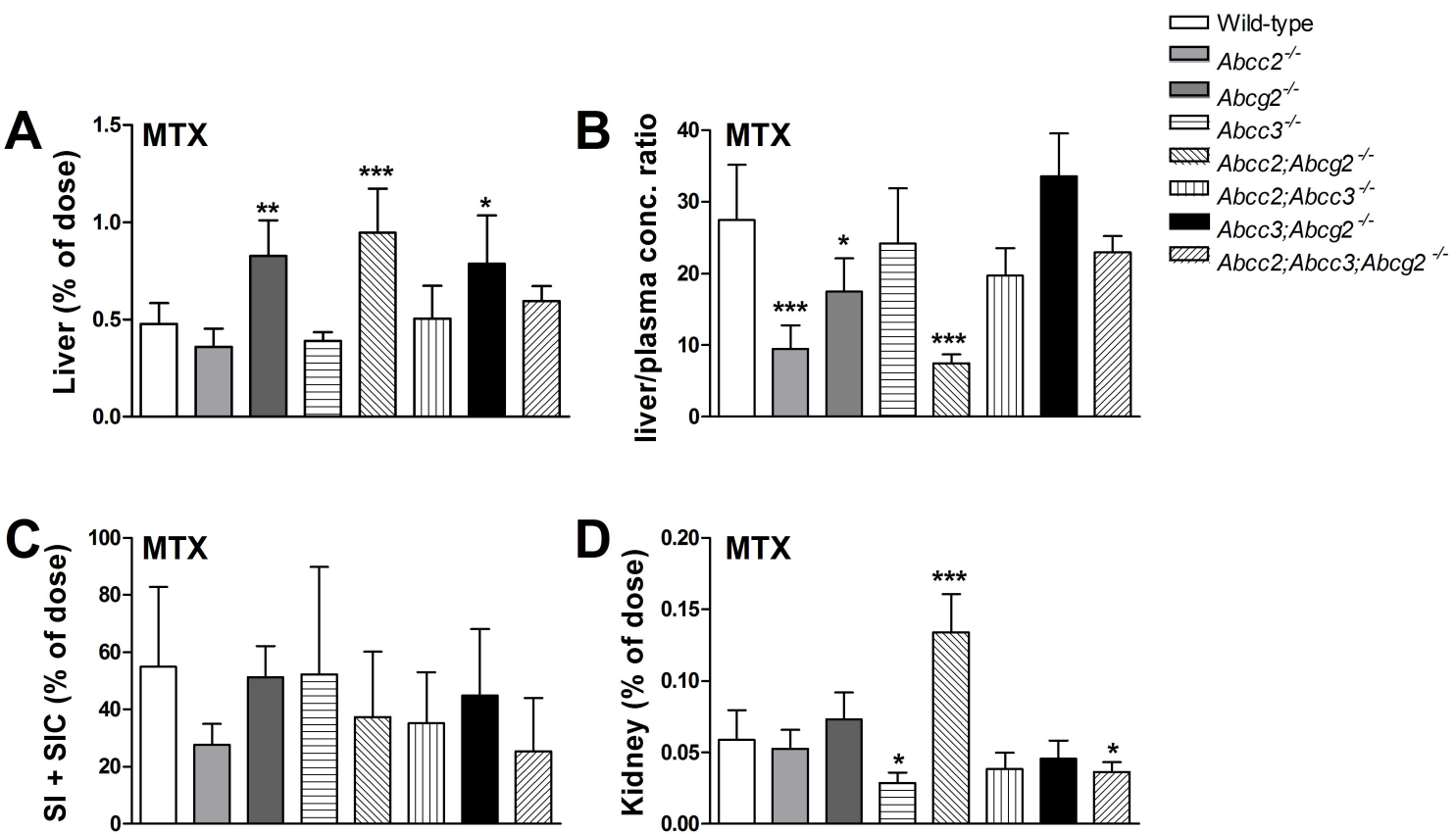
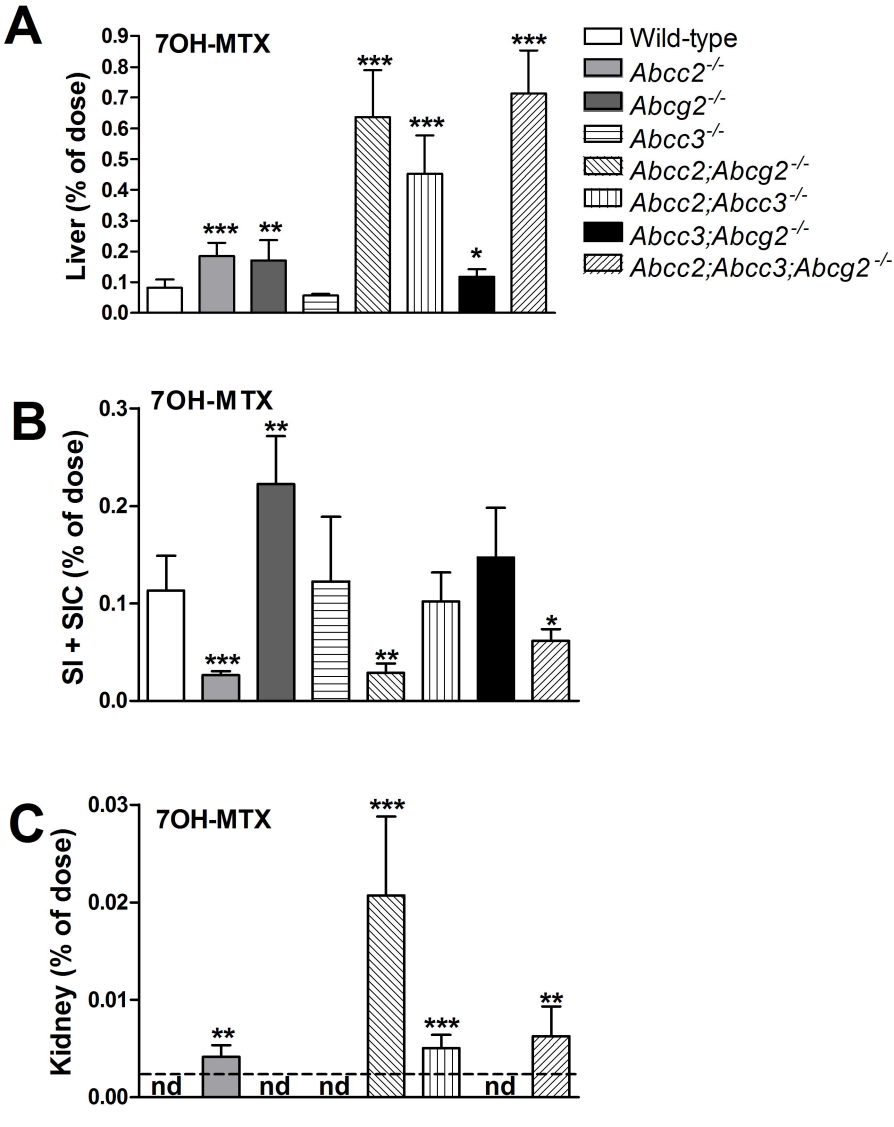

A
MTX
Liver (% of dose)
1.5
1.0
0.5
0.0
**
***
*
B
MTX
liver/plasma conc. ratio
40
30
20
10
0
***
*
***
C
MTX
SI + SIC (% of dose)
100
80
60
40
20
0
D
MTX
Kidney (% of dose)
0.20
0.15
0.10
0.05
0.00
***
*
*
Wild-type
Abcc2-/-
Abcg2-/-
Abcc3-/-
Abcc2;Abcg2-/-
Abcc2;Abcc3-/-
Abcc3;Abcg2-/-
Abcc2;Abcc3;Abcg2-/-

Figure 3


A
7OH-MTX
Liver (% of dose)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
**
***
***
*
***
Wild-type
Abcc2-/-
Abcg2-/-
Abcc3-/-
Abcc2;Abcg2-/-
Abcc2;Abcc3-/-
Abcc3;Abcg2-/-
Abcc2;Abcc3;Abcg2-/-
B
7OH-MTX
SI + SIC (% of dose)
0.3
0.2
0.1
0.0
**
***
**
*
C
7OH-MTX
Kidney (% of dose)
0.03
0.02
0.01
0.00
**
***
***
**
nd
nd
nd
nd