0090-9556/11/3911-2155–2161$25.00
DRUG METABOLISM AND DISPOSITION
DMD 39:2155–2161, 2011

Vol. 39, No. 11
41228/3724841
Printed in U.S.A.

Dose-Dependent Disposition of Methotrexate in *Abcc2* and *Abcc3* Gene Knockout Murine Models

Zhan Wang, Qingyu Zhou, Gary D. Kruh, and James M. Gallo

Department of Pharmaceutical Sciences, Temple University, Philadelphia, Pennsylvania (Z.W.); Mount Sinai School of Medicine, New York, New York (Q.Z., J.M.G.); and Department of Medicine and Cancer Center, University of Illinois at Chicago, Chicago, Illinois (G.D.K.)

Received June 16, 2011; accepted August 8, 2011

ABSTRACT:

Methotrexate (MTX) is a substrate for numerous human ATP-binding cassette (ABC) efflux transporters, yet the impact of these transporters on MTX pharmacokinetics (PK) over a large dose range has not been examined. To investigate the effects of two transporters—ABC subfamily C member 2 (Abcc2; multidrug resistance protein 2) and ABC subfamily C member 3 (Abcc3; multidrug resistance protein 3)—involved in MTX hepatobiliary disposition in vivo, MTX plasma, urine, and feces concentrations were analyzed after 10, 50, and 200 mg/kg i.v. doses to groups of wild type (WT), *Abcc2*(−/−), and *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 7-hydroxymethotrexate (7OH-MTX). In *Abcc3*(−/−) mice, total clearance was elevated at the two lower dose levels and 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 because 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 ATP-binding cassette (ABC) superfamily are recognized as critical determinants of drug disposition (Fromm, 2003; Chan et al., 2004) that transport various 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 through IV of the U.S. Food and Drug Administration's Biopharmaceutics Classification System (Amidon et al., 1995) or the extended Biopharmaceutics Drug Disposition Classification System (Wu and Benet, 2005), require accurate analysis of their complex pharmacokinetic (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 organ models (Xia et al., 2007), provides 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 provides an opportunity to characterize not only how specific transporters impact drug disposition, but they also may be used as a tool to examine how specific inhibitors influence drug disposition (Sikic et al., 1997).

Methotrexate (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, 1962) and rheumatoid arthritis (Weinblatt et al., 1985) and high doses for different types of cancers, such as primary central nervous system lymphoma (Deangelis and Iwamoto, 2006), acute lymphoblastic leukemia (Chessells et al., 1987), and other neoplastic diseases (Rizzoli et al., 1985). On the basis of several in vitro experiments, MTX is a known substrate for numerous membrane transporters, including the multidrug resistance proteins (MRP) ABC subfamily C member 2 (ABCC2/MRP2) and member 3 (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 (canalicular multispecific organic anion transporter), which was consistent with in vitro studies on canalicular membrane vesicles prepared from normal and Abcc2-deficient rats (Masuda et al., 1997). Recent in vivo studies that used single [*Abcc2*(−/−), *Abcc3*(−/−), and *Abcg2*(−/−)], double [*Abcc2;Abcc3*(−/−), *Abcc2;Abcg2*(−/−), and *Abcc3;Abcg2*(−/−)], and triple [*Abcc2;Abcc3;Abcg2*(−/−)] knockout

This work was supported by the National Institutes of Health National Cancer Institute [Grant CA114574].

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.111.041228.

ABBREVIATIONS: ABC, ATP-binding cassette; ABCC2, ABC subfamily C member 2; ABCC3, ABC subfamily C member 3; ANOVA, analysis of variance; AUC, area under the concentration-time curve; CL, total systemic clearance; 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; PK, pharmacokinetic; V_d, volume of distribution; WT, wild type.

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, 2009a,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 after 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. On the basis of these considerations, the current project was undertaken to elucidate the impact of Abcc2 and Abcc3 on the PKs of MTX by using different dose levels in normal mice and *Abcc2*(−/−) and *Abcc3*(−/−) mouse strains.

Materials and Methods

Animals. Three strains of male mice—C57BL/6 (wild type [WT]), *Abcc2*(−/−), and *Abcc3*(−/−) mice (20–30 g, age 10–15 weeks) 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, 2005). All of the animals were maintained on an alternating 12-h 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, while under anesthesia (intraperitoneal dose [0.01 ml/10 g 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 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 = 10–14) blood samples of 20 μl were collected for up to 8 h at low and medium doses and approximately for 2 days at the high dose level. The samples were then centrifuged, and the resultant plasma was stored at −80°C until analysis 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 h at low and medium doses and for 49 h at the high 200 mg/kg dose and then stored at −80°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 (API 4000; Applied Biosystems, Foster City, CA) 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/1 mM 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 m/z 441.1 → 294.3 with a dwell time of 800 ms for each ion transition. The limit of quantitation was 1.3 and 2.6 ng/ml for MTX and 7OH-MTX, respectively. Intra- and interday precisions were all less than 15% for plasma concentrations over a range of 1.3 to 1021.6 ng/ml for MTX and 2.6 to 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 a 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. Intra- and interday precisions were all less than 15% over a range of 2.744 to 2000 μg/ml for urine concentrations and 2.31 to 300 μg/ml for feces concentrations of MTX.

Data Analysis. MTX plasma concentrations of each individual mouse were analyzed by noncompartmental methods with WinNonlin Professional Version 5.2 (Pharsight, Mountain View, CA) to obtain estimates of the area under the MTX plasma concentration-time curve (AUC) and the terminal disposition rate constant that allowed for 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 the total amounts eliminated and were then used to calculate the renal and fecal fraction of the dose (f_u and f_f, respectively) eliminated. Comparisons between knockout and WT mice were conducted based on the mean and S.D. 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) was conducted with significant differences based on 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, 50, and 200 mg/kg in WT, *Abcc2*(−/−), and *Abcc3*(−/−) mouse strains. MTX plasma concentration-time profiles for each strain and dose level are illustrated in Fig. 1, in which several differences are apparent. In general, plasma MTX concentrations were highest in the *Abcc2*(−/−) strain, followed by the WT strain and then the *Abcc3*(−/−) 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 elimination. Humans also use 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 (Balis et al., 1983). The PK parameters are summarized in Table 1.

Interstrain Analysis. Interstrain analysis indicated several differences in the PK parameters. Relative to WT mice, *Abcc2*(−/−) 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 and renal proximal tubules, to perform its normal excretory function. At the 50 mg/kg dose level, the mean AUC value in *Abcc2*(−/−) 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 with 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 % $AUC_{m/p}$, Table 1), being 3- to 8-fold compared with the WT strain over the 10 to 200 mg/kg dose range,

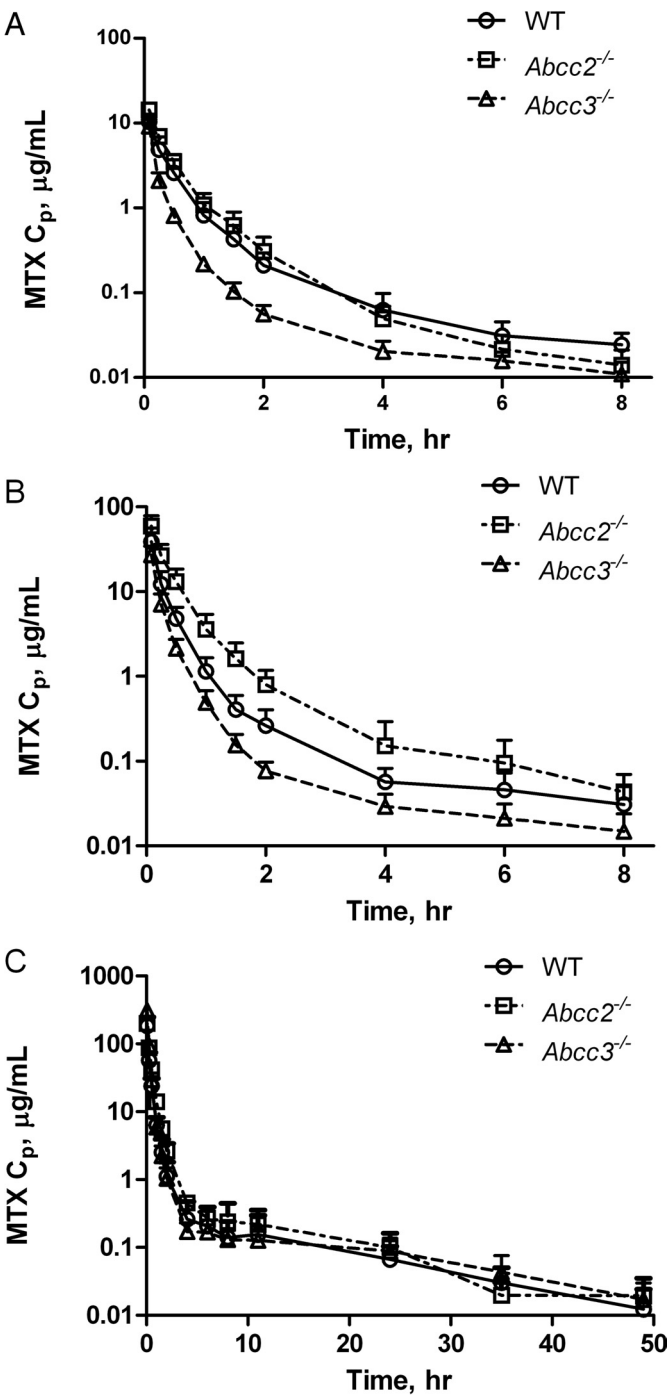


FIG. 1. Plasma concentrations (C_p, mean ± S.D.; semi-log scale) of MTX as a function of time after IV bolus administration of single doses of MTX at 10 (A), 50 (B), and 200 (C) mg/kg to male WT (○), *Abcc2*(−/−) (□), or *Abcc3*(−/−) (△) mice.

could reflect both reduced clearance and 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 V_d between the WT and *Abcc2*(−/−) strains were difficult to decipher. At the two lower dose levels, mean V_d 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 V_d ($P < 0.05$) 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 several significant differences. Total clearances were significantly elevated in the *Abcc3*(−/−) strain compared with the WT strain at the 10 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 the *Abcc2*(−/−) strain, are absent or compromised because of 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 with the wild-type mice (see Table 1). The reduced formation of 7OH-MTX is also consistent with hastened biliary excretion of this metabolite because 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, because 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 V_d in the *Abcc3*(−/−) mice at the two lower doses compared with 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 V_d 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 to 200 mg/kg (see Table 1 and Figs. 2 and 3). WT mice exhibited an approximate 2-fold increase in CL ($P < 0.05$) in going from the 10 to 50 mg/kg dose level, indicating that Michaelis-Menten kinetics is 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 because there was no change in clearance or fecal excretion from the 50 mg/kg dose level. Interestingly, there was no enhanced conversion of MTX to 7OH-MTX in the WT strain, and at the high 200 mg/kg dose only renal excretion appeared to be a compensatory mechanism. Dose-dependent elevation in V_d ($P < 0.05$) 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 to 50 mg/kg; however, the elevated

TABLE 1

Pharmacokinetic parameters (mean ± S.D.) of MTX after IV administration of MTX at three dose levels for WT, Abcc2(−/−), and Abcc3(−/−) mice

Parameters	Dose		
	10 mg/kg	50 mg/kg	200 mg/kg
WT mice	$n = 8$	$n = 10$	$n = 11$
CL (ml · min^{-1} · kg^{-1})	33.8 ± 10.2†	68.1 ± 31.1†	53.8 ± 16.4
V_d (l/kg)	11.5 ± 6.2	21.1 ± 14.0	58.1 ± 29.8‡
$t_{1/2}$ (min)	229.2 ± 92.6	211.7 ± 97.6	730.2 ± 101.0‡
AUC_{MTX} (min · μg/ml)	308.4 ± 79.0	842.9 ± 298.2	4051.1 ± 1304.3
$AUC_{7OH\text{-}MTX}$ (min · μg/ml)	13.1 ± 5.1	23.2 ± 12.1	96.1 ± 43.9
$AUC_{m/p}$ (%)	4.13 ± 0.94†	2.99 ± 1.51	2.04 ± 0.73†
Renal fraction of dose (f_u, %)	35.3 ± 15.0	43.4 ± 2.95	59.7 ± 6.94‡
Fecal fraction of dose (f_f, %)	1.10 ± 0.72‡	12.4 ± 6.84	8.55 ± 5.86
Abcc2(−/−) mice	$n = 9$	$n = 14$	$n = 7$
CL (ml · min^{-1} · kg^{-1})	23.0 ± 5.7*,‡	35.1 ± 12.4**	35.3 ± 5.4*
V_d (l/kg)	4.37 ± 1.49**	8.01 ± 3.07**	95.8 ± 34.7*,‡
$t_{1/2}$ (min)	131.7 ± 37.4**	166.2 ± 70.7	1928.8 ± 738.2**,‡
AUC_{MTX} (min · μg/ml)	412.5 ± 62.7**	1572.5 ± 520.3**	5607.1 ± 686.0**
$AUC_{7OH\text{-}MTX}$ (min · μg/ml)	50.9 ± 15.7**	227.3 ± 94.1**	955.1 ± 202.4**
$AUC_{m/p}$ (%)	11.3 ± 3.5**	15.8 ± 6.2**	17.0 ± 2.6**
Renal fraction of dose (f_u, %)	101.1 ± 9.87**,‡	79.8 ± 19.8**	76.8 ± 12.2
Fecal fraction of dose (f_f, %)	0.70 ± 0.49‡	6.08 ± 3.34*	7.84 ± 4.14
Abcc3(−/−) mice	$n = 7$	$n = 8$	$n = 6$
CL (ml · min^{-1} · kg^{-1})	55.2 ± 8.3**,†	100.0 ± 22.8*,†	32.6 ± 2.5**,†
V_d (l/kg)	21.4 ± 8.6**	35.4 ± 14.0*	30.3 ± 7.5*
$t_{1/2}$ (min)	264.8 ± 88.1	269.9 ± 150.7	646.5 ± 166.0‡
AUC_{MTX} (min · μg/ml)	180.8 ± 31.8**	516.7 ± 11.4*	6153.8 ± 472.5**
$AUC_{7OH\text{-}MTX}$ (min · μg/ml)	3.71 ± 0.83**	2.57 ± 1.11**	43.0 ± 10.4*
$AUC_{m/p}$ (%)	2.12 ± 0.61**,‡	0.49 ± 0.18**	0.70 ± 0.14**
Renal fraction of dose (f_u, %)	33.3 ± 12.6	34.1 ± 3.3**	46.0 ± 4.39**,‡
Fecal fraction of dose (f_f, %)	5.88 ± 4.89*,†	24.9 ± 9.9**,†	16.0 ± 9.1

* $P < 0.05$ and ** $P < 0.01$, significant difference between WT and knockout strains at that dose level.

†,‡ significant difference among dose levels within the same strain ($P < 0.05$), with † indicating differences between designated dose levels and ‡ indicating differences from the other two dose levels.

biliary excretion was also supplemented by compensatory metabolism to 7OH-MTX, although the latter was insignificant ($P > 0.05$) among 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 because 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 with 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 because of the discovery that several membrane transporters are involved in its disposition that affect not only its systemic PK properties but also pharmacologically active concentrations at the cellular level that are not measured in whole animals. Extensive studies using various in vitro and in vivo models have been performed 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 (van de Wetering et al., 2007; Vlaming et al., 2008; Lagas et al., 2010a,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 of coordinately exporting MTX and furthermore that hepatic metabolism can be altered 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, MRP1–5, and breast cancer resistance protein are low-affinity and moderate-to-high MTX transporters with an average K_m in the millimolar range and an average V_{max} ranging from ~0.2 to 2.9 nmol · min^{-1} · mg protein^{-1}, respectively (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 to 50 mg/kg caused an increase in total clearance that is consistent with Michaelis-Menten kinetics, and when one considers interstrain differences it also suggests altered transporter capacity most likely through increased protein expression. Depending on the background strain (FVB versus C57BL/6) in *Abcc2*(−/−) mice, Abcc3 and Abcc4 expression in kidney and liver ranged from no different than WT to 2-fold elevation (Chu et al., 2006; Vlaming et al., 2006). Similar expression data are not available in the *Abcc3*(−/−) 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

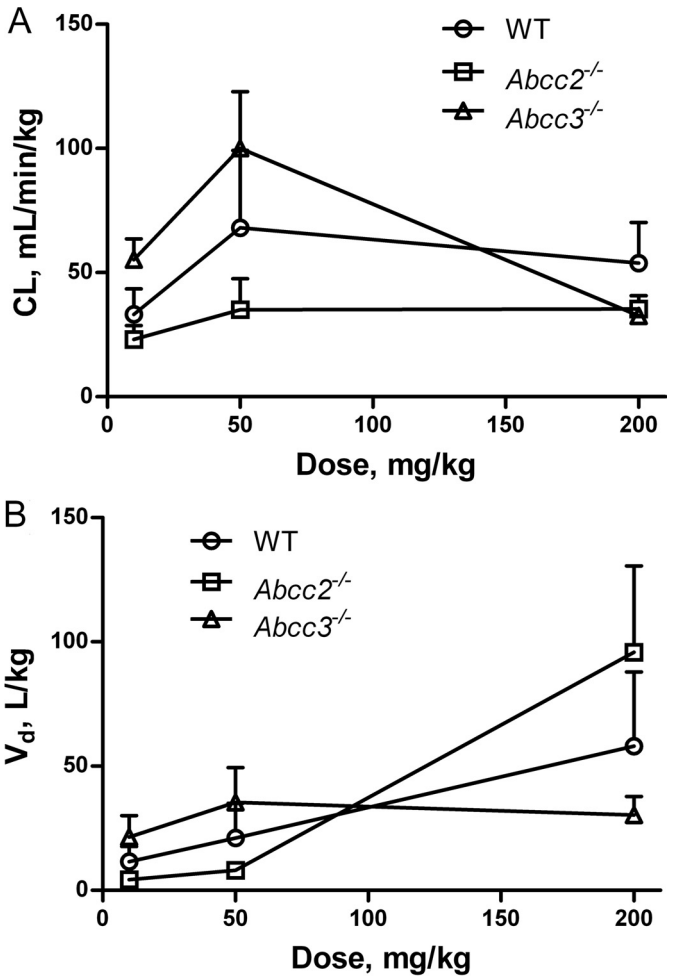


FIG. 2. (A) Total clearance (CL) and (B) volume of distribution (V_d) (mean ± S.D.) of MTX as a function of dose to different mouse strains.

biliary excretory pump. The studies by Vlaming et al. (2008, 2009a) in single and double [*Abcc2;Abcg2*(−/−)] knockout mice have demonstrated the key role of Abcg2 that plays in MTX's biliary clearance. We observed additional compensatory elimination methods for MTX in the *Abcc2*(−/−) 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*(−/−) group, other compensatory pathways were unnecessary.

Although there were concordant increases in MTX's CL in going from 10 to 50 mg/kg among 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*(−/−) strain, active transport of MTX into bile was saturated and accompanied by a significant yet modest increase in renal excretion, which was clearly insufficient to achieve the clearance rates obtained at the lower two dose levels. The *Abcc3*(−/−) strain distinguishes itself at the high dose in its inability to increase the formation of 7OH-MTX as a compensatory mechanism as observed in the *Abcc2*(−/−) group. The wild-type strain had no need to increase metabolic conversion of MTX because all biliary transporters were functioning, albeit at saturation, and furthermore because of 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*(−/−) 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*(−/−) strain is quite different from what was observed in the *Abcc2*(−/−) strain, in which substantial conversion to 7OH-MTX was seen [~20-fold increase in the $AUC_{7OH\text{-}MTX}$ compared with the *Abcc3*(−/−) 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*(−/−) mice suggests that patients with low ABCC2 activity may have increased risk of MTX-induced hepatic and renal toxicity. In fact, there are several clinical studies that have identified polymorphisms in ABCC2 in different patient populations that led to increased MTX toxicity, particularly hepatotoxicity and nephrotoxicity (Hulot et al., 2005; Ranganathan et al., 2008).

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

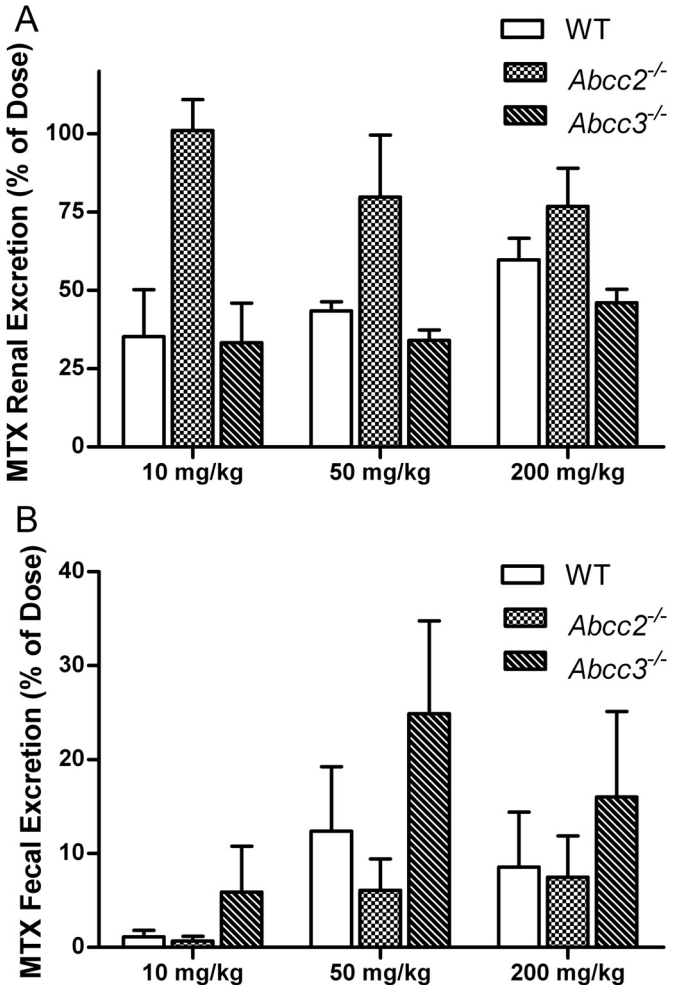
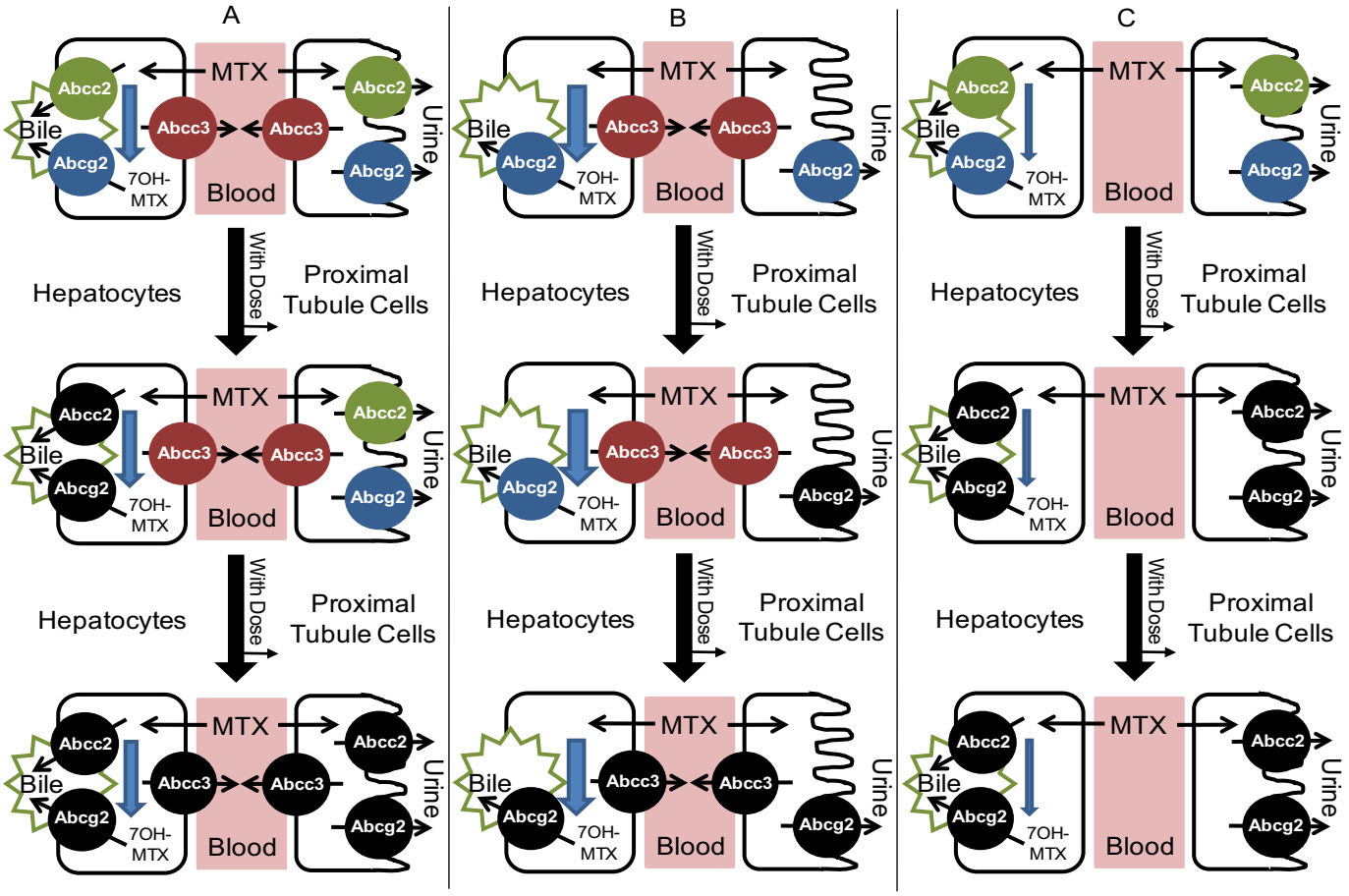


FIG. 3. Renal (A) and fecal excretion (B) of MTX (expressed as % of dose) in WT, *Abcc2*(−/−) and *Abcc3*(−/−) mice at three different dose levels.



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*(−/−) (B), and *Abcc3*(−/−) (C) strains. For each strain, three states of transporter function are shown that correspond to our low-, medium-, and high-dose MTX groups. Nonblack, colored transporters are operating below saturation, whereas black color 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,b). The extent of conversion of MTX to 7OH-MTX in hepatocytes is indicated by the thickness of the arrows.

that 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 for patients through the design of individualized therapy and monitoring of renal and hepatic function to avoid toxicity.

Acknowledgments

The scientific insight of Gary D. Kruh (deceased) is greatly appreciated and he will forever be missed.

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

Amidon GL, Lennernäs H, Shah VP, and Crison JR (1995) A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. *Pharm Res* **12:**413–420.
Assaraf YG (2006) The role of multidrug resistance efflux transporters in antifolate resistance and folate homeostasis. *Drug Resist Updat* **9:**227–246.
Balis FM, Holcenberg JS, and Bleyer WA (1983) Clinical pharmacokinetics of commonly used anticancer drugs. *Clin Pharmacokinet* **8:**202–232.
Belinsky SA (2005) Silencing of genes by promoter hypermethylation: key event in rodent and human lung cancer. *Carcinogenesis* **26:**1481–1487.
Borst P, de Wolf C, and van de Wetering K (2007) Multidrug resistance-associated proteins 3, 4, and 5. *Pflugers Arch* **453:**661–673.
Borst P and Elferink RO (2002) Mammalian ABC transporters in health and disease. *Annu Rev Biochem* **71:**537–592.
Braun J and Rau R (2009) An update on methotrexate. *Curr Opin Rheumatol* **21:**216–223.
Chan LM, Lowes S, and Hirst BH (2004) The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. *Eur J Pharm Sci* **21:**25–51.
Chessells JM, Leiper AD, Tiedemann K, Hardisty RM, and Richards S (1987) Oral methotrexate is as effective as intramuscular in maintenance therapy of acute lymphoblastic leukaemia. *Arch Dis Child* **62:**172–176.
Chu XY, Strauss JR, Mariano MA, Li J, Newton DJ, Cai X, Wang RW, Yabut J, Hartley DP, Evans DC, et al. (2006) Characterization of mice lacking the multidrug resistance protein MRP2 (ABCC2). *J Pharmacol Exp Ther* **317:**579–589.
Deangelis LM and Iwamoto FM (2006) An update on therapy of primary central nervous system lymphoma. *Hematology Am Soc Hematol Educ Program* 311–316.
Fromm MF (2003) Importance of P-glycoprotein for drug disposition in humans. *Eur J Clin Invest* **33 (Suppl 2):**6–9.
Fuskevåg OM, Kristiansen C, Olsen R, Aarbakke J, and Lindal S (2000) Microvascular perturbations in rats receiving the maximum tolerated dose of methotrexate or its major metabolite 7-hydroxymethotrexate. *Ultrastruct Pathol* **24:**325–332.
Gerk PM and Vore M (2002) Regulation of expression of the multidrug resistance-associated protein 2 (MRP2) and its role in drug disposition. *J Pharmacol Exp Ther* **302:**407–415.
Guo P, Wang X, Liu L, Belinsky MG, Kruh GD, and Gallo JM (2007) Determination of methotrexate and its major metabolite 7-hydroxymethotrexate in mouse plasma and brain tissue by liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal* **43:**1789–1795.
Hulot JS, Villard E, Maguy A, Morel V, Mir L, Tostivint I, William-Faltaos D, Fernandez C, Hatem S, Deray G, et al. (2005) A mutation in the drug transporter gene ABCC2 associated with impaired methotrexate elimination. *Pharmacogenet Genomics* **15:**277–285.
Hunter GA (1962) The use of methotrexate in the treatment of psoriasis. *Aust J Dermatol* **6:**248–254.
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.
Lagas JS, Fan L, Wagenaar E, Vlaming ML, van Tellingen O, Beijnen JH, and Schinkel AH (2010a) P-glycoprotein (P-gp/Abcb1), Abcc2, and Abcc3 determine the pharmacokinetics of etoposide. *Clin Cancer Res* **16:**130–140.
Lagas JS, Sparidans RW, Wagenaar E, Beijnen JH, and Schinkel AH (2010b) Hepatic clearance of reactive glucuronide metabolites of diclofenac in the mouse is dependent on multiple ATP-binding cassette efflux transporters. *Mol Pharmacol* **77:**687–694.
Masuda M, I'izuka Y, Yamazaki M, Nishigaki R, Kato Y, Ni'inuma K, Suzuki H, and Sugiyama Y (1997) Methotrexate is excreted into the bile by canalicular multispecific organic anion transporter in rats. *Cancer Res* **57:**3506–3510.
Pratt WB, Ruddon RW, Ensminger WD, and Maybaum J (1994) *The Anticancer Drugs*, 2nd ed, Oxford University Press, New York.
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.
Rizzoli V, Mangoni L, Caramatti C, Degliantoni G, and Costi D (1985) High-dose methotrexate-leucovorin rescue therapy: selected application in non-Hodgkin's lymphoma. *Tumori* **71:**155–158.
Sand TE and Jacobsen S (1981) Effect of urine pH and flow on renal clearance of methotrexate. *Eur J Clin Pharmacol* **19:**453–456.
Scheffer GL, Kool M, de Haas M, de Vree JM, Pijnenborg AC, Bosman DK, Elferink RP, van der Valk P, Borst P, and Scheper RJ (2002) Tissue distribution and induction of human multidrug resistant protein 3. *Lab Invest* **82:**193–201.
Sikic BI, Fisher GA, Lum BL, Halsey J, Beketic-Oreskovic L, and Chen G (1997) Modulation and prevention of multidrug resistance by inhibitors of P-glycoprotein. *Cancer Chemother Pharmacol* **40 (Suppl):**S13–S19.
Steele WH, Lawrence JR, Stuart JF, and McNeill CA (1979) The protein binding of methotrexate by the serum of normal subjects. *Eur J Clin Pharmacol* **15:**363–366.
van de Wetering K, Zelcer N, Kuil A, Feddema W, Hillebrand M, Vlaming ML, 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.
Vlaming ML, Mohrmann K, Wagenaar E, de Waart DR, Elferink RP, Lagas JS, van Tellingen O, Vainchtein LD, Rosing H, Beijnen JH, et al. (2006) Carcinogen and anticancer drug transport by Mrp2 in vivo: studies using Mrp2 (Abcc2) knockout mice. *J Pharmacol Exp Ther* **318:**319–327.
Vlaming ML, Pala Z, van Esch A, Wagenaar E, de Waart DR, van de Wetering K, van der Kruijssen CM, Oude Elferink RP, 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 ML, Pala Z, van Esch A, Wagenaar E, van Tellingen O, de Waart DR, Oude Elferink RP, 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 ML, 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.
Weinblatt ME, Coblyn JS, Fox DA, Fraser PA, Holdsworth DE, Glass DN, and Trentham DE (1985) Efficacy of low-dose methotrexate in rheumatoid arthritis. *N Engl J Med* **312:**818–822.
Wu C-Y and Benet LZ (2005) Predicting drug disposition via application of BCS: transport/absorption/elimination interplay and development of a Biopharmaceutics Drug Disposition Classification System. *Pharmacol Res* **22:**11–23.
Xia CQ, Liu N, Yang D, Miwa G, and Gan LS (2005) Expression, localization, and functional characteristics of breast cancer resistance protein in Caco-2 cells. *Drug Metab Dispos* **33:**637–643.
Xia CQ, Milton MN, and Gan LS (2007) Evaluation of drug-transporter interactions using in vitro and in vivo models. *Curr Drug Metab* **8:**341–363.

Address correspondence to: Dr. James M. Gallo, Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, NY 10029. E-mail: james.gallo@mssm.edu