ABC transporter compound knockout mice: physiological and pharmacological characterization
Vlaming, M.L.H.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 4

Impact of Abcc2 (Mrp2) and Abcc3 (Mrp3) on the \textit{in vivo} elimination of methotrexate and its main toxic metabolite 7-hydroxymethotrexate

Maria L.H. Vlaming, Zeliha Pala, Anita van Esch, Els Wagenaar, Olaf van Tellingen, Dirk R. de Waart, Ronald P.J. Oude Elferink, Koen van de Wetering and Alfred H. Schinkel

\textit{Modified from Clin Cancer Res (2008) 14: 8152-8160}
Impact of Abcc2 (Mrp2) and Abcc3 (Mrp3) on the \textit{in vivo} elimination of methotrexate and its main toxic metabolite 7-hydroxymethotrexate

Maria L.H. Vlaming\textsuperscript{1}, Zeliha Pala\textsuperscript{2}, Anita van Esch\textsuperscript{1}, Els Wagenaar\textsuperscript{1}, Olaf van Tellingen\textsuperscript{3}, Dirk R. de Waart\textsuperscript{4}, Ronald P.J. Oude Elferink\textsuperscript{4}, Koen van de Wetering\textsuperscript{5} and Alfred H. Schinkel\textsuperscript{1}

Divisions of \textsuperscript{1}Experimental Therapy, \textsuperscript{3}Clinical Chemistry and \textsuperscript{5}Molecular Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands; \textsuperscript{2}Faculty of Pharmacy, Istanbul University, Istanbul, Turkey; \textsuperscript{4}AMC Liver Center, Academic Medical Center, Amsterdam, The Netherlands.

Purpose: ABCC2 (MRP2) and ABCC3 (MRP3) mediate the elimination of toxic compounds such as drugs and carcinogens and have a large overlap in substrate specificity. We investigated the roles of Abcc2 and Abcc3 in the elimination of the anti-cancer drug methotrexate (MTX) and its toxic metabolite 7-hydroxymethotrexate (7OH-MTX) \textit{in vivo}.

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

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

Conclusions: Abcc2 is important for (biliary) excretion of MTX and its toxic metabolite 7OH-MTX. When Abcc2 is absent, Abcc3 transports MTX and 7OH-MTX back from the liver into the circulation, leading to increased plasma levels and urinary excretion. Variation in ABCC2 and/or ABCC3 activity may therefore have profound effects on the elimination and severity of toxicity of MTX and 7OH-MTX after MTX treatment of patients.
INTRODUCTION
The multidrug resistance proteins ABCC2 (MRP2) and ABCC3 (MRP3) are members of the ATP-binding cassette (ABC) transporter superfamily. ABCC2 is present in apical membranes of hepatocytes and epithelial cells of small intestine and kidney and is involved in the elimination of both endogenous and exogenous compounds from the body (1). ABCC3 is also found in liver, kidney and small intestine, as well as in adrenal glands and pancreas. In contrast to ABCC2, ABCC3 localizes to the basolateral membrane of polarized cells (2). Both transporters are expressed in various tumors and can transport a range of (anti-cancer) drugs (1;2).

There is a large overlap in the substrate specificity of ABCC2 and ABCC3. They can for example both transport bilirubin glucuronides (3-5), and in patients with the Dubin-Johnson syndrome, who have functionally deficient ABCC2 (6;7), both plasma bilirubin glucuronide levels and ABCC3 protein levels are increased. It has been speculated that these two findings are related: ABCC3 upregulation in absence of ABCC2 would allow increased basolateral efflux of bilirubin glucuronides from the liver (7). Since Abcc2 and Abcc3 are both involved in the elimination of xenobiotics, absence of each or both of them can have a profound effect on the pharmacokinetics of drugs, or their glucuronide conjugates, as has been shown using single knockout mice for Abcc2 and Abcc3 (8), as well as with the recently generated Abcc2;Abcc3 +/- mice (9).

Methotrexate (MTX), a widely used anti-cancer and anti-rheumatic drug, is a substrate for both ABCC2 and ABCC3 in vitro (2). Abcc2-deficient mice and rats are hampered in the (biliary) elimination of ([3H])MTX (10;11), whereas the effect of Abcc3 on the pharmacokinetics of MTX in vivo thus far has not been studied. In the treatment of cancer, MTX is given intravenously at relatively high doses (>15 mg/m²), which sometimes leads to severe and even lethal toxicity in patients (12). Interestingly, mutations in the ABCC2 gene have recently been associated with increased methotrexate toxicity in patients (13-15). Furthermore, crystalline deposits of the toxic metabolite 7-hydroxymethotrexate (7OH-MTX) (which has a very low aqueous solubility) in renal tubules have been implicated in kidney failure after high-dose MTX treatment (12;16). The effect of ABCC2 and ABCC3 on the disposition of 7OH-MTX has not yet been studied, although it was shown by Breedveld et al. (2007) that ABCC2 transports 7OH-MTX in vitro (17).

To investigate the hypothesized functional relationship between Abcc2 and Abcc3 in vivo, we have generated and characterized Abcc2;Abcc3 +/- mice and analyzed plasma, bile and urine of these mice. We further used the mice to investigate the relative roles of Abcc2 and Abcc3 in the elimination of MTX and its toxic metabolite 7OH-MTX, which is primarily formed by aldehyde oxidase in the liver (18-20). We show here that Abcc2 has a major impact on the pharmacokinetics of both MTX and 7OH-MTX and that, when Abcc2 is absent, Abcc3 transports these toxic compounds from the liver back into the circulation, allowing an
alternative route of elimination via the urine. Our data illustrate the importance of the functionally overlapping and complementary roles of Abcc2 and Abcc3 in vivo.

**METHODS**

**Animals.** Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Abcc2 WT (10) and Abcc3 WT (5) mice have been described. Abcc2;Abcc3 WT mice were generated by cross-breeding. 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) and 7OH-MTX from Toronto Research Chemicals Inc. (North York, ON, Canada). Ketamine was from Parke-Davis (Hoofddorp, The Netherlands), xylazine from Sigma Chemical Co. (St. Louis, MO) and methoxyflurane (Metofane) from Medical Developments Australia Pty. Ltd. (Springvale, Victoria, Australia). MRPr1, M4I-80 and M5II-54 were kind gifts of Dr. G.L. Scheffer (Free University Hospital, Amsterdam, The Netherlands), K12 was kindly provided by Dr. Bruno Stieger (University Hospital, University of Zürich, Zürich, Switzerland). BXP-53 was described before (21).

**Western analysis.** Crude membrane fractions from tissues were prepared as described (21;22). Western blotting was performed as described (23). Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer (not shown). Abcc1 (Mrp1), Abcc4 (Mrp4), Abcc5 (Mrp5), Abcg2 (Bcrp1) and Abcb11 (Bsep) were detected with Abs MRPr1 (dilution 1:1000), M4I-80 (dilution 1:400), M5II-54 (dilution 1:1), BXP-53 (dilution 1:400) and K12 (dilution 1:2000), respectively. Bound primary antibodies were detected by incubating the blot with HRP-labelled rabbit anti-rat IgG (1:1000, DAKO) (Abcc1, Abcc4, Abcc5 and Abcg2) or HRP-labelled goat anti-rabbit IgG (1:2000, DAKO) (Abcb11).

**Histological, clinical-chemical and hematological analysis.** Histological analysis of mouse tissues, standard clinical chemistry analyses on serum and standard hematological analysis (twice within a time span of 1.5 years) were performed as described (10).

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

**Plasma and tissue pharmacokinetic experiments.** MTX was administered to female mice by injecting 5 μl/g body weight of a 10 mg/ml MTX in 0.9% NaCl solution into the tail vein. Animals were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia and organs were removed. Intestinal contents (feces) and tissue were separated.

**Biliary excretion of MTX and 7OH-MTX.** Gall bladder cannulations in female mice were performed as described (24). After cannulation, 50 mg/kg MTX was administered i.v. as described above. Bile was collected in 15 min fractions for 60 min. Subsequently, mice were killed by cardiac puncture and blood and organs were collected.

**Fecal and urinary excretion of MTX and 7OH-MTX.** Female mice were individually housed in Ruco Type M/1 stainless steel metabolic cages (Valkenswaard, The Netherlands), and allowed 24 hours to adapt before 50 mg/kg MTX was injected into the tail vein, as described above. Feces and urine were collected over 0-24 and 24-48 hours. 48 hours after injection, mice were killed by terminal bleeding through cardiac puncture under methoxyflurane anesthesia. Organs were removed and intestinal contents (feces) and tissue were separated.

**HPLC analysis of MTX and 7OH-MTX.** Collected organs were homogenized in an ice-cold 4% BSA solution before HPLC analysis. MTX and 7OH-MTX concentrations in plasma, urine and tissue homogenates were determined as described (27).

**Statistical analysis.** Unless otherwise indicated, the two-sided unpaired Student's t-test was used to assess statistical significance of differences between two sets of data. When more than two groups were compared, one-way ANOVA followed by Tukey's multiple comparison test was used, as indicated in text and/or figure legends. Results are presented as the means ± standard deviations (SD). Differences were considered statistically significant when \( P < 0.05 \). Averaged concentrations for each time-point were used to calculate the area under the plasma-concentration versus time curve (AUC) from \( t = 0 \) to the last sampling point by the linear trapezoidal rule; standard errors (SE) were calculated by the law of propagation of errors (28). Results of AUC measurements are presented as means ± SD.
Figure 1. Characterization of $Abcc2;Abcc3^{-/-}$ mice. A, liver weight (as percentage of body weight) of male (left panel) and female (right panel) wild-type, $Abcc2^{-/-}$, $Abcc3^{-/-}$ and $Abcc2;Abcc3^{-/-}$ mice (means ± SD, n = 5, **, P < 0.01, ***, P < 0.001, ANOVA). B, levels of $Abcc4$ protein in crude membrane fractions of liver samples from two independent female $Abcc2;Abcc3^{-/-}$ and wild-type mice. C, levels of $Abcc4$ protein in crude membrane fractions of kidney samples from two independent female $Abcc2;Abcc3^{-/-}$ and wild-type mice. Equal protein loading was confirmed by Ponceau S staining of the membranes after transfer. In B and C, the lane with the positive control (Sf-9 vesicles containing ABCC4 (40)) is indicated with “+”. Underglycosylation of ABCC4 in the Sf-9 cells causes the faster migration compared to the murine $Abcc4$. The amount of protein loaded is noted above the lanes.
RESULTS

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

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

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

All other clinical-chemical parameters measured showed no significant differences between wild-type and knockout strains (not shown). Also hematological analysis did not yield any consistent differences between Abcc2;Abcc3−/− and wild-type mice.
Figure 2. Bilirubin levels in plasma of Abcc2;Abcc3−/− mice. A, plasma values of total bilirubin in wild-type, Abcc2−/−, Abcc3−/− and Abcc2;Abcc3−/− mice (means ± SD, n = 3-9, ***, P < 0.001). B, plasma values of conjugated bilirubin in wild-type, Abcc2−/−, Abcc3−/− and Abcc2;Abcc3−/− mice (means ± SD, n = 3-9). The values for wild-type, Abcc3−/− and Abcc2;Abcc3−/− mice were below the detection limit of the analyzer (<1 µM).

**Biliary and urinary excretion and composition in Abcc2;Abcc3−/− mice.** We analyzed the bile and urine composition of wild-type, Abcc2−/−, Abcc3−/− and Abcc2;Abcc3−/− mice after ligation of the common bile duct and gall bladder cannulation. We previously found that Abcc2−/− mice have a significantly reduced bile flow (Figure 3A and (10)). Bile flow in the Abcc2;Abcc3−/− mice was significantly reduced as well, to about 76% of wild-type bile flow (Figure 3A). Surprisingly, this was still significantly higher than in Abcc2−/− mice (P<0.01, ANOVA), although this was not the case after correction for differences in liver weight (ANOVA) (not shown). The biliary excretion of total bilirubin in Abcc2;Abcc3−/− mice was significantly reduced compared to wild-type mice (Figure 3B). The reduced total biliary bilirubin excretion in Abcc2;Abcc3−/− mice was caused by a reduction in bilirubin monoglucuronide output, which was also reduced in Abcc2−/− mice (Figure 3B). Interestingly, the (much lower) biliary bilirubin diglucuronide output in both strains lacking Abcc2 was increased compared to wild-type and Abcc3−/− mice (Figure 3B). The (modest) output of unconjugated bilirubin in bile of Abcc2−/−, Abcc3−/− and Abcc2;Abcc3−/− mice was not significantly different.
from wild-type mice (Figure 3B). Analysis of the urine of the cannulated mice showed that bilirubin monoglucuronide was found in urine of Abcc2/–, but not of Abcc2;Abcc3/– mice, consistent with the increased bilirubin glucuronide levels in plasma of Abcc2/– mice (Figure 3C and 2B).

**Figure 3.** Analysis of bile and urine from male wild-type, Abcc2/–, Abcc3/– and Abcc2;Abcc3/– mice after gall bladder cannulation and ligation of the common bile duct. Bile was collected in 15 min fractions over 1 h. Bile collected in the first 15 min was analyzed for bilirubin concentration (Panel B). A, average bile flow in the various mouse strains (0-60 min). B, output of total bilirubin, bilirubin monoglucuronides (BMG), bilirubin diglucuronides (BDG) and unconjugated bilirubin (UCB) in the bile of the mouse strains (first 15 min fractions) (BW, body weight). C, BMG concentration in urine of the mouse strains. BDG and UCB were not detected in any of the urine samples. Data are means ± SD (n = 5, *, P < 0.05; **, P < 0.01, ***, P < 0.001) (n.q., not quantifiable).

Biliary output of cholesterol and bile acids was not significantly different in any of the knockout strains (not shown). Biliary phospholipid output was significantly increased in Abcc2/– and Abcc2;Abcc3/– mice compared to wild-type mice (Supplementary Figure 1SA). However, after correction for liver weight, no significant differences were seen in any of the strains (Supplementary Figure 1SB).

**Influence of Abcc2 and Abcc3 on methotrexate pharmacokinetics in vivo.** To investigate the relative roles of Abcc2 and Abcc3 in the pharmacokinetics of MTX, we administered MTX i.v. to female wild-type, Abcc2/–, Abcc3/– and
Abcc2;Abcc3\(^{-/-}\) mice at a dose of 50 mg/kg (comparable to 154 mg/m\(^2\) in man (31)) and measured MTX levels in plasma and a set of organs at different time points. The results for plasma are shown in Figure 4A and Supplementary Table 1S. Compared to wild-type mice, the MTX plasma AUC between 7.5 and 120 min was 2-fold increased in Abcc2\(^{-/-}\) mice (870 ± 103 min·µg/ml vs. 444 ± 44 min·µg/ml for wild-type, P = 6.2 \times 10^{-4}). In contrast, in the Abcc2;Abcc3\(^{-/-}\) mice the MTX plasma AUC was not significantly different from wild-type (435 ± 47 min·µg/ml, P = 0.76), and 2-fold lower than in single Abcc2\(^{-/-}\) mice (P = 2.2\times10^{-3}). In Abcc3\(^{-/-}\) mice the MTX plasma AUC was not significantly different from wild-type either, although there was a tendency of a reduced AUC\textsubscript{plasma} in these mice (368 ± 34 min·µg/ml, P = 0.084). This suggests that in the presence of Abcc2 there is only a minor influence of Abcc3 on the pharmacokinetics of MTX. However, the role of Abcc3 becomes important when Abcc2 is absent.

Figure 4. Pharmacokinetics of MTX after i.v. administration of 50 mg/kg MTX to female wild-type, Abcc2\(^{-/-}\), Abcc3\(^{-/-}\) and Abcc2;Abcc3\(^{-/-}\) mice. A, MTX plasma concentration versus time curves of the various strains (means ± SD, n = 3-11). B, MTX liver level versus time curves (means ± SD, n = 4-9). C, liver-to-plasma ratio versus time curves (means ± SD, n = 4-9). D, MTX small intestinal tissue + contents (SI tissue + contents) level versus time curves (means ± SD, n = 5-9).
We also measured liver levels of MTX between 7.5 and 120 min after administration (Figure 4B and Supplementary Table 1S). Already 7.5 min after administration, livers contained high amounts of MTX (44-55% of the dose), which decreased thereafter. Interestingly, the liver-to-plasma ratios of MTX over 120 min (Figure 4C and Supplementary Table 1S) were lower in Abcc2−/− mice than in wild-type mice, whereas Abcc3+/− mice tended to have increased MTX liver-to-plasma ratios. In Abcc2;Abcc3−/− mice the liver-to-plasma ratios were similar to those found in wild-type. These results, combined with the MTX plasma vs. time curves, suggest that Abcc2;Abcc3+/− mice had reduced sinusoidal elimination of MTX from the liver compared to Abcc2−/− mice.

We next determined the MTX levels in small intestinal tissue and contents of the different strains (Figure 4D and Supplementary Table 1S). MTX levels in all strains steadily increased up to 60 min after administration. At all time points MTX levels in small intestine of Abcc2−/− mice were significantly lower compared to wild-type mice (Supplementary Table 1S), indicating an important role for Abcc2 in the elimination of MTX, most likely via hepatobiliary excretion (see also below). At 60 min after administration, MTX small intestinal levels in Abcc2;Abcc3+/− mice were significantly reduced as well, compared to wild-type and Abcc3+/− mice (P<0.01, ANOVA). However, MTX levels in Abcc2;Abcc3−/− mice were significantly higher than in Abcc2−/− mice at this time point (P<0.01, ANOVA). Collectively, these data suggest that apical elimination mechanisms different from Abcc2 still mediate substantial hepatobiliary excretion of MTX in the Abcc2−/− and Abcc2;Abcc3−/− mice. Tendencies of increased liver MTX concentrations in the Abcc2;Abcc3+/− mice (Figure 4B), and higher MTX hepatobiliary excretion compared to Abcc2−/− mice (see below), likely explain the higher small intestinal values in the Abcc2;Abcc3+/− mice.

Kidney toxicity is a clinically relevant dose-limiting factor for (high-dose) MTX treatment (32). Compared to wild-type mice, kidney levels of MTX were significantly increased in Abcc2+/− mice 15-60 min after MTX administration, whereas levels in kidneys of Abcc2;Abcc3+/− mice were not (Supplementary Table 1S). MTX kidney levels appeared to correlate with MTX plasma levels in all strains.

Subsequent gall bladder cannulation experiments revealed that excretion of MTX into bile the first 60 min after i.v. MTX administration (50 mg/kg) was reduced in Abcc2+/− mice to about 27% of that found in wild-type mice. Surprisingly, in Abcc2;Abcc3−/− mice, the biliary excretion of MTX over the first 60 min did not differ from that found in wild-type or Abcc3+/− mice (ANOVA) (Figure 5A). MTX levels in small intestinal contents and tissue after gall bladder cannulation were very low (<1% of the dose) in all strains and did not differ between the strains (not shown), indicating that direct intestinal excretion is a negligible route for the elimination of MTX in mice.
Figure 5. Effect of Abcc2 and Abcc3 on biliary and urinary excretion of MTX in female mice. A, biliary excretion of MTX (as % of dose) after i.v. administration of 50 mg/kg MTX to common bile duct ligated and gall bladder cannulated female wild-type, Abcc2\(-/-\), Abcc3\(-/-\) and Abcc2;Abcc3\(-/-\) mice (means ± SD, n = 3-5, **, P < 0.01, ANOVA). B, urinary and fecal excretion of MTX (as % of dose) in the first 24 hrs after i.v. administration of 50 mg/kg MTX to mice of the various strains (means ± SD, n = 3-9, **, P < 0.01, ANOVA). Urinary and fecal excretion of MTX 24-48 hrs after i.v. administration were around 1% of the dose and not significantly different from wild-type in any of the strains (not shown). The levels of MTX in liver, small intestine and kidney 48 hrs after i.v. administration were below 0.03% in all strains and plasma levels were below the LLQ (24 nM) (not shown).

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

Influence of Abcc2 and Abcc3 on 7-hydroxymethotrexate pharmacokinetics in vivo.

In patients receiving high-dose MTX a significant amount of the toxic metabolite 7OH-MTX, which is primarily formed in the liver (18-20), is detected in the urine
The effects of Abcc2 and Abcc3 on the pharmacokinetics of 7OH-MTX after i.v. administration of 50 mg/kg MTX are shown in Figure 6 and Supplementary Table 2S. The plasma concentration-time curves for 7OH-MTX in wild-type, Abcc2\(^{-/-}\), Abcc3\(^{-/-}\) and Abcc2;Abcc3\(^{-/-}\) mice (Figure 6A) suggest a very important role for Abcc2 in the elimination of this toxic metabolite. Whereas the plasma AUCs for 7OH-MTX in wild-type and Abcc3\(^{-/-}\) mice were quite low and not significantly different from each other (wild-type: 17 ± 3 min·µg/ml, Abcc3\(^{-/-}\): 12 ± 1 min·µg/ml, P = 0.084), the plasma AUC for 7OH-MTX in Abcc2\(^{-/-}\) mouse was 6-fold increased compared to wild-type mice (106 ± 11 min·µg/ml, P = 3.7 \times 10^{-5}). At early time-points (0-30 min), plasma levels of 7OH-MTX in Abcc2;Abcc3\(^{-/-}\) mice were not different from wild-type and Abcc3\(^{-/-}\) mice, and clearly lower than in Abcc2\(^{-/-}\) mice. This suggests that in the absence of Abcc2, Abcc3 efficiently transports 7OH-MTX from liver towards the circulation. Interestingly, at later time points after MTX administration (> 30 min) plasma 7OH-MTX levels were substantially increased in Abcc2;Abcc3\(^{-/-}\) mice, leading to a 4.3-fold higher plasma AUC compared to wild-type mice (73 ± 20 min·µg/ml, P = 7.1 \times 10^{-5}). The concomitant liver levels of 7OH-MTX in Abcc2\(^{-/-}\) mice were markedly higher than in wild-type mice at all time points tested, and the 7OH-MTX levels in livers of Abcc2;Abcc3\(^{-/-}\) mice were even more increased, especially between 30 and 120 min after administration of MTX (Figure 6B and Supplementary Table 2S). This suggests an important role for Abcc2 in clearing the liver from the toxic metabolite 7OH-MTX. Abcc3 only plays a role in the absence of Abcc2, as the 7OH-MTX levels in liver of Abcc3\(^{-/-}\) mice were not different from those found in wild-type mice. Kidney levels of 7OH-MTX in Abcc2\(^{-/-}\) (0-60 min) and Abcc2;Abcc3\(^{-/-}\) (15-60 min) mice were 3-11 fold and 4-7 fold increased compared to wild-type, respectively, correlating with the increased 7OH-MTX plasma levels (Supplementary Table 2S).

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

Whether Abcc2 and Abcc3 affect urinary and fecal excretion of 7OH-MTX in the first 24 hr after i.v. administration of MTX (50 mg/kg) was subsequently investigated (Figure 6D). Compared to control mice, urinary excretion of 7OH-MTX was markedly higher in both Abcc2\(^{-/-}\) and Abcc2;Abcc3\(^{-/-}\) mice, consistent with the increased 7OH-MTX plasma levels in these strains (Figure 6D). Urinary 7OH-MTX output in Abcc3\(^{-/-}\) mice was not significantly different from wild-type output (Figure 6D). The fecal output of 7OH-MTX was similar to wild-type in both Abcc2\(^{-/-}\) and Abcc3\(^{-/-}\) mice, but 2-fold increased in Abcc2;Abcc3\(^{-/-}\) mice (Figure 6D).
Chapter 4

Figure 6. Pharmacokinetics of 7OH-MTX after i.v. administration of 50 mg/kg MTX to female wild-type, Abcc2−/−, Abcc3−/− and Abcc2;Abcc3−/− mice. A, 7OH-MTX plasma concentration versus time curves of the various strains (means ± SD, n = 3-11). B, 7OH-MTX liver level (as % of MTX dose) versus time curves (means ± SD, n = 4-9). C, biliary excretion of 7OH-MTX (as % of MTX dose) after ligation of the common bile duct and gall bladder cannulation, followed by i.v. administration of 50 mg/kg MTX (means ± SD, n = 3-5, *, P < 0.05). D, urinary and fecal excretion of 7OH-MTX (as % of MTX dose) in the first 24 hrs after i.v. administration of 50 mg/kg MTX (means ± SD, n = 3-9, ***, P < 0.001). The urinary excretion of 7OH-MTX 24-48 hours after MTX administration was below 1% of the given dose in all strains, and 7OH-MTX was not detected in feces over that time period (not shown). 48 hrs after administration, the 7OH-MTX concentrations in liver and small intestinal contents were below 0.15% and 7OH-MTX was undetected in plasma, kidneys and small intestinal tissue of all strains (not shown).

DISCUSSION

Abcc2 and Abcc3 have overlapping substrate specificities and Abcc3 protein expression is often increased when Abcc2 is absent (7;10). Using Abcc2−/− and the here described Abcc2;Abcc3−/− mice we show that in the absence of Abcc2, Abcc3 transports bilirubin glucuronide across the sinusoidal membrane, resulting in increased plasma levels and urinary excretion. Similar results were obtained for the pharmacokinetics of the anti-cancer and anti-rheumatic drug MTX: Abcc2 transports MTX into bile whereas in the absence of Abcc2, Abcc3 transports MTX back into
the circulation, allowing increased urinary excretion. Using Abcc2+/- and Abcc2;Abcc3-/- mice we additionally show that Abcc2 is important for the biliary excretion of 7OH-MTX, the main (toxic) metabolite of MTX. In the absence of Abcc2, Abcc3 markedly influences the sinusoidal elimination of 7OH-MTX from the liver, and the absence of both ABC transporters leads to significant liver accumulation of 7OH-MTX. Absence of Abcc2 leads to a profound increase in plasma and kidney levels of MTX and especially 7OH-MTX, which is partly dependent on presence of Abcc3. Analysis of Abcc3-/- mice alone would have suggested little impact of Abcc3 on either MTX or 7OH-MTX pharmacokinetics. Our findings suggest that differences in ABCC2 and (when ABCC2 is absent or reduced) ABCC3 activity could profoundly influence plasma and tissue levels, as well as MTX and 7OH-MTX related toxicity in patients who are treated with MTX.

It is quite surprising that in the absence of both Abcc2 and Abcc3, the health of the mice does not seem to be affected. This may be caused by the fact that the mice live in a relatively protected environment and therefore may not be confronted with many toxic compounds during their life. Other elimination mechanisms possibly (partly) compensate for the absence of these ABC transporters, for example Abcc4, which is 2-fold increased in liver and kidney of female Abcc2;Abcc3-/- mice. Although no spontaneous health problems were seen in untreated Abcc2;Abcc3-/- mice, drug treatment can clearly lead to substantial accumulation of potentially toxic metabolites in liver and plasma, as we show here for 7OH-MTX after MTX treatment, and as was previously shown for morphine-3-glucuronide upon morphine treatment of Abcc2;Abcc3-/- mice (9).

Analysis of the plasma and urine of the Abcc2;Abcc3-/- mice (Figures 2 and 3C) showed that Abcc3 is necessary for the increased bilirubin glucuronide levels in blood and urine of Abcc2-/- mice. This indicates that Abcc3 functions as a back-up pathway for Abcc2: when toxic compounds in the liver cannot be excreted into the bile, Abcc3 transports them into the blood and the compounds are alternatively excreted via the urine. Surprisingly, in Abcc2;Abcc3-/- mice both biliary and urinary excretion of bilirubin are relatively low, raising the question how the bilirubin formed is handled in these mice. Abcc2;Abcc3-/- mice do not display obvious pathologies, suggesting that alternative bilirubin elimination pathways are involved. Bilirubin accumulating in the liver might for example be degraded by CYP1A enzymes (34), but also (possibly unknown) other mechanisms could play a role.

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

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

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

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

The severities of MTX-related toxicities in humans appear to correlate with MTX serum levels (12,16) and might, based on our results, be related to the expression levels and/or activity of ABCC2 and ABCC3. High-dose MTX therapy additionally leads to persistent renal dysfunction in nearly 30% of all patients (32). Renal failure is thought to be caused by crystalline deposits of MTX and especially 7OH-MTX (12). In rats, 7OH-MTX is much more toxic than MTX (38), and after high dose MTX treatment ($>50$ mg/kg) a significant amount of 7OH-MTX is
detected in patient urine (33). This suggests that MTX-related toxicity may correlate with 7OH-MTX plasma and tissue levels. Insight into in vivo mechanisms that affect plasma levels of 7OH-MTX is therefore important. Recently, it was shown in vitro that ABCG2 and ABCC2 can transport 7OH-MTX (17) and we now show in mice that hepatic Abcc2 and Abcc3 are involved in the excretion of 7OH-MTX from the liver by transporting it into bile and circulation, respectively. Especially the absence of Abcc2 affects plasma and kidney levels of 7OH-MTX. Abcc3 provides an alternative (sinusoidal) route of 7OH-MTX elimination from the liver when Abcc2 is absent and combined absence leads to substantial accumulation of 7OH-MTX in liver and, probably secondary to that, also in plasma. Abcc3 is not exclusively responsible for transport of 7OH-MTX into the circulation and subsequent urinary excretion. Especially at somewhat later time points (>30 min) after administration another mechanism appears to be relevant as well, as was seen in the Abcc2;Abcc3−/− mice. This mechanism becomes apparent when liver concentrations of 7OH-MTX are high (Figure 6B). A possible candidate for this would be Abcc4, which is expressed at the basolateral membrane of hepatocytes and the apical membrane in renal cells, and about 2-fold upregulated in liver of female Abcc2+/− and Abcc2;Abcc3−/− mice.

In Abcc2+/− and especially Abcc2;Abcc3−/− mice, the total amount of excreted 7OH-MTX (via urine and feces) is higher than in the other strains (Figure 6D). It could be that MTX accumulation in the liver of these strains causes more 7OH-MTX formation. Another possible explanation is increased liver expression of the enzyme aldehyde oxidase, which is responsible for 7OH-MTX formation (18-20). Indeed, preliminary microarray analysis showed that mRNA expression of aldehyde oxidase in Abcc2+/− and Abcc2;Abcc3−/− mice is 1.4-2.4-fold increased. The 7OH-MTX formed is subsequently (slowly) eliminated from the liver and body, leading to a high 7OH-MTX exposure in Abcc2-deficient mice. It would be interesting to know whether the increased expression of aldehyde oxidase is also seen in patients with ABCC2 mutations, as this could make them more vulnerable to 7OH-MTX-related toxicity.

In this study we administered an i.v. bolus injection of 50 mg/kg MTX to mice, which is comparable to a dose of 154 mg/m² in man (31). It cannot be excluded that the effects of Abcc2 and/or Abcc3 found in this study might differ when higher doses or different routes of administration (e.g. oral or constant infusion) are used. However, as associations between ABCC2 mutations and MTX-related toxicity in patients have been found previously after high-dose (3-5 g/m²) MTX infusions as well (13;15), it is likely that at least the impact of ABCC2 is similar even at very high doses and longer infusion times.

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

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

ACKNOWLEDGEMENTS
We thank our colleagues for critical reading of the manuscript, Rob Lodewijks, Enver Delic and Hans Tensen for excellent technical assistance, Martin van der Valk, Ji-Ying Song and Nadine Meertens for histological analysis, and George Scheffer and Bruno Stieger for kindly providing antibodies.

REFERENCES


(34) Rifkind AB. CYP1A in TCDD toxicity and in physiology – with particular reference to CYP dependent arachidonic acid metabolism and other endogenous substrates. Drug Metab Rev 2006;38:291-335.


**Supplementary Table 1S.** Levels of MTX in tissues and plasma of female wild-type, *Abcc2*/*, Abcc3*/* and *Abcc2;Abcc3*/* mice at different time-points after iv administration of 50 mg/kg MTX.

<table>
<thead>
<tr>
<th>Biological matrix</th>
<th>Time (min)</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild-type</td>
</tr>
<tr>
<td><strong>Plasma (µg/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>23.3 ± 2.7</td>
<td>36.2 ± 4.9***</td>
</tr>
<tr>
<td>15</td>
<td>9.1 ± 2.7</td>
<td>18.9 ± 6.4**</td>
</tr>
<tr>
<td>30</td>
<td>4.3 ± 0.7</td>
<td>11.9 ± 2.2**</td>
</tr>
<tr>
<td>60</td>
<td>1.4 ± 0.4</td>
<td>2.7 ± 0.4**</td>
</tr>
<tr>
<td>120</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td><strong>Liver (% of dose)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>49.5 ± 4.4</td>
<td>44.4 ± 4.9*</td>
</tr>
<tr>
<td>15</td>
<td>36.0 ± 1.3</td>
<td>28.1 ± 4.7**</td>
</tr>
<tr>
<td>30</td>
<td>16.4 ± 5.3</td>
<td>23.2 ± 3.7</td>
</tr>
<tr>
<td>60</td>
<td>4.8 ± 0.8</td>
<td>4.0 ± 1.9</td>
</tr>
<tr>
<td>120</td>
<td>1.0 ± 0.4</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td><strong>Liver/plasma concentration ratio</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>20.0 ± 2.3</td>
<td>9.6 ± 1.8***</td>
</tr>
<tr>
<td>15</td>
<td>36.8 ± 5.9</td>
<td>12.0 ± 3.3***</td>
</tr>
<tr>
<td>30</td>
<td>37.6 ± 13.5</td>
<td>22.5 ± 8.0</td>
</tr>
<tr>
<td>60</td>
<td>34.9 ± 9.4</td>
<td>11.5 ± 4.4***</td>
</tr>
<tr>
<td>120</td>
<td>42.1 ± 16.5</td>
<td>19.9 ± 5.4</td>
</tr>
<tr>
<td><strong>SI tissue + content (% of dose)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>5.1 ± 1.5</td>
<td>1.5 ± 0.4***</td>
</tr>
<tr>
<td>15</td>
<td>26.9 ± 2.8</td>
<td>10.6 ± 4.3***</td>
</tr>
<tr>
<td>30</td>
<td>39.5 ± 9.4</td>
<td>14.8 ± 1.3**</td>
</tr>
<tr>
<td>60</td>
<td>47.7 ± 5.3</td>
<td>24.5 ± 8.3***</td>
</tr>
<tr>
<td><strong>Kidney (% of dose)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>3.3 ± 1.6</td>
<td>5.3 ± 2.3</td>
</tr>
<tr>
<td>15</td>
<td>0.9 ± 0.3</td>
<td>2.6 ± 0.7**</td>
</tr>
<tr>
<td>30</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.2**</td>
</tr>
<tr>
<td>60</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.03**</td>
</tr>
</tbody>
</table>

Note: MTX tissue levels are expressed as percentage of the dose (means ± SD, n = 3-9) and MTX plasma levels are presented as µg/ml (means ± SD, n = 3-12). *P < 0.05, **P < 0.01, ***P < 0.001, compared to wild-type mice (Student’s t-test was used for statistical analysis).
**Supplementary Table 2S.** Levels of 7OH-MTX in tissues and plasma of female wild-type, *Abcc2<sup>-/-</sup>, *Abcc3<sup>-/-</sup> and *Abcc2;Abcc3<sup>-/-</sup> mice at different time-points after iv administration of 50 mg/kg MTX.

<table>
<thead>
<tr>
<th>Biological matrix</th>
<th>Time (min)</th>
<th>Wild-type</th>
<th>*Abcc2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>*Abcc3&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>*Abcc2;Abcc3&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma (µg/ml)</strong></td>
<td>7.5</td>
<td>0.23 ± 0.07</td>
<td>0.38 ± 0.16**</td>
<td>0.19 ± 0.03</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.25 ± 0.07</td>
<td>1.08 ± 0.38**</td>
<td>0.19 ± 0.03</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.28 ± 0.10</td>
<td>1.43 ± 0.37**</td>
<td>0.16 ± 0.04</td>
<td>0.50 ± 0.17**</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.10 ± 0.03</td>
<td>1.00 ± 0.11**</td>
<td>0.08 ± 0.04</td>
<td>0.82 ± 0.32**</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.05 ± 0.02</td>
<td>0.46 ± 0.06**</td>
<td>0.04 ± 0.01</td>
<td>0.68 ± 0.45**</td>
</tr>
<tr>
<td><strong>Liver (% of dose)</strong></td>
<td>7.5</td>
<td>4.4 ± 1.0</td>
<td>7.3 ± 1.9**</td>
<td>4.1 ± 1.3</td>
<td>9.1 ± 0.5**</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>4.5 ± 1.0</td>
<td>8.9 ± 0.7**</td>
<td>5.7 ± 0.8</td>
<td>8.9 ± 1.2**</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2.3 ± 0.9</td>
<td>6.2 ± 0.6**</td>
<td>2.2 ± 1.3</td>
<td>12.3 ± 4.1**</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>2.2 ± 0.2</td>
<td>4.8 ± 1.6**</td>
<td>1.6 ± 0.9</td>
<td>13.9 ± 6.2**</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>0.4 ± 0.1</td>
<td>1.8 ± 0.7**</td>
<td>0.3 ± 0.2</td>
<td>8.0 ± 1.6**</td>
</tr>
<tr>
<td><strong>SI tissue + content</strong></td>
<td>7.5</td>
<td>0.08 ± 0.02</td>
<td>0.02 ± 0.01**</td>
<td>0.06 ± 0.02</td>
<td>0.02 ± 0.01**</td>
</tr>
<tr>
<td>(% of dose)</td>
<td>15</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.1**</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.04**</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.6 ± 0.5</td>
<td>0.3 ± 0.1**</td>
<td>1.2 ± 0.9</td>
<td>0.8 ± 0.1**</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>3.0 ± 0.5</td>
<td>0.8 ± 0.2**</td>
<td>2.8 ± 0.8</td>
<td>1.8 ± 0.6**</td>
</tr>
<tr>
<td><strong>Kidney (% of dose)</strong></td>
<td>7.5</td>
<td>0.009 ± 0.003</td>
<td>0.03 ± 0.02**</td>
<td>0.007 ± 0.003</td>
<td>0.012 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.01 ± 0.002</td>
<td>0.11 ± 0.03**</td>
<td>0.02 ± 0.004</td>
<td>0.04 ± 0.01**</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.02 ± 0.005</td>
<td>0.09 ± 0.01**</td>
<td>0.02 ± 0.006</td>
<td>0.05 ± 0.01**</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.01 ± 0.008</td>
<td>0.11 ± 0.01**</td>
<td>0.01 ± 0.003</td>
<td>0.07 ± 0.01**</td>
</tr>
</tbody>
</table>

Note: 7OH-MTX tissue levels are expressed as percentage of the dose (means ± SD, n = 3-9) and 7OH-MTX plasma levels are presented as µg/ml (means ± SD, n = 3-12). *P < 0.05, **P < 0.01, ***P < 0.001, compared to wild-type mice (Student’s t-test was used for statistical analysis).
Supplementary Figure 1. Analysis of phospholipid output into bile of male wild-type, Abcc2\(^{-/-}\), Abcc3\(^{-/-}\) and Abcc2;Abcc3\(^{-/-}\) mice after gall bladder cannulation and ligation of the common bile duct. Bile was collected in 15 min fractions over 1 h. Bile collected in the first 15 min was analyzed for phospholipid concentration. A, biliary phospholipid output in the various mouse strains when corrected for body weight (BW). B, biliary phospholipid output in the various strains when corrected for liver weight. Data are means ± SD (\(n = 5\), *, P < 0.05, **, P < 0.01).