Dose-Dependent Disposition of Methotrexate in Abcc2 and Abcc3 gene knockout murine models

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Running title: Impact of Abcc2 and Abcc3 on MTX PK

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List of nonstandard abbreviations:
ABC, ATP-binding cassette; ABCC2/MRP2, ABC sub-family C member 2; ALL, acute lymphoblastic leukemia; ANOVA, analysis of variance; AUC, area under the concentration-time curve; BCRP/ABCG2, Breast Cancer Resistance Protein; BCS, Biopharmaceutics Classification System; BDDCS, Biopharmaceutics Drug Disposition Classification System; CL, total systemic clearance; cMOAT, canalicular multispecific organic anion transporter; CMV, canalicular membrane vesicles; f_f, fecal fraction of dose; f_u, renal fraction of dose; IV, intravenous; LC/MS/MS, liquid chromatography/tandem mass
spectrometry; MRP, multidrug resistance proteins; MTX, Methotrexate; 7OH-MTX, 7-hydroxymethotrexate; P-gp, P-glycoprotein; PK, pharmacokinetic; V_d, volume of distribution; WT, wild type
ABSTRACT

Methotrexate (MTX) is a substrate for numerous human ATP-binding cassette (ABC) efflux transporters, yet the impact of these transporters on MTX PK over a large dose range has not been examined. To investigate the effects of two transporters, Abcc2 (Mrp2) and Abcc3 (Mrp3), involved in MTX hepatobiliary disposition \textit{in vivo}, MTX plasma, urine and feces concentrations were analyzed after 10, 50, and 200 mg/kg intravenous (IV) doses to groups of wild type (WT), \textit{Abcc2}^{−/−} and \textit{Abcc3}^{−/−} mice. The absence of Abcc2 caused a decrease in total clearance of MTX relative to WT mice at all dose levels yet was accompanied by compensatory increases in renal excretion and metabolism to 7OH-MTX. In \textit{Abcc3}^{−/−} mice total clearance was elevated at the two lower dose levels that was attributed to stimulation of biliary excretion and confirmed by elevated fecal excretion; however at the high 200 mg/kg dose clearance was severely retarded and could be attributed to hepatotoxicity as conversion to 7OH-MTX was diminished. The findings confirmed that both Abcc2 and Abcc3 significantly influenced the PK properties of MTX, and depending on the MTX dose and strain alternate elimination pathways were elicited and saturable.
INTRODUCTION

Membrane transporters in the ABC superfamily are recognized as critical determinants of drug disposition (Fromm, 2003; Chan et al., 2004) that transport a variety of endogenous and exogenous compounds against concentration gradients at the expense of ATP hydrolysis (Borst and Elferink, 2002). Drugs that are substrates for such membrane transporters, especially those falling into Class II-IV of the FDA’s Biopharmaceutics Classification System (BCS) (Amidon et al., 1995) or the extended Biopharmaceutics Drug Disposition Classification System (BDDCS) (Wu and Benet, 2005), require accurate analysis of their complex PK behavior to properly assess their suitability as emerging drug candidates. The use of established in vitro permeability and in situ/ex vivo models, such as perfused organs models (Xia et al., 2007) provide useful yet isolated information as to the potential impact of transporters on drug disposition. Given that drugs may be substrates for multiple transporters of overlapping function (Vlaming et al., 2009a; Lagas et al., 2010a), whole-body animal models are required to provide a comprehensive analysis of the PKs of a drug. In this regard, the use of gene knockout animal models provide an opportunity to characterize not only how specific transporters impact drug disposition, but also may be used as a tool to examine how specific inhibitors influence drug disposition (Sikic et al., 1997).

MTX is a folate antagonist widely-used for the treatment of different diseases that require a large therapeutic dose range (Braun and Rau, 2009); low doses for autoimmune diseases, such as psoriasis (Hunter et al., 1962) and rheumatoid arthritis (Weinblatt et al., 1985), and high doses for different types of cancers, such as primary CNS lymphoma (Deangelis and Iwamoto, 2006), acute lymphoblastic leukemia (ALL) (Chessells et al., 1987), and other neoplastic diseases (Rizzoli et al., 1985). Based on a number of in vitro experiments, MTX is a known substrate for numerous membrane transporters including the multidrug resistance proteins (MRP) ABC sub-family C member 2 (ABCC2/MRP2) and ABCC3/MRP3 (Assaraf, 2006). These two transporters have a role in the hepatobiliary axis, with ABCC2 located on the apical side of bile canaliculi whereas ABCC3 is located on the basolateral surface of hepatocytes (Gerk and Vore, 2002; Scheffer et al., 2002). An early report revealed limited MTX biliary
excretion in rats lacking Abcc2 (cMOAT, canalicular multispecific organic anion transporter), which was consistent with in vitro studies on canalicular membrane vesicles (CMV) prepared from normal and Abcc2 deficient rats (Masuda et al., 1997). Recent in vivo studies that utilized single (Abcc2+/−, Abcc3+/− and Abcg2+/−), double (Abcc2;Abcc3+/−, Abcc2;Abcg2+/− and Abcc3;Abcg2+/−), and triple (Abcc2;Abcc3;Abcg2+/−) knockout mouse models in conjunction with 50 mg/kg intravenous administrations of MTX established the importance of Abcc2 and Abcc3 in the disposition of MTX and its major metabolite, 7-hydroxymethotrexate (7OH-MTX) (Kitamura et al., 2008; Vlaming et al., 2008, 2009 a&b). Although these investigations were instrumental in implicating the function of relevant transporters the use of a single dose level prevented an understanding of the complex behavior associated with saturable kinetics and compensatory elimination pathways that are revealed following dose-dependent investigations. The analysis of MTX PKs over a large dose range is pertinent to the wide dose range used in patients, and could add insight to interindividual PK variability. Based on these considerations, the current project was undertaken to elucidate the impact of Abcc2 and Abcc3 on the PKs of MTX by utilizing different dose levels in normal mice, Abcc2+/− and Abcc3+/− mouse strains.
MATERIAL AND METHODS

Animal

Three strains of male mice, C57BL/6 (wild type, WT), Abcc2\(^{-/-}\) and Abcc3\(^{-/-}\) mice (20 – 30 g, age 10 – 15 wk) were used in the study after confirmation of their genotype by regular polymerase chain reaction. Generation of the Abcc2\(^{-/-}\) or Abcc3\(^{-/-}\) mice was described elsewhere (Belinsky et al, 2005). All the animals were maintained on an alternating 12 hr light/dark cycle with free access to water and rodent chow. The Institutional Animal Care and Use Committee at Temple University approved all animal procedures.

Pharmacokinetic studies of MTX

One day before the administration of MTX, under anesthesia [i.p. dose (0.01 mL/10g body weight) of a 3:2:1 (v/v/v) mixture of ketamine hydrochloride (100 mg/mL), acepromazine maleate (10 mg/mL) and xylazine hydrochloride (20 mg/mL)] mice had an indwelling cannula placed in the right carotid artery for serial blood sampling. Pharmacokinetic experiments were conducted the next day on conscious freely-mobile mice placed in metabolic cages (Nalgene®, Braintree Scientific Inc., Braintree, MA) that allowed for the separate collection of urine and feces. MTX dissolved in saline adjusted to a pH of 7.0 was administered to groups of mice at doses of 10, 50, and 200 mg/kg as an IV bolus via a tail vein. Serial (n = 10 to 14) blood samples of 20 µL were collected for up to 8 hours at low and medium doses and approximately for 2 days at the high dose level, centrifuged with the resultant plasma stored at -80 \(^\circ\)C until analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS). To avoid blood volume depletion 10 µL of saline was replaced after each blood collection. Urine and feces of each animal were collected for 24 hours at low and medium doses and for 49 hours at the high 200 mg/kg dose, and then stored at -80 \(^\circ\)C until analysis.
LC/MS/MS assay for MTX and 7OH-MTX

MTX and its major metabolite, 7OH-MTX, in plasma were measured using an electrospray ionization LC/MS/MS system (Applied Biosystems, API 4000) described previously (Guo et al., 2007). In brief, each plasma sample (10 µL) was deproteinized by a 4-fold volume of methanol containing the internal standard aminopterin (3 ng/ml). After centrifugation (14,000 rpm × 15 min), a 20 µL aliquot of the supernatant was diluted 6 times with double distilled water and mixed followed by injection of a 30 µL aliquot into the LC/MS/MS system. The chromatographic system consisted of a C18 guard cartridge (4.0 × 2.0 mm, Phenomenex, Torrance, CA) and analytical column (50 × 2.0 mm, 3 µm particle size, Phenomenex) set at an operation temperature of 35 °C, in which an isocratic mobile phase of acetonitrile/1mM ammonium formate containing 0.1% formic acid (18:82, v/v) was pumped at a flow rate of 0.2 mL/min. The column effluent was monitored in positive ion scan mode at the following transitions: MTX m/z 455.4 → 308.0, 7OH-MTX m/z 471.0 → 324.2, and aminopterin 441.1 → 294.3 with a dwell time of 800 ms for each ion transition. The limit of quantitation was 1.3 ng/mL and 2.6 ng/mL for MTX and 7OH-MTX, respectively. Both intra- and inter-day precision were all less than 15% for plasma concentrations over a range of 1.3 – 1021.6 ng/mL for MTX and 2.6 – 2062.9 ng/mL for 7OH-MTX.

MTX concentrations in urine and feces were quantitated using the same LC/MS/MS system as for plasma. In brief, each clean urine sample obtained by centrifugation was diluted 500 times with double distilled water followed by transfer of an aliquot (10 µL) that was deproteinized by 4-fold volume of methanol containing the internal standard aminopterin (1 µg/mL). After centrifugation (14,000 rpm × 5 min), an aliquot of 10 µL of supernatant was further diluted 100 times with the mobile phase and a 5 µL aliquot was injected into the LC/MS/MS system. Each sample of feces was first homogenized in a 5% (w/v) solution of double distilled water. A 20 µL aliquot of the resultant homogenate was deproteinized with 180 µL of methanol containing the internal standard aminopterin (2 µg/mL). After centrifugation (14,000 rpm × 5 min), an aliquot of 2 µL of supernatant was further diluted 500 times with double distilled water and then mixed with a 5 µL aliquot injected into the LC/MS/MS system. The limits of quantitation were 2.74 and 2.31 µg/mL of MTX for urine and feces concentrations, respectively. Both
intra- and inter-day precision were all less than 15% over a range of 2.744 – 2000 µg/mL for urine concentrations and of 2.31 – 300 µg/mL for feces concentrations of MTX.

Data Analysis

Each individual mouse MTX plasma concentrations were analyzed by noncompartmental methods with WinNonlin Professional Version 5.2 (Pharsight Co, Mountain View, CA) to obtain estimates of area under the MTX plasma concentration-time curve (AUC) and terminal disposition rate constant that allowed calculation of the total systemic clearance (CL), volume of distribution (V_d) and the terminal elimination half-life. The analyzed MTX concentrations in urine and feces samples of each individual mouse were converted to total amounts eliminated and then used to calculate the renal and fecal fraction of the dose (f_u and f_f) eliminated. Comparisons between knockout and WT mice were conducted based on the mean and standard deviation values from two independent groups using unpaired t tests in which a P value of less than 0.05 was considered statistically significant. To compare PK parameters within each strain as a function of dose, one-way analysis of variance (ANOVA) were conducted with significant differences based upon P values of less than 0.05.
RESULTS

The PK properties of MTX were assessed based on its measurement in plasma, urine and feces at IV doses of 10 mg/kg, 50 mg/kg and 200 mg/kg in WT, Abcc2<sup>−/−</sup> and Abcc3<sup>−/−</sup> mouse strains. MTX plasma concentration-time profiles for each strain and dose level are illustrated in Figure 1, in which a number of differences are apparent. In general, plasma MTX concentrations were highest in the Abcc2<sup>−/−</sup> strain followed by the WT strain and then the Abcc3<sup>−/−</sup> strain but the magnitude of the differences was also a function of dose. Regardless of the dose and strain, renal excretion of unchanged MTX is the primary elimination pathway, always greater than biliary or metabolic. Humans also utilize renal excretion as the primary route of unchanged MTX elimination and support the use of mice as a relevant animal model for MTX's PK behavior (Balas et al., 1983). The PK parameters are summarized in Table 1.

Interstrain analysis

Interstrain analysis indicated a number of differences in the PK parameters. Relative to WT mice, Abcc2<sup>−/−</sup> mice exhibited significantly higher AUC (P < 0.01) and reduced CL (P < 0.05 for low and high doses; P < 0.01 for middle dose) values at all dose levels that are attributed to the inability of Abcc2, located on the apical membrane of bile canaliculi as well as renal proximal tubules to perform its normal excretory function. At the 50 mg/kg dose level, the mean AUC value in Abcc2<sup>−/−</sup> mice doubled relative to WT mice, which was comparable to an earlier report that had used a different genetic background of FVB mice (Vlaming et al., 2008). The loss of Abcc2 function, as evidenced by reduced fecal excretion of MTX, coupled to the efflux action of Abcc3 on hepatocytes led to elevated MTX plasma concentrations. These elevated plasma concentrations resulted in a significant compensatory increase in the fractional renal excretion of MTX at all dose levels (P < 0.01 at low and middle doses), which serves as the primary elimination pathway regardless of strain. Given that 7OH-MTX is also a substrate for Abcc2 (Vlaming et al., 2008), its elevated AUC and fractional conversion to MTX (see % AUC<sub>mip</sub>, Table 1), being 3-fold to 8-fold compared to the WT strain over the 10 mg/kg to 200 mg/kg dose range, could reflect both reduced
clearance as well as a compensatory mechanism of increased metabolism of MTX to 7OH-MTX. The latter may be attributed to the accumulation or stasis of MTX in hepatocytes even in the presence of Abcc3, and the potential of increased aldehyde oxidase enzyme expression, the enzyme responsible for MTX to 7OH-MTX conversion (Vlaming et al., 2009b). Even though compensatory clearance mechanisms of MTX in the Abcc2−/− strain were apparent they were insufficient to match the clearance rates in WT mice.

The changes in the Vd between WT and Abcc2−/− strains were difficult to decipher. At the two lower dose levels, mean Vd values were less than in the WT strain and suggested that the higher plasma MTX concentrations are unable to saturate plasma protein binding, mainly albumin (Steele et al., 1979), as well as any apically-directed vascular pumps that limit extravascular distribution. However, at a dose of 200 mg/kg, the significantly elevated Vd (P < 0.05) in Abcc2−/− mice relative to the WT group appears to be sufficient to saturate such efflux pumps and permit MTX to distribute into tissues.

Comparison of the PK parameters in the Abcc3−/− strain to those in the WT strain revealed a number of significant differences. Total clearances were significantly elevated in the Abcc3−/− strain compared to the WT strain at the 10 mg/kg and 50 mg/kg dose levels (P < 0.01 at low dose; P < 0.05 at middle dose), but at the high 200 mg/kg dose level CL was decreased to less than that obtained in the high dose WT group (P < 0.01, see Table 1). Abcc3, being a basolateral efflux pump on hepatocytes, has a protective function for hepatocytes apparent when normal biliary excretion mechanisms, such as those for Abcc2−/− strain, are absent or compromised due to genetic manipulation, disease or saturated at high MTX doses. The enhanced elimination of MTX in the absence of Abcc3 supports a compensatory process of biliary excretion at least until these apical pumps are overwhelmed at the high 200 mg/kg dose level. This enhanced biliary elimination may be attributed to increased expression of the biliary pumps and is coupled to increased fecal excretion noted at the 50 mg/kg dose level that was also associated with reduced formation of 7OH-MTX compared to the wild-type mice (see Table 1). The reduced formation of 7OH-MTX is also consistent with hastened biliary excretion of this metabolite as it is also an Abcc2 substrate (Vlaming et al., 2008). The 200 mg/kg MTX dose in the Abcc3−/− strain resulted in a lower total
clearance and correspondingly an elevated plasma AUC relative to that in the WT group, which could be attributed to the saturation of the bile excretory pumps. However, in conjunction with saturable biliary excretion the absence of Abcc3 may likely have caused hepatocyte toxicity, as a compensatory enhancement of enzymatic conversion to 7OH-MTX was not observed. Without the normal compensatory protective function of Abcc3, hepatocyte MTX concentrations would be expected to be quite high.

The increase in the Vd in the Abcc3−/− mice at the two lower doses compared to the WT strain could be attributable to the absence of Abcc3 on the basolateral membranes of epithelial cells allowing enhanced distribution to liver, kidney, intestine, adrenals and pancreas (Borst et al., 2007). However, at the 200 mg/kg dose level, no further change in the Vd was seen in the Abcc3−/− group.

**Intrastrain analysis**

Intrastrain analysis revealed dose-dependent changes in the PK variables in each strain over the dose range of 10 – 200 mg/kg (see Table 1, Figure 2). WT mice exhibited an approximate 2-fold increase in CL (P < 0.05) in going from the 10 mg/kg to 50 mg/kg dose level that is indicative of Michaelis-Menten kinetics are operative in biliary elimination and supported by increased fecal excretion. The activity of these low affinity-high capacity pumps plateaued at the 200 mg/kg dose as there was no change in clearance or fecal excretion from the 50 mg/kg dose level. Interestingly there was not enhanced conversion of MTX to 7OH-MTX in the WT strain, and at the high 200 mg/kg dose only renal excretion appeared as a compensatory mechanism. Dose-dependent elevation in Vd (P < 0.05) indicative of enhanced tissue distribution in the WT group at the 200 mg/kg dose level is consistent with saturation of the capacity of efflux pumps located at the blood-tissue interface that would be more likely when expression of such pumps is low.

The Abcc2−/− strain also demonstrated elevated total clearance as the dose increased from 10 mg/kg to 50 mg/kg; however the elevated biliary excretion was also supplemented by compensatory metabolism to 7OH-MTX, although the latter were insignificant (P > 0.05) amongst the three dose levels.
Dose-dependent elevations in $V_d$ ($P < 0.05$) at the 200 mg/kg dose level is consistent with saturation of the capacity of efflux pumps located at the blood-tissue interface that results in greater tissue distribution.

Finally for the $Abcc3^{+/−}$ group, a 2-fold increase in total clearance was observed in going from 10 to 50 mg/kg doses that was attributed to engagement of compensatory bile efflux pumps as there was not an increase in renal excretion or 7OH-MTX conversion; however the precipitous fall in CL at the 200 mg/kg dose, without compensatory increases in metabolism or renal excretion, is suggestive of hepatotoxicity coupled to the inability of Abcc3 to perform its normal protective function in hepatocytes. There was an absence of dose-dependent changes in $V_d$ in the $Abcc3^{+/−}$ group.
DISCUSSION

The investigation of MTX PK characteristics is of interest due to the discovery that several membrane transporters are involved in its disposition that affects not only its systemic PK properties but also pharmacologically active concentrations at the cell level that are not measured in whole animals. Extensive studies using various in vitro and in vivo models have been carried out to determine the impact of transporters on the pharmacokinetics of MTX, which are considered important mechanisms contributing to the wide interindividual variability in patients (Xia et al., 2005; Vlaming et al., 2008, 2009a&b). Our studies confirm previous reports that Abcc2 and Abcc3 play an important role in the PKs of MTX and that alternative elimination pathways are available in the absence of either Abcc2 or Abcc3 (Vlaming et al., 2008; van de Wetering et al., 2007; Lagas et al., 2010 a&b). Our studies extend these past analyses in that we completed dose-dependent MTX studies in each strain and found that depending on the strain compensatory elimination mechanisms of MTX were elicited as well as saturable PK processes. In essence, the studies demonstrate that ABC transporters with shared substrate specificity are capable to export MTX coordinately and further that hepatic metabolism can be altered in order to maintain the organism’s homeostasis, albeit with limitations.

The multiple ABC transporters that accept MTX as a substrate are categorized as low affinity high capacity transporters. Uptake experiments with membrane vesicles have demonstrated that P-glycoprotein (P-gp), MRP1-5 and Breast Cancer Resistance Protein (BCRP/ABCG2) are low affinity MTX transporters with an average $K_m$ in the millimolar range with moderate-to-high capacity with an average $V_{max}$ ranging from 0.2 ~ 2.9 nmol/min/mg protein (Assaraf., 2006). When transporters of overlapping function exist it would be expected that high affinity transporters would preferentially contribute to transport and as substrate concentrations are elevated lower affinity transporters would be coordinately engaged and contribute to the overall transport rate. In the current investigation, regardless of the strain, an increase in the MTX dose from 10 mg/kg to 50 mg/kg caused an increase in total clearance that is consistent with Michaelis-Menten kinetics, and when one considers interstrain differences also suggests altered transporter capacity most likely through increased protein expression.
Depending on the background strain (FVB vs. C57BL/6) in Abcc2\textsuperscript{−/−} mice, Abcc3 and Abcc4 expression in kidney and liver ranged from no different than WT to 2-fold elevations (Chu et al., 2006; Vlaming et al., 2006). Similar expression data is not available in the Abcc3\textsuperscript{−/−} strain, but given that this group showed the greatest compensatory increase in clearance at the 50 mg/kg dose level it suggests altered transporter expression underlies the higher elimination capacity.

The increased MTX clearance observed at the 50 mg/kg dose is accompanied by increased fecal excretion that implicates Abcg2 as a biliary excretory pump. The recent studies by Vlaming et al. (2008, 2009a) in both single and double (Abcc2;Abcg2\textsuperscript{−/−}) knockout mice have demonstrated the key role Abcg2 plays in MTX’s biliary clearance. We observed additional compensatory elimination methods for MTX in the Abcc2\textsuperscript{−/−} strain with elevated renal excretion and intrahepatic conversion to 7OH-MTX. Of certain interest, the absence of Abcc3 was not accompanied by increased renal excretion or metabolism at the 50 mg/kg dose, but rather elevated biliary excretion. Given that the clearance of MTX was highest at this dose level in the Abcc3\textsuperscript{−/−} group, other compensatory pathways were unnecessary.

Although there were concordant increases in MTX’s CL in going from 10 mg/kg to 50 mg/kg amongst the three mouse strains, there was either a reversal or no change at the high 200 mg/kg MTX dose depending on the strain. At this high dose level in the Abcc3\textsuperscript{−/−} strain active transport of MTX into bile was saturated and accompanied by a significant, yet modest, increase in renal excretion and clearly insufficient to achieve the clearance rates obtained at the lower two dose levels. The Abcc3\textsuperscript{−/−} strain distinguishes itself at the high dose in the inability to increase the formation of 7OH-MTX as a compensatory mechanism as observed in the Abcc2\textsuperscript{−/−} group. The wild-type strain had no need to increase metabolic conversion of MTX as all biliary transporters were functioning, albeit at saturation, and further due to the function of Abcc3 high hepatocyte concentrations of MTX could be mitigated by efflux into plasma and be available for renal elimination. Without an adequate efflux capacity into plasma for the Abcc3\textsuperscript{−/−} strain it seems likely that high MTX hepatocyte concentrations were lethal to microsomal enzymes. The inability to convert MTX to 7OH-MTX in the Abcc3\textsuperscript{−/−} strain is quite different than what
was observed in the *Abcc2*<sup>-/-</sup> strain where substantial conversion to 7OH-MTX was seen (~20-fold increase in the AUC<sub>7OH-MTX</sub> compared to *Abcc3*<sup>-/-</sup> strain).

The 7OH-MTX metabolite is a main toxic metabolite of MTX, and in fact, in rats, it is reported to be more toxic than the parent drug at doses up to 5 g/kg for MTX (Fuskevåg et al., 2000). Moreover, MTX-induced renal dysfunction has been associated with the precipitation of both MTX and the less soluble metabolite 7OH-MTX in acidic urine (Sand and Jacobsen, 1981; Pratt et al., 1994). Our observation of increased hepatic formation of 7OH-MTX and urinary excretion of MTX in *Abcc2*<sup>-/-</sup> mice suggests that patients with low ABCC2 activity may have increased risk of MTX-induced hepatic and renal toxicity. In fact, there are a number of clinical studies that have identified polymorphisms in ABCC2 in different patient populations that led to increased MTX toxicity particularly hepatotoxicity and nephrotoxicity (Ranganthan et al., 2008; Hulot et al., 2005).

In summary, our study revealed the complexity of MTX disposition due to the presence of multiple transporters with overlapping functions that lead to compensatory elimination pathways in bile and urine as well as in hepatocytes (see Scheme 1). The central role of Abcc2 as a determinant of biliary excretion is confirmed as is the pivotal role Abcc3 plays in hepatic protection. It was also noted that high MTX doses may surpass the body’s ability to eliminate MTX and cause toxicity. The compensatory and saturable elimination mechanisms of MTX observed over a large dose range may have implications to patients through the design of individualized therapy and monitoring of renal and hepatic function to avoid toxicity.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Wang, Kruh, and Gallo.

Conducted experiments: Wang.

Contributed new reagents or analytic tools: Kruh.

Performed data analysis: Wang.

Wrote or contributed to the writing of the manuscript: Wang, Zhou, and Gallo.
REFERENCES


FOOTNOTES

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LEGENDS FOR SCHEMES

Scheme 1.

Proposed action of key ABC transporters involved in the pharmacokinetics of MTX in liver hepatocytes and renal proximal tubules as a function of dose in WT (A), Abcc2<sup>−/−</sup> (B) and Abcc3<sup>−/−</sup> (C) strains. For each strain three states of transporter function are shown that correspond to our low, medium and high dose MTX groups. Non-black colored transporters are operating below saturation whereas black colored indicates saturation. It is understood that Abcc2, Abcc3 and Abcg2 operate in a similar manner (i.e. apical or basolateral directions) in enterocytes as hepatocytes, and the apical transporters could contribute to fecal elimination of MTX. Other transporters may be involved, such as Abcc4, and are not shown, but the current illustration is most consistent with our data and others highlighting the key transporters (Vlaming et al., 2006, 2008, 2009a&amp;b). The extent of conversion of MTX to 7OH-MTX in hepatocytes is indicated by the thickness of the arrow.
LEGENDS FOR FIGURES

Figure 1.
Plasma concentrations \( \left( C_p, \text{ mean } \pm \text{ SD; semi-log scale} \right) \) of MTX as a function of time after IV bolus administration of single doses of MTX at 10 (A), 50 (B) and 200 (C) mg/kg to male WT (diamond), \( Abcc2^{-/-} \) (square) or \( Abcc3^{-/-} \) (triangle) mice.

Figure 2.
(A) Total clearance (CL) and (B) volume of distribution \( \left( V_d \right) \) (mean \( \pm \) SD) of MTX as a function of dose to different mouse strains.

Figure 3.
Renal (A) and fecal excretion (B) of MTX (expressed as \% of dose) in WT, \( Abcc2^{-/-} \) and \( Abcc3^{-/-} \) mice at three different dose levels.
Table 1. Pharmacokinetic parameters (mean ± SD) of MTX following IV administration of MTX at three dose levels for WT, *Abcc2−/−* and *Abcc3−/−* mice.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT mice</th>
<th>Dose = 10 mg/kg</th>
<th>Dose = 50 mg/kg</th>
<th>Dose = 200 mg/kg</th>
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<td>n = 8</td>
<td>n = 10</td>
<td>n = 11</td>
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<tr>
<td>CL (mL/min/kg)</td>
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<td>68.1 ± 31.1   †</td>
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<td>Vd (L/kg)</td>
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<td>t\textsubscript{1/2} (min)</td>
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<td>211.7 ± 97.6</td>
<td>730.2 ± 101.0‡</td>
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<td>AUC\textsubscript{MTX} (min*µg/mL)</td>
<td>308.4 ± 79.0</td>
<td>842.9 ± 298.2</td>
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<tr>
<td>AUC\textsubscript{7OH-MTX} (min*µg/mL)</td>
<td>13.1 ± 5.1</td>
<td>23.2 ± 12.1</td>
<td>96.1 ± 43.9</td>
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<td>AUC\textsubscript{m/p} (%)</td>
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<td>2.99 ± 1.51</td>
<td>2.04 ± 0.73†</td>
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<td>Renal fraction of dose (%)</td>
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<td>43.4 ± 2.95</td>
<td>59.7 ± 6.94‡</td>
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<td>Fecal fraction of dose (%)</td>
<td>1.10 ± 0.72†</td>
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<td>8.55 ± 5.86</td>
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<table>
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<th>Abcc2−/− mice</th>
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<th>n = 14</th>
<th>n = 7</th>
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<tbody>
<tr>
<td>CL (mL/min/kg)</td>
<td>23.0 ± 5.7*‡</td>
<td>35.1 ± 12.4**</td>
<td>35.3 ± 5.4*</td>
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<td>Vd (L/kg)</td>
<td>4.37 ± 1.49**</td>
<td>8.01 ± 3.07**</td>
<td>95.8 ± 34.7**‡</td>
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<td>t\textsubscript{1/2} (min)</td>
<td>131.7 ± 37.4**</td>
<td>166.2 ± 70.7</td>
<td>1928.8 ± 738.2**‡</td>
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<td>AUC\textsubscript{MTX} (min*µg/mL)</td>
<td>412.5 ± 62.7**</td>
<td>1572.5 ± 520.3**</td>
<td>5607.1 ± 686.0**</td>
</tr>
<tr>
<td>AUC\textsubscript{7OH-MTX} (min*µg/mL)</td>
<td>50.9 ± 15.7**</td>
<td>227.3 ± 94.1**</td>
<td>955.1 ± 202.4**</td>
</tr>
<tr>
<td>AUC\textsubscript{m/p} (%)</td>
<td>11.3 ± 3.5**</td>
<td>15.8 ± 6.2**</td>
<td>17.0 ± 2.6**</td>
</tr>
<tr>
<td>Renal fraction of dose (%)</td>
<td>101.1 ± 9.87**‡</td>
<td>79.8 ± 19.8**</td>
<td>76.8 ± 12.2</td>
</tr>
<tr>
<td>Fecal fraction of dose (%)</td>
<td>0.70 ± 0.49†</td>
<td>6.08 ± 3.34‡</td>
<td>7.84 ± 4.14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Abcc3−/− mice</th>
<th>n = 7</th>
<th>n = 8</th>
<th>n = 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL (mL/min/kg)</td>
<td>55.2 ± 8.3**†</td>
<td>100.0 ± 22.8**</td>
<td>32.6 ± 2.5**‡</td>
</tr>
<tr>
<td>Vd (L/kg)</td>
<td>21.4 ± 8.6**</td>
<td>35.4 ± 14.0†</td>
<td>30.3 ± 7.5*</td>
</tr>
<tr>
<td>t\textsubscript{1/2} (min)</td>
<td>264.8 ± 88.1</td>
<td>269.9 ± 150.7</td>
<td>646.5 ± 166.0†</td>
</tr>
<tr>
<td>AUC\textsubscript{MTX} (min*µg/mL)</td>
<td>180.8 ± 31.8**</td>
<td>516.7 ± 11.4*</td>
<td>6153.8 ± 472.5**</td>
</tr>
<tr>
<td>AUC\textsubscript{7OH-MTX} (min*µg/mL)</td>
<td>3.71 ± 0.83**</td>
<td>2.57 ± 1.11**</td>
<td>43.0 ± 10.4*</td>
</tr>
<tr>
<td>AUC\textsubscript{m/p} (%)</td>
<td>2.12 ± 0.61**‡</td>
<td>0.49 ± 0.18**</td>
<td>0.70 ± 0.14**</td>
</tr>
<tr>
<td>Renal fraction of dose (%)</td>
<td>33.3 ± 12.6</td>
<td>34.1 ± 3.3**</td>
<td>46.0 ± 4.39**‡</td>
</tr>
<tr>
<td>Fecal fraction of dose (%)</td>
<td>5.88 ± 4.89**†</td>
<td>24.9 ± 9.9**‡</td>
<td>16.0 ± 9.1</td>
</tr>
</tbody>
</table>

* † significant difference between WT and knockout strains at that dose level (* P < 0.05, † P < 0.01).
** ‡ significant difference among dose levels within the same strain (** P < 0.01), with * † indicating differences between designated dose levels, and **‡ indicating differences from the other two dose levels.
Scheme 1

(A) Proximal Tubule Cells

Blood

MTX

Bile

Hepatocytes

7OH-MTX

Abcc2

Abcc3

Abcg2

Urine

(B) Proximal Tubule Cells

Blood

MTX

Bile

Hepatocytes

7OH-MTX

Abcc2

Abcc3

Abcg2

Urine

(C) Proximal Tubule Cells

Blood

MTX

Bile

Hepatocytes

7OH-MTX

Abcc2

Abcc3

Abcg2

Urine

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Figure 2

A) 

CL, mL/min/kg

Dose, mg/kg

WT

Abcc2^-/-

Abcc3^-/-

B) 

V_d, L/kg

Dose, mg/kg

WT

Abcc2^-/-

Abcc3^-/-
Figure 3

A) MTX Renal Excretion (% of Dose)

- WT
- Abcc2<sup>−/−</sup>
- Abcc3<sup>−/−</sup>

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>WT</th>
<th>Abcc2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Abcc3&lt;sup&gt;−/−&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>10</td>
<td></td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>200</td>
<td></td>
<td>50</td>
<td>25</td>
</tr>
</tbody>
</table>

B) MTX Fecal Excretion (% of Dose)

- WT
- Abcc2<sup>−/−</sup>
- Abcc3<sup>−/−</sup>

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>WT</th>
<th>Abcc2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Abcc3&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td></td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>200</td>
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<td>20</td>
<td>10</td>
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</tbody>
</table>