

**IMPACT OF MULTIDRUG RESISTANCE-ASSOCIATED
PROTEIN 2 (MRP2/ABCC2) AND 3 (MRP3/ABCC3) ON THE
PHARMACOKINETICS OF METHOTREXATE**

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ABSTRACT

This dissertation presents an investigation of the impact of Multidrug Resistance-associated Protein 2/ATP-binding cassette superfamily C member 2 (Mrp2/Abcc2) and 3 (Mrp3/Abcc3) on the pharmacokinetics (PKs) of methotrexate (MTX) using gene knockout murine models.

MTX is a substrate for numerous human ATP-binding cassette (ABC) efflux transporters, yet the impact of these transporters on the pharmacokinetics of MTX 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 *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), *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 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.

We also sought to characterize the effects of Abcc2 and Abcc3, on the PKs of MTX after oral dosing. Plasma, urine, and fecal concentrations of MTX were measured after 10, 50, and 200 mg/kg oral doses to cohorts of WT, *Abcc2*^{-/-}, and *Abcc3*^{-/-} mouse

strains. The absence of *Abcc2* caused an approximate 2-fold increase in system exposure and a slight increase in oral bioavailability of MTX relative to WT mice at all dose levels. These elevations were accompanied by compensatory increases in conversion to 7OH-MTX, and based on $AUC_{7OH-MTX}/AUC_{MTX}$ (area under the curve ratio of metabolite and parent drug) that ranged from 3% to 9% in WT mice increased to a range of 16% to 26% in *Abcc2*^{-/-} mice. Renal excretion of unchanged MTX was unaltered in the *Abcc2*^{-/-} strain; fraction urinary excretion (f_r) ranged from about 4% to 11% in WT mice, whereas in *Abcc2*^{-/-} mice f_r ranged from about 7% to 23%. *Abcc3*^{-/-} mice exhibited more than a 2-fold decrease in C_{max} and significant reductions in AUC_{MTX} when compared to WT mice at all dose levels. There were no compensatory increases in either metabolism or in renal and biliary excretion, which suggests future studies for investigating a potential unknown mechanism. Regardless of the mouse strain, increases in the MTX dose were not accompanied by proportional increases in AUC_{MTX} .

The PKs of MTX in different mouse strains was successfully modeled by a nonlinear semi-mechanistic 3-compartmental conditional model incorporating key efflux transporters. The model employed population-based analysis and conditional transport terms to well capture the nonlinear properties of MTX systemic disposition for a wide dose range of 10 – 200 mg/kg in WT and knockout strains. The model correlates the mechanistic nature of the nonlinear phenomenon with the key efflux transporters effects on MTX PKs and provides insight for preclinical therapeutic study design.

Overall, the information obtained in this investigation underscores the significance of efflux transporters, Abcc2 and Abcc3, for they significantly influence the pharmacokinetics of MTX and their impact can be reflected by a nonlinear semi-mechanistic 3-compartmental conditional model. The studies also provide implication in the preclinical therapeutic study design and insights on the source of inter-patient variability as well as on the combination drug regimens to maximize drug activity yet without toxicity.

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DELICATION

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CHAPTER 1

INTRODUCTION

1.1 Background

Pharmacokinetics (PKs) is defined as the study of drug absorption, distribution, metabolism, and excretion (ADME) evolved mostly from the field of chemical kinetics and was formalized in the 1970's and 1980's (Gibaldi & Perrier, 1982). The field of PKs, although integrated, could be considered as consisting of subdisciplines, one concerned with methodologies designed to advance drug discovery efforts and another concerned with mechanisms of drug disposition. The study of drug transport across biological membranes is one area that warrants consideration both in drug discovery efforts, as drugs subject to active transport could be eliminated from further development, and in mechanistic PKs, as characterization of drug-transporter interactions provides a foundation for model building and important insights into a drug's properties. Passive diffusion, facilitated passive diffusion and active transport across biological membranes are the molecular processes involved in drug absorption, distribution, and elimination.

My project concerns two members of the ATP-binding cassette (ABC) family of transporters. The first ABC transporter, P-glycoprotein (P-gp, ABCB1), was discovered about over 30 years ago (Juliano & Ling, 1976). This discovery provided the stimulus to establish the field of drug transporters that consists of genetic, biochemical, and

pharmacological approaches to not only discover new transporters and their endogenous functions, but also to characterize drug-transporter interactions. There are two major classes of drug transporters, the 9 members of the ABC family and the 30 members of the solute carrier (SLC) family are found to influence drug pharmacokinetics, pharmacodynamics, and toxicity (Scherrmann, 2009). ABC family members are associated with active transport by transporting their substrates against concentration gradients, while SLC family members are usually involved in facilitated passive diffusion to transport their diverse substrates. The importance of drug transporters in drug disposition has been amply demonstrated for numerous classes of drugs including many anticancer drugs. For example, significantly reduced oral bioavailability of taxol can be directly attributed to P-gp efflux transporter (Malingre et al., 2001; Chiou, Wu, Ma, & Jeong, 2002). In addition, a drug may inhibit or induce a certain transporter that may be involved in ADME processes of a second drug. In such case, the pharmacokinetic property of the second drug can be altered due to drug-drug interaction. Although such interaction was initially considered to be related only to drug metabolism (cytochrome P-450 [CYP]-mediated process), it is now recognized that transporters may also be involved (Christians, Schmitz, & Haschke, 2005; Holtzman, Wiggins, & Spinler, 2006).

The potential influence of transporters on drug pharmacokinetics can be assessed using *in silico*, *in vitro* and *in vivo* approaches and concerned in clinical as well. *In vitro* cell systems are regular components of the drug discovery process that seek means to rapidly screen large numbers of compounds for their propensity to be substrates of active transport. Although quite suitable for this purpose *in vivo* methods are required to both

validate *in silico* and *in vitro* findings but are needed to understand how transporters function in whole organs and species. Gene-disrupted or gene-knockout rodent models are the mainstay of *in vivo* PK investigations to elucidate the role of the drug transporters. Comparative PK drug concentration-time profiles in wild-type and gene knockout mice will provide a quantitative basis to determine the role of single and at time multiple transporters on drug disposition. Clinically, drug transporters are also considered important, as they may mediate intracellular drug concentrations, which is a reflection of pharmacological activity or toxicity after treatment of drugs.

1.2 Literature Review

1.2.1 The Significance of Studies of Membrane Transporters in Drug Discovery and Development

Membrane transporters, potentially a major factor in drug pharmacokinetics and disposition, must be considered during the process of drug discovery and development. Being localized on cellular membranes, drug transporters sometimes get involved in absorption, distribution and elimination by active delivery and responsible for transporting drugs through intestine for absorption, into the liver for metabolism, and into bile or urine for elimination. Yet currently, no single mechanism explains how drugs cross associated cell membranes.

In order to enhance the efficiency of drug discovery and development in terms of drug pharmacokinetic behavior, Wu and Benet (2005) modified the Food and Drug Administration's (FDA's) Biopharmaceutics Classification System (BCS) (Amidon,

Lennernas, Shah, & Crison, 1995), into the Biopharmaceutics Drug Disposition Classification System (BDDCS). The BDDCS not only links the physicochemical properties of drug candidates, to the pharmacokinetic concerns, such as metabolic involvement and excretory pathways, but takes into account the potential interaction of drug candidates with membrane transporters (influx and efflux transporters) and drug metabolizing enzymes. Thus, the scientific rationalization of BDDCS provides guidance for predicting the influence of transporters on oral absorption as well as post-absorption systemic levels following different administration routes at the earliest stages of drug discovery and development. However, this guidance is not sufficient to quantify the extent of those interactions and for late phases in drug development, due to different facts—overlapping substrates profiles and genetic polymorphism of transporters leading to difficulty for quantitative characterization. Therefore, for better study design, a comprehensive understanding of membrane transporters is a necessity.

1.2.1.1 Membrane transporters: classification, structure and mechanism of action

Membrane transporters have been classified into three categories based on mechanism of actions: (1) primary active transport system, relying directly on the hydrolysis of ATP to move solutes across the membrane, such as ABC superfamily proteins (Gottesman & Pastan, 1993; Barrand, Bagrij, & Neo, 1997; Bates et al, 2001); (2) secondary active transporters, using electrochemical gradients typically from the movement of sodium or hydrogen ions to move compounds across membranes, including sodium-dependent amino acid transporters in the intestine and blood-brain barrier (BBB) (Castagna et al., 1997), and the hydrogen ion-dependent peptide transporters (PepT1 and

PepT2) in the intestine, kidneys, lungs, and blood-cerebrospinal fluid barrier (BCSFB) (Daniel & Herget, 1997; Groneberg et al., 2001; Teuscher, Novotny, Keep, & Smith, 2000); and (3) tertiary transport system, allowing compounds to move across the lipid barrier via facilitated diffusion through interaction with conformational changes in the protein, such as SLCs (Stein, 1986). Transporters with name standardized by the Human Gene Nomenclature Committee are listed as Table 1 (<http://www.gene.ucl.ac.uk/nomenclature/genefamily.shtml>).

Based on their functions, transporters are also broadly separated into two major groups: uptake and efflux transporters. Uptake transporters include members of the organic anion-transporting polypeptide (OATP/SLCO) family, organic anion transporter (OAT/SLC22A) and organic cation transporter (OCT/SLC22A) family, organic cation/carnitine transporter (OCTN/SLC22A) family, and peptide transporter (PEPT/SLC15A) family in secondary and tertiary transport systems. ABC transporters are a group of efflux transporters being extensively studied. By utilizing the energy from ATP hydrolysis, transporters in this category move substrates against a concentration gradient, subsequently being responsible for the cellular extrusion of xenobiotics. Thus, as important self-protectants, they may potentially influence ADME of certain drugs (see Figure 1, modified from International Transporter Consortium [ITS], 2010).

Being primary transporters, most members of ABC superfamily contain a highly conserved (about 30% sequence identity) ABC of 200–250 amino acids with higher mean molecular weight of 150–200 kDa. The conserved ABC structure generally consists of 12

membrane-spanning domains (MSD), which split into two halves with each half containing a nucleotide-binding domain (NBD) and share extensive sequence homology and domain organization (Higgins et al., 1986; Hyde et al., 1990; Barrand et al., 1997; Bates et al., 2001; Borst, Zelcer, & van Helvoort, 2000, Chan, Lowes, & Hirst, 2004). Yet there are a number of exceptions to this structural arrangement. For example, ABCC/MRP1-3 (ABC subfamily C member 1-3) and ABCC/MRP6-7 have five additional MSDs at the N-terminus, while BCRP contains only six MSDs and one NBD (called half-transporters) and functions only after dimerization (Özvegy et al., 2001; see Figure 2, modified from Borst et al., 2000).

1.2.1.2 Key ABC transporters involved in ADME of anticancer drugs

As aforementioned, ABC transporters are a family of large membrane proteins that can transport a variety of compounds against concentration gradients by the combination of conformational changes within MSDs and the consumption of energy from ATP hydrolysis within NBD. Yet genetic deficiency in specific ABC transporters may cause loss of associated functions and lead to inherited diseases (Ho & Kim, 2005) such as Dubin-Johnson syndrome, with ABCC2 deficiency. Tissue localization and substrate specificity of ABC transporters also imply their potential physiological and therapeutic functions. Therefore, increasing ABC transporters are being investigated for their roles in pharmacokinetics and disposition of xenobiotics (See Table 2, modified from Ambudkar, Kimchi-Sarfaty, Sauna, & Gottesman, 2003; Kruh, Guo, Hopper-Borge, Belinsky, & Chen, 2007; Chen & Tiwari, 2011). The two important efflux transporters, ABCC2 and ABCC3, also our interested ones in the project, will be discussed in detailed as follows.

Table 1. Some standardized names of transporters by the Human Gene Nomenclature Committee

Abbreviation	Symbol
MDR1/P-gp	ABCB1
MDR3	ABCB3
BSEP/SPGP	ABCB11
MRP1	ABCC1
MRP2/cMOAT	ABCC2
MRP3	ABCC3
MRP4	ABCC4
MRP5	ABCC5
MRP6	ABCC6
MRP7	ABCC10
MRP8	ABCC11
MRP9	ABCC12
BCRP	ABCG2
NTCP	SLC10A1
ASBT	SLC10A2
PEPT1	SLC15A1
PEPT2	SLC15A2
OATP-A	SCL21A3
OATP-C/OATP2/LST-1	SCL21A6
OATP8	SCL21A8
OATP-B	SCL21A9
OATP-D	SCL21A11
OATP-E	SCL21A12
OATP-F	SCL21A14
OCT1	SCL22A1
OCT2	SCL22A2
OCT3	SCL22A3
OCTN1	SCL22A4
OCTN2	SCL22A5
OAT1	SCL22A6
OAT2	SCL22A7
OAT3	SCL22A8
OAT4	SCL22A9

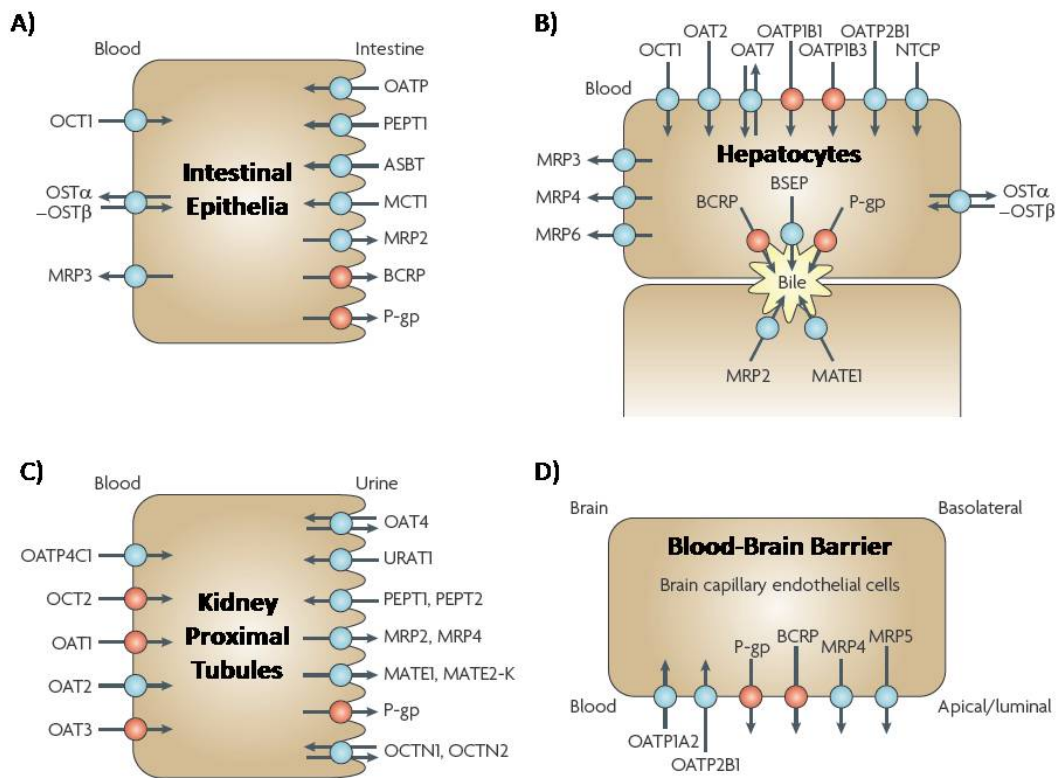


Figure 1. Localization of selected human membrane transporters in organs typically involved in drug ADME (modified from ITS, 2010).

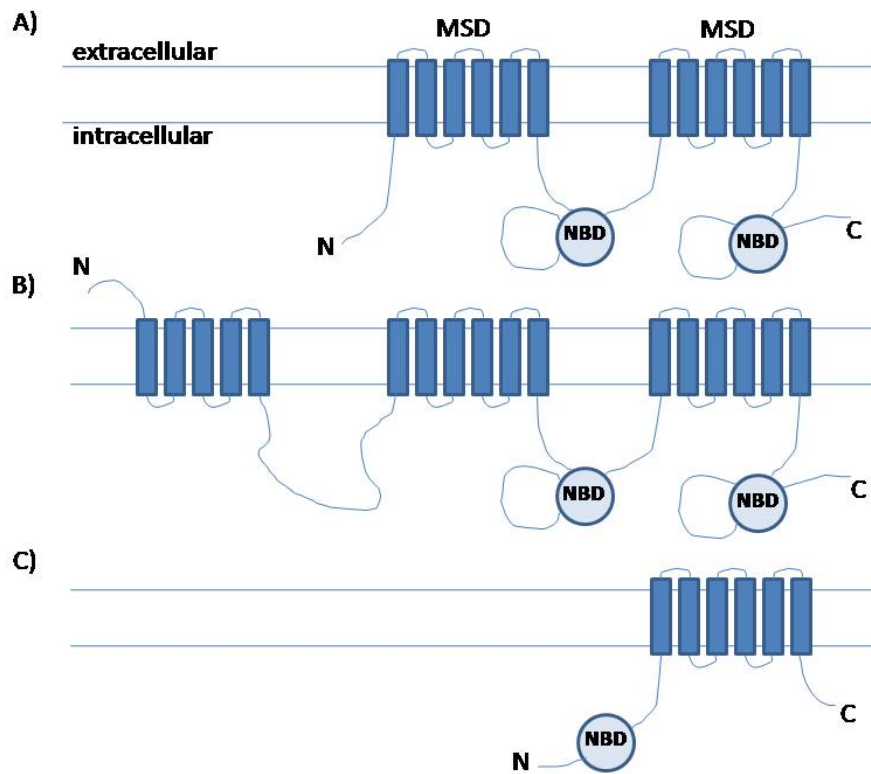


Figure 2. Transmembrane arrangement of ABC efflux transporters (modified from Özvegy et al., 2001). (A) Pgp/MDR1, MDR3, BSEP/SPGP, MRP4-5, and MRP8-9 have 12MSDs and two NBDs. (B) Typical MRP transporters (MRP1-3 and 6–7) have five extra MSDs near the N terminus. (C) Half-transporters such as BCRP have only six MSDs and one NBD and function only after dimerization.

ABCC2 (MRP2). *ABCC2* is expressed in several critical organs for absorption (intestine), elimination (liver and kidney) and distribution. (Kruh & Belinsky, 2003; Doyle & Ross, 2003; Mottino, Hoffman, Jennes, & Vore, 2000; Schaub et al., 1997; Buchler et al., 1996; St-Pierre et al., 2000; van Aabel, Hartog, Bindels, Van Os, & Russel, 2000). It has been proposed to not only prevent accumulation of noncancer agents by effluxing at absorption sites but also play an important role in biliary excretion of amphiphilic anions and endogenous metabolites. Overexpression of *ABCC2* in the BBB of patients with epilepsy was suggested to be a reason behind drug resistance (Nagel, 1999). During the course of pregnancy, elevated *ABCC2* level in the placenta is found to protect the fetus from endogenous and xenobiotic toxins (St-Pierre et al., 2000).

While regarding to cancer chemotherapeutic agents, *ABCC2* exhibits a broad substrate spectrum, including anthracyclines, vincas, epipodophyllotoxins, mitoxantrone, and camptothecins, although certain camptothecins (irinotecan and topotecan) are weaker substrates to P-gp (Kawabe et al., 1999; Cui et al., 1999; Chen et al., 1999). MTX was later demonstrated as a good substrate of *ABCC2* as well (Hooijberg et al, 1999; Zeng, Chen, Belinsky, Rea, & Kruh, 2001). Obviously, the broad substrate profile together with apical localization indicates *ABCC2* may be a major factor in drug PK and disposition of a large number of chemotherapeutic agents. In this regard, Eisai hyperbilirubinemic (EHBR) rat strain was early used as a model for Dubin-Johnson syndrome (Gerk & Vore, 2002; Kartenbeck, Leuschner, Mayer, & Keppler, 1996). As with *Abcc2*-deficiency the hepatobiliary elimination of drugs, such as lipid lowering agents and antibiotics (Gerk & Vore, 2002; Konig, Nies, Cui, Leier, & Keppler, 1999) as well as anticancer agents

(Masuda et al., 1997; Chu, Kato, & Sugiyama, 1997) may be influenced. However, the rat model has not been used to evaluate the functions of Abcc2 as a molecular determinant of PK and disposition of cancer chemotherapeutics until the recent availability of *Abcc2*^{-/-} mice (Vlaming et al., 2006, 2008, 2009a&b). The *Abcc2*^{-/-} single knock-out mice revealed doubled plasma AUC, reduced hepatobiliary elimination and elevated renal excretion of unchanged MTX in first couple of hours after intravenous administration. Furthermore, only few studies showed that Abcc2 is involved in hepatobiliary extrusion of certain anti-cancer drugs. There is no report evaluating Abcc2 as a molecular determinant, especially in terms of the extent of involvement, with respect to a whole PK profile of anti-cancer drugs. Especially regarding to the oral bioavailability, only one recent study showed less important role of that pump (Vlaming et al., 2011). The answer to this question would still depend on a thorough understanding of the physiological and pathological function of Abcc2, a missing yet critical building block.

ABCC3 (MRP3). Although apically localized ABC transporters, such as ABCC2, are generally emphasized due to its excretory functions, basolateral ones may also play a vital role, although few studies were conducted to identify such transporters. With expression in the vital organs for absorption and elimination, ABCC3 is now investigated as a good candidate (Kruh & Belinsky, 2003). Firstly, ABCC3 is highly expressed on the basolateral surfaces of enterocytes in human and rodents (Rost, Mahner, Sugiyama, & Stremmel, 2002; Scheffer et al., 2002), which suggests that ABCC3 may promote the absorption of anticancer agents orally administered. Besides bile acid and glucuronides (Zeng, Liu, Rea, & Kruh, 2000; Hirohashi, Suzuki, & Sugiyama, 1999; Hirohashi, Suzuki,

Table 2. Tissue localization and substrate specificity of ABC transporters

Transporters	Tissue expression	Physiological or nonchemotherapy substrates	Chemotherapeutic substrates
ABCA2	Brian, monocytes	Steroid derivatives, lipids	Estramustine
ABCB1/MDR1 /P-gp	Intestine, liver, kidney, placenta, BBB	Neutral and cationic organic compounds, many commonly used drugs	Doxorubicin, daunorubicin, vincristine, vinblastine, actinomycin-D, paclitaxel, docetaxel, etoposide, teniposide, bisantrene, homoharringtonine, Gleevec (STI-571)
ABCB4/MDR2	Liver	Phosphatidylcholine, some hydrophobic drugs	Paclitaxel, vinblastine
ABCB11/BSEP /SP-gp	Liver	Bile salts	Paclitaxel
ABCC1/MRP1	All tissues	LTC ₄ , E ₂ 17βG, sulfated bile acids, folic acid, bilirubin, GSH conjugates	Anthracyclines, etoposide, SN-38, irinotecan, topotecan, vinca alkaloids, antifolateneoplastics (MTX, edatrexate)
ABCC2/MRP2 /cMOAT	Liver, kidney, intestine	Bilirubin conjugates, GSH, LTC ₄ , LTD ₄ , LTE ₄	MTX, etoposide, anthracyclines, cisplatin, vinca alkaloids, itoxantrone, irinotecan, camptothecins
ABCC3/MRP3	Pancreas, kidney, intestine, liver, placenta, adrenal glands	Taurocholate, glycocholate, cholate, LTC ₄ , E ₂ 17βG, nucleoside analogues	Etoposide, teniposide, MTX, cisplatin, vincristine, doxorubicin

Table 2. (Continued)

Transporters	Tissue expression	Physiological or nonchemotherapy substrates	Chemotherapeutic substrates
ABCC4/MRP4	Prostate, testis, ovary, intestine, pancreas, lung, hepatocytes	cGMP, cAMP, PGE1, PGE2, E ₂ 17βG, DHEAS, GSH, and GSH conjugated bile acid, LTB ₄ , LTC ₄	MTX, thiopurines, SN-38, irinotecan, topotecan, PMEA
ABCC5/MRP5	Most tissues	cGMP, cAMP, DNP-SG, GSH	6-Mercaptopurine, 6-thioguanine, fluorouracil, MTX, cisplatin, PMEA, Azathioprine, daunorubicin, doxorubicin, gemcitabine, Ara-C
ABCC6/MRP6	Liver, kidney	LTC ₄ , DNP-SG, anionic cyclic pentapeptide	Doxorubicin, daunorubicin, etoposide, teniposide, cisplatin, actinomycin-D
ABCC10/MRP7	Most tissues	E ₂ 17βG, LTC ₄	Docetaxel, paclitaxel, vinblastine, vincristine, Ara-C, gemcitabine, epothilone-B
ABCC11/MRP8	Testis, breasts	cGMP, cAMP, E ₂ 17βG, DHEAS, LTC ₄ , bile acids, estrone 3-sulfate, DNP-SG, folic acids	PMEA, fluorouracil, ddC, Ara-C, MTX
ABCC12/MRP9	Testis, breasts, ovary, brain, skeletal muscles	Unknown	Unknown
ABCG2/BCRP	Placenta, intestine, breast, liver	Prazosin	Doxorubicin, daunorubicin, mitoxantrone, topotecan, SN-38

Takikawa, & Sugiyama, 2000), etoposide and MTX are ABCC3 substrates as well (Zeng, Bain, Belinsky, & Kruh, 1999; Kool et al., 1999), which further raises the level of importance to clarify ABCC3's role in PK and drug disposition. Recent studies demonstrated a decreased oral bioavailability of MTX in *Abcc3*^{-/-} mice, and this was attributed to reduced fraction of absorption after an oral dose (Kitamura, Hirouchi, Kusuhara, Schuetz, & Sugiyama, 2008). Secondly, ABCC3 expression level in liver is significantly increased (~ 50-fold) under cholestatic conditions, when the normal canalicular (i.e., biliary) route of detoxification is impaired (Donner & Keppler, 2001; Kiuchi, Suzuki, Hirohashi, Tyson, & Sugiyama, 1998; Konig et al., 1999; Ogawa et al., 2000; Soroka, Lee, Azzaroli, & Boyer, 2001). These discussed features suggest that ABCC3 may function as a back-up system to protect the liver from anticancer agents during liver dysfunction. In addition, *Abcc3*^{-/-} mice demonstrated a reduced basolateral efflux from hepatocytes after intravenous infusion of MTX, a substrate of ABCC2 as well. This result indicates that even at normal function status of liver, *Abcc3* is involved in basolateral extrusion of anticancer drugs. However, very recent studies complicated the understanding of ABCC3 function in PK and drug disposition (Vlaming et al., 2008, 2011) by showing controversial observations of insignificant or less important role of *Abcc3* in absorption and elimination of MTX at a higher intravenous dose within first few hours, compared to *Abcc2*. Therefore, ABCC3 function in oral bioavailability and in liver protection of anti-cancer agents are still obscure and need further elucidation, especially when the investigational substances are common substrates of ABCC3 and other apical efflux pumps, such as ABCC2.

1.2.1.3 Pharmacokinetic aspects regarding the involvement of ABC transporters in anti-cancer drugs

The statement that key ABC transporter would influence PK and disposition of anticancer drugs seems obvious given the following facts. First, specific ABC transporters act as self-protectants to extrude toxic xenobiotics or their metabolites at different sites through the passage of ADME (Szakács, Váradi, Ozvegy-Laczka, & Sarkadi, 2008; see Figure 3, modified from Undevia, Gomez-Abuin, & Ratain, 2005). Second, most anticancer agents are substrates of transporters, especially ABC transporters. Oral absorption of most anti-cancer drugs is a result of enteric transport by membrane transporters, including ABC transporters, in addition to first-pass effect and intestinal metabolism. Since modulation of cellular entry into and exit from the metabolic organs of either unmodified or metabolized compound are mediated by transporter proteins ABC transporters have been recognized of the similar importance in metabolism. Finally, anti-cancer agents are mainly eliminated through biliary tract and kidney by a range of transport systems, especially ABC transporters, in addition to those quantitatively negligible routes (lungs, skin, sweat and tears). Although normal intravenous dose regimen of anticancer drugs leads to rapid and complete bioavailability, distribution, metabolism and elimination of drugs are still connected with those transporters as mentioned above.

Given that ABC transporters are theoretically involved in each process in pharmacokinetics of anticancer drugs, it may be understandable that such proteins are sources of pharmacokinetic variability. As known, there is a 2~10-fold variation of

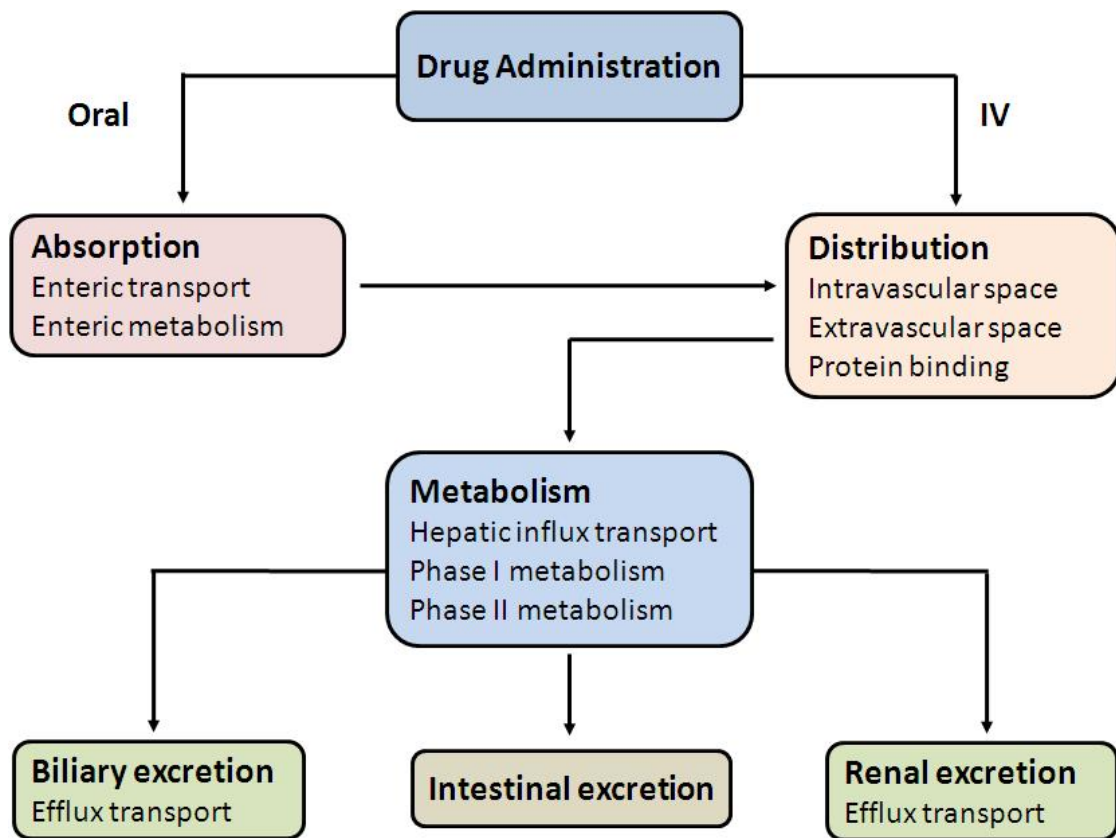


Figure 3. Possible transporters involvement in drug transport along ADME processes in pharmacokinetics (modified from Undevia et al., 2005).

systemic exposure after standard doses of anticancer drugs among patients. Here, if transporters are involved in any ADME process, their effects on pharmacokinetic variability should not be ignored. Expression levels of ABC transporters at critical sites for ADME processes show a large intersubject variability. This variation relies on pharmacogenomic information (gender difference or mutations, such as single nucleotide polymorphism (SNP)) and physiological status among patients. Moreover, some transporters tend to serve as backup system, when the primarily engaged transporters are saturated or dysfunctional, which brings more complexity to drug PK profile. Although a few studies have been carried out to investigate these aspects further on knock-out mouse models (Vlaming et al., 2009; Wang, Zhou, Kruh, & Gallo, 2010), to date, these investigations are still at a very early stage.

Ideally, when the individual variability is taken into account, doses of anti-cancer drugs should be tailored accordingly. Obviously, the traditional way of individualized dosing by normalizing of doses to body surface area (BSA), based on height and weight of patients (Baker et al., 2002), is not adequate. Therefore, strategies to characterize the transporter involvement are needed to provide rationale and guidance for dosing, such as dose adjustment methods based on the correlation of pharmacokinetic and genetic-based methods or mechanism-based mathematical models.

1.2.2 Preclinical Methods in Characterization of ABC Transporters in Drug Development

Since identification and characterization of ABC transporters roles on pharmacokinetics processes are essential for interpretation and prediction of pharmacokinetic properties of chemotherapeutic agents in drug discovery and early development, a variety of tools have been established to probe transporter interactions of agents. These include cell-based uptake, absorption and transport assays, in situ models that can isolate the pertinent absorption, distribution, or elimination tissues and computational models which can simulate and/or predict transporter interactions. In addition, transgenic mouse models have made it possible to examine the impact of selected transporter systems on the ADME process at the whole animal level. In this section, strategies incorporating *in vitro* and *in vivo* transporter studies are discussed.

1.2.2.1 In vitro systems and methods for identification of transporter interactions

Various cell-based *in vitro* systems have been established as a standardized process for high throughput screening of transporter interaction with drug entities in drug early development. Those systems include isolated cell or membrane preparations from various tissues, such as Caco-2 (resembling the morphology of GI epithelial cells), and other transfected cell lines. They are applied in different ways based on their mechanisms. Several approaches encompass widely used permeability assay (to model different transport mechanisms) and accumulation assay (to identify substrates or inhibitors and quantitatively determine transporter kinetics). Other methods, such as oocyte expression and uptake method (to express and measure the properties of transporters of interest) and ATPase assay for ABC transporters (to measure ATP hydrolysis for high throughput analysis) can also be employed. By the establishment of a wide range of concentrations,

monolayer (Yamashita et al., 2002) or bidirectional (Troutman & Thakker, 2003; Wang, Cao, & Zeng, 2005) permeability studies can determine the impact of the transport system on the permeability of the investigational compound and its absorptive, distributional, or excretory transport properties (Li et al., 2006). The cellular accumulation assays, including inverted vesicle assay for examining ABC efflux transporters and Calcein-AM assay for Pgp inhibitor screening, have become the mandatory assessment requested by FDA in the early drug development for a new drug application. The results of these assays can directly tell whether drugs are potential inhibitors of drug efflux transporters (such as P-gp and BCRP). Although those assays provide powerful tools for drug screening and quantitative determination of transporter kinetics, large variability was found among different cellular systems and laboratories for same type of transporters. For instance, different values of K_m and V_{max} were reported in the various BBB models for MRP related transport (Bachmeier, Trickler, & Miller, 2006). Therefore, correlation of *in vitro* assay results with *in vivo* or clinical test results are questionable. To obtain reliable translational results, *in vivo* studies at the whole animal basis would be the next step to further investigate the impact of transporters on ADME properties.

1.2.2.2 In vivo methods for characterization of transporters

Examination of drug transporter interactions at the whole animal level is much more complex than cell-based assays. Yet such examination is necessary to further assess the transport properties or impact of appropriate transporters on drug ADME. Such studies may confirm *in vitro* observations and provide greater confidence and

understanding of the effect of the transporter in a dynamic living system. Normally, a cassette dosing is applied on rodents first to provide an early glimpse of the ADME properties of a drug in the *in vivo* settings (Korfmacher et al., 2001; Raynaud et al., 2004; Smith, Hayes, Nutley, Raynaud, & Workman, 2004). After that, single dose PK studies using normal or transgenic animals with “knockout” of selective transporters (Schinkel et al., 1994) may be applied to demonstrate such transporters involvement *in vivo*. The latter seems an emerging tool for studying ADME-related transport process. For instance, studies with these mice have established quite conclusively the influence of P-gp on the oral absorption, brain penetration, and elimination of selected drugs (Schinkel, Wagenaar, van Deemter, Mol, & Borst, 1995). However, the use of transgenic animal models has its limitation, especially dealing with transporters with structural similarity and subsequent overlapping functions (Borst, Evers, Kool, & Wijnholds, 1999; Kruh, Belinsky, Gallo, & Lee, 2007). The other issue is the aforementioned dynamic functional status (upregulation) of compensatory transport systems in the various transgenic knockout models. For example, *mdr1* knockout mice deficient in P-gp appear to have enhanced BCRP transport, suggested by the studies of Salama, Kelly, Bui, & Ho (2005), who evaluated the tissue accumulation of the HIV protease inhibitor nelfinavir in both wild-type (WT) and *Mdr1* knockout mice.

In addition to transgenic knockout models, physiological function of transporters under investigation can be blocked by coadministering or preadministering an inhibitor of a transporter to create so called chemical knockout models. In use of those models, the specificity of the employed inhibitor directly determines the validity of the results.

Besides, dose of the inhibitors should ensure sufficient inhibiting activity. By comparing the difference in compound performance pharmacokinetically and/or pharmacodynamically, with inhibitor coadministration versus no inhibitor, one is able to know whether the transporter would effect on the compound's *in vivo* ADME-Tox properties (Polli et al., 1999; Letrent, Pollack, Brouwer, & Brouwer, 1998; Luker, Fracasso, Dobkin, & Piwnica, 1997). Chemical knockout experiments can be performed together with the animal efficacy/pharmacology model, which led to better correlation of the tested results with observations in regular *in vivo* biology experiments. The two types of *in vivo* models can be applied in combination, which is exemplified by successfully elucidation of the priority of P-gp over BCRP in etoposide efflux (Allen, van Dort, Buitelaar, van Tellingen, & Schinkel, 2003).

1.2.2.3 Complexity of ABC transporters and the limitation of the current methods

Coupling various cell-based assays with *in vivo* studies of ADME, one may be able to optimize the identification of transporters and evaluate their potential impact on the ADME properties of drugs in the developmental pipeline. Because the former helps to pre-identify potential compounds that interact with transport systems mimicking different ADME transport barriers and facilitates the latter. However, there is still gap between correlation of the *in vitro* assays results into *in vivo* and human studies. Neither the level of expressed transporter in the isolated *in vitro* systems reflects the actual levels under the physiological environment, nor do the test results show reliable inter-laboratory repeatability and consistency. *In vivo* investigation of transporters using animal models however may represent more closely to conditions in human, at least, under which all

associated types of the transporters are functioning as an integral after drug administration.

Nevertheless, it is because of such functioning concomitantly that leads to the difficulties of uncovering the transporters bodily behavior in ADME processes. Putting aside the different expression levels mentioned above, the reasons can be as follows: 1) for a luckily available inhibitor to a particular type of transporter, its specificity sometimes may be questionable, as it may more or less inhibit other transporters with resembling structures; 2) Similarly, even with the availability of specific knockout animal models, common substrates for transporters make it hard to discrete the impact of a specific transporter on drugs' PK profiles; 3) To compensate the functional loss of knocked out transporters, the possible up-regulated expression levels of backup transporters may further complicate the results; 4) Even with unchanged expression levels, some backup transporters may be more actively involved in the knockout animal models. In one word, in order to make a clear but scientific sound elucidation of the results using knockout models, these concerns should be taken into account during experiment design and mathematic models buildup.

Overall, although transporters are recognized as molecular determinants for drug pharmacokinetics and disposition, the complexity of the living transporter systems and the limitation of the current *in vitro* and *in vivo* tools are insufficient to unwind the mystery. Also, the increasing cost in the drug development may be partially due to such failure of describing and predicting the impact of transporters on drug pharmacokinetics.

The less accuracy of drug PK assessment, the more likelihood of under-/over-estimation of drug pharmacodynamics (PD) profiles leading to subsequently under-therapeutic efficacy or toxicity as well as large inter-individual variability. Therefore, development of tools capable of accurately predicting ADME properties of drug candidates becomes a necessity for reducing the cost for drug development and improving the chances of designing drug candidates with optimal ADME characteristics. Moreover, for the drugs widely available for decades, a clear picture of transporters' effects on their ADME can help obtain a predictable PK profile, which would improve treatment outcome and finally render personalized dosing strategies and therapeutics a possible mission.

1.2.3 MTX as A Model Drug for Characterization of ABC Transporters

For the characterization of the impact of certain ABC transporters on PK profiles of their substrates, MTX is chosen as a model drug due to its following properties. On one hand, MTX is a widely-used antifolate and antimetabolite drug with a large therapeutic dose range, with low dose for autoimmune diseases, such as psoriasis and rheumatoid arthritis (RA) and high dose for various cancers (e.g., acute lymphoblastic leukemia (ALL), osteosarcoma, breast cancer and choriocarcinoma). On the other hand, being a Class III drug in BCS and extended BDDCS systems, MTX is known to be involved with ABC transporter interactions at brush boarder of organs for oral absorption, distribution and elimination through urine or bile. Therefore, ABC transporters appear to influence MTX pharmacokinetics.

1.2.3.1 Mechanism of action of MTX

MTX is classified as an antifolate that is widely used in neoplastic and non-malignant diseases due to its antiproliferative and immunosuppressive functions. The biochemical scheme in which MTX acts is illustrated in Figure 4 (modified from Assaraf, 2006). When MTX, a structure analogue of folic acid, is transported into the cell, it acts to inhibit dihydrofolatereductase (DHFR) and thymidylate synthase (TS), the key enzymes in folate metabolism (Johnson et al., 1997; Chu, Drake, Boarman, Baram, & Allegra, 1990). Such inhibition to the enzymes in folic acid metabolism leads to the cessation of DNA replication and cell death by disrupting purine and pyrimidine nucleotide biosynthesis. A recent study also proposed that MTX may have a novel function of inhibiting histone deacetylase (HDAC), a class of enzymes associated with epigenetic silence of tumor suppressor genes (Pan, Lu, & Huang, 2007), as a potential HDAC inhibitor for cancer treatment (Yang et al., 2010). Inhibition of those HDACs may thus restore the expression of tumor suppressor genes, resulting in cell differentiation, growth arrest and apoptosis. Once intracellular MTX undergoes an anabolic process of polyglutamylation, additional glutamate residues are sequentially added and catalyzed by folate polyglutamylate synthase (FPGS) (see Figure 4). The polyglutamylation process produces MTX polyglutamylate conjugates (MTX-PG), which can no longer cross the lipid bilayers as substrates of those transporters that deliver MTX. MTX-PGs exert similar or enhanced inhibitory function of de novo purine synthesis, increasing cellular retention and subsequent toxicity. Hence, leucovorin (5-CHO-THF, a metabolite of folic acid) is often co-administrated with high dose MTX regimens in cancer patients to rescue the depleted folic acid pools.

In addition to MTX anticancer actions mainly related to folate homeostasis, its use at lower doses as an anti-inflammatory agent has been related to other mechanisms, such as the increase of apoptosis of mitogen-activated T cells (Genestier et al., 1998), the increase of endogenous adenosine release (Cronstein, Naime, & Ostad, 1993; Morabito, Montesinos, & Schreiber, 1998), the alteration of expression of cellular adhesion molecules (Johnston, Gudjonsson, Sigmundsdottir, Ludviksson, & Valdimarsson, 2005; Yamasaki, Soma, Kawa, & Mizoguchi, 2003; Dolhain et al., 1998), and the influence on production of cytokines (Neurath et al., 1999; Herman, Zurgil, Langevitz, Ehrenfeld, & Deutsch, 2008), humoral responses (Segal, Yaron, & Tartakovsky, 1990), and bone formation (Carbone et al., 1999; El Miedany, Abubakr, & El Baddini, 1998).

1.2.3.2 Pharmacokinetic characteristics of MTX: ADME-tox

To understand the pharmacokinetic properties of MTX, a brief review of its physicochemical properties is needed. As is said earlier, MTX belongs to BCS and BDDCS class III with high solubility, low permeability, and predictive transporters involvement especially in oral absorption. Being a weak dicarboxylic acid with pKa of about 4.8 and 5.5, MTX is mostly ionized at physiologic pH, but not in GI tract, thus affecting oral absorption. IC₅₀ of MTX toward human or murine leukemia (McGuire et al., 1994; Zhao, Assaraf, & Goldman, 1998) and human neck squamous cell carcinoma cell lines (Van der Laan et al., 1992; Braakhuis, Jansen, Noordhuis, Kegel, & Peters, 1993) decreased as a function of exposure time, indicative of low permeability of MTX

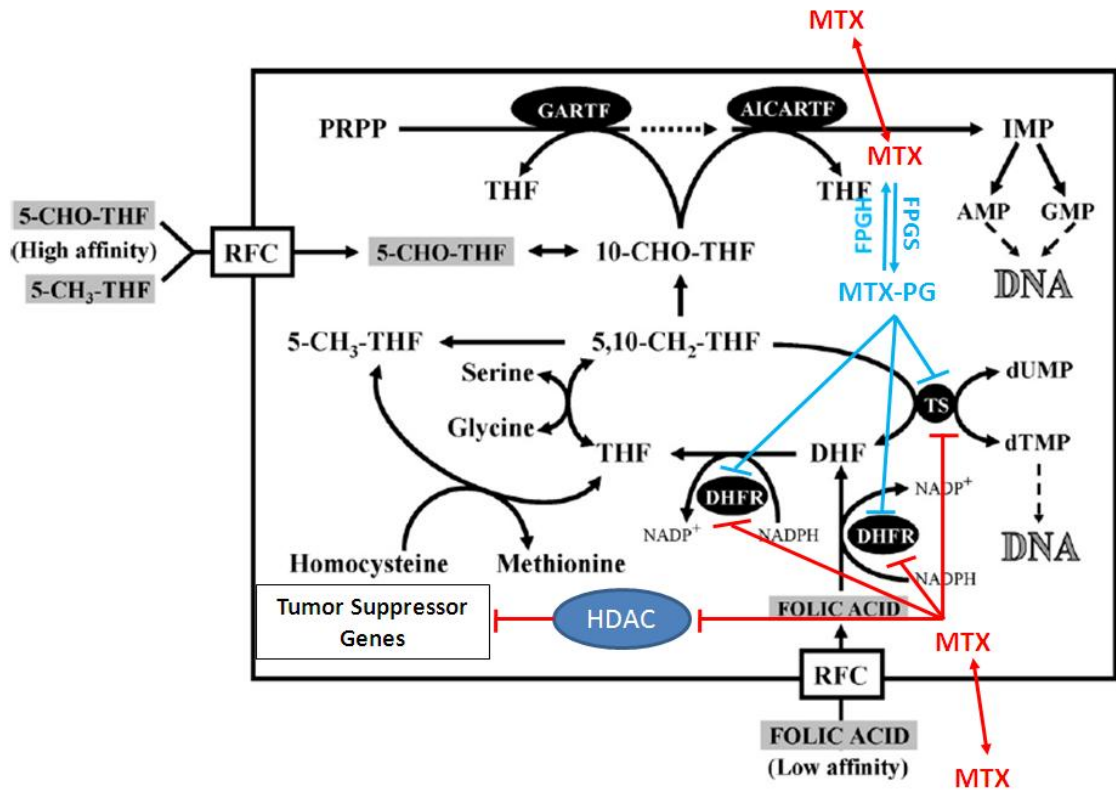


Figure 4. Mechanisms of action of MTX as an anti-cancer drug (modified from Assaraf, 2006).

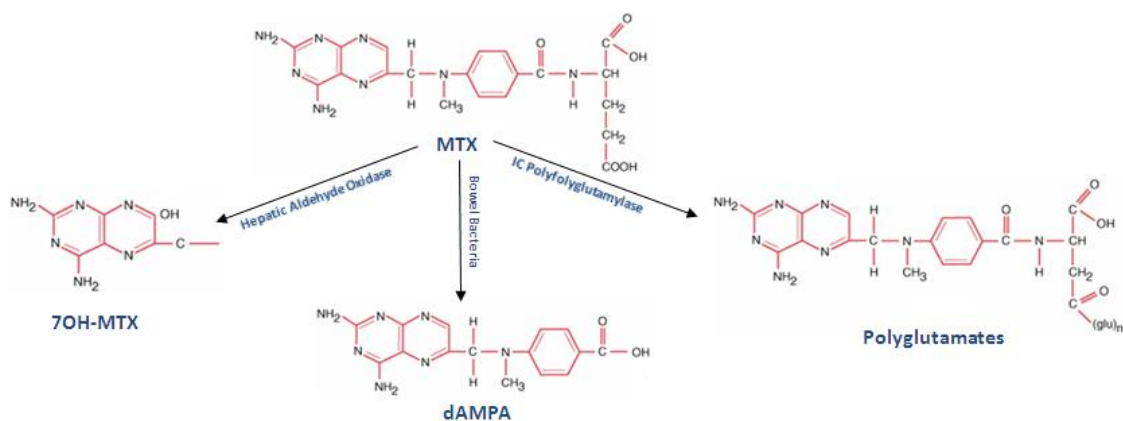


Figure 5. Metabolic pathways of MTX.

after oral doses possibly due to interaction with specific transporters, which was in agreement of its classification in class III of BDDCS system.

Oral absorption of MTX is low and variable, based on its low permeability and interaction with transporters. Following IV injections, the steady-state volume of distribution of MTX is 0.4 to 0.8 L/kg of body weight (Huffman et al., 1973), due to ~50% of albumin binding and highest tissue distribution in kidney and liver. The variability in kinetics after high IV dose of MTX may also be attributed to interaction with apical ABCC2. For example, at IV doses of 30 to 80 mg/m², 0.4% to 20% of the administered dose undergoes ABCC2-mediated biliary excretion, one of the major

elimination routes of MTX. Less than 10% of MTX is recovered in the feces collected over 24 hours (Creaven et al., 1973). Renal elimination is another major and maybe primary elimination route for MTX; usually large variability of unchanged MTX was found in urine. Whereas, based on a bile-ligation study on rodents, it is also speculated that some of MTX may be recycled under enterohepatic pathway which elongate its elimination time (Said, Strum, & Hollander, 1984).

MTX can be hydrolyzed in the GI tract by bacterial flora to pterate (4-amino-4-deoxy-N¹⁰-methyl pteric acid, dAMPA), an inactive metabolite (Shen & Azarnoff, 1978); while in its major site of metabolism, hepatic aldehyde oxidase (a phase I enzyme) is responsible for the formation of another pharmacologically inactivate yet toxic metabolite—7OH-MTX (Figure 5). Due to lower solubility compared with MTX, 7OH-MTX contributes to renal toxicity associated with high dose MTX regimen. Another metabolite, polyglutamate forms, also contributes to MTX toxicity due to the largely enhanced cellular retention, especially after MTX high dose regimen. Such cellular retention of polyglutamate coupled with folate metabolism inhibition leads to liver dysfunction or intestinal injury, the two major types of clinical toxicity of MTX. However, the molecular mechanisms of such effects are still under investigation. Another type of MTX's toxicity may come from its harm to hematopoietic system and central nervous system (CNS). Based on all the above toxicity that MTX can cause, clinical application of MTX has to be monitored for safety purpose.

1.2.3.3 The interactions of MTX with influx transporters

Being a folic acid analogue, MTX was verified to be transported through RFC (SLC19A1) in L1210 leukemia cell lines with a high affinity yet low capacity (Henderson, Grzelakowska-Sztabert, Zevely, & Huennekens, 1980; Spinella, Brigle, Sierra, & Goldman, 1995), which indicated that other transporters in key eliminating organs with relative high capacities may have more clinical relevance for MTX PKs. With the help of studies on rat kidney slice, it was found that rOat3, together with rRfc-1, may most likely account for the basolateral MTX uptake into proximal tubule with high affinity (Nozaki, Kusuhara, Endou, & Sugiyama, 2004). Human hOAT1, hOAT3 and hOAT4 also transport MTX. hOAT1 and hOAT3 were demonstrated by uptake experiments through S2 cells containing OATs genes derived from the human proximal tubule (Takeda et al., 2002). Indirect inhibition of uptake studies also showed inhibited uptake of OAT4 substrate (i.e. EE2, 17 α -Ethinylestradiol) with incubation of different concentrations of MTX in HEK-293 cells which overexpressed OAT4 transporters (Han et al., 2010). Other OATs in kidney, such as OAT-K1 and OAT-K2 of the rat SCL21 family, were indicated their possible roles in urinary secretion of MTX given the transport capability in MDCK cells (Masuda, 2003). Hepatic OATP1B1 were also found to transport MTX *in vitro* (Abe et al., 2001; Sasaki et al., 2004) and further confirmed *in vivo*. The *in vivo* study exhibited a 1.5-fold decrease of plasma AUC and ~2-fold increased liver disposition in transgenic mice compared to WT mice (van de Steeg et al., 2009). Another OATP member, OATP1A2 transports MTX with a Michaelis Menten curve in oocytes expressing OATP1A2. OATP1A2 was thus speculated for active tubular reabsorption of MTX and MTX-induced toxicity because of its localization on distal

tubule and other tissues pertinent to MTX disposition and toxicity (Badagnani et al., 2006).

Intestinal proton coupled folate transporter/haem carrier protein (PCFT/HCP1), using H⁺ gradient as a driving force, was recently discovered as an apically expressed influx transporter for folate and its analogues, including MTX (Selhub & Rosenberg, 1981; Said & Redha, 1987; Said, Ghishan, & Redha, 1987; Qiu et al., 2006; Mason, Shoda, Haskell, Selhub, & Rosenberg, 1990; Chiao, Roy, Tolner, Yang, & Sirotnak, 1997; Li, Tomimatsu, Ito, & Horie, 2003; Nakai et al., 2007), which suggests an important role for PCFT/HCP1 in the intestinal absorption of MTX like RFC (Selhub & Rosenberg, 1981; Qiu et al, 2006; Yokooji, Murakami, Yumoto, Nagai, & Takano, 2007). Further observations that consisted of an elevated mucosal MTX influx rate at pH 5.5 in everted rat intestine and significantly higher expression level in proximal small intestine relative to that in distal intestine indicated a site-specific contribution of this transporter in intestinal absorption of MTX (Yokooji, Mori, & Murakami, 2009).

1.2.3.4 The interactions of MTX with efflux transporters

The first direct evidence that certain members of the MRP (ABCC) family may be able to transport the hydrophilic antifolate MTX was documented about 15 years ago, when Masuda et al. (1997) demonstrated that MTX underwent biliary excretion in WT rats but not in EHBR rats that are deficient in ABCC2 function. This breakthrough aroused further in vitro investigations to screen other MRP (ABCC) family members. ABCC1-5 were demonstrated to transport MTX according to Michaelis Menten kinetics

in accumulation studies with various MRP overexpressing cells (Hooijberg et al., 1999; Wielinga et al., 2005; Assaraf, 2006). For instance, compared to parental HEK293 cells, HEK293 cells overexpressing ABCC5 exhibited 2.3-fold less accumulation of [³H]MTX after 24h (Wielinga et al., 2005). Furthermore, it was shown that MTX transport could be appreciably altered when the amino acid composition of the transmembrane helices of MRP members were changed. Substitution or mutation of a highly conserved Trp1254 (tryptophan residue at position 1254) in the last transmembrane segment (TM17) resulted in complete loss of MTX transport by ABCC1 and ABCC2 (Ito et al., 2001). In contrast to short-time drug exposure used for the above transporter identification studies, long-period (72 h) exposure produced insignificant difference to many anti-folates including MTX. This strongly indicated the occurrence of intracellular polyglutamylation (Hooijberg et al., 1999; Wijnholds et al., 2000; Wielinga et al., 2005), because the longer the polyglutamate chain, the poorer MRP-dependent antifolate efflux would be. Except ABCC5 can transport MTX di-glutamate, the members of ABCC1-4 are only able to extrude monoglutamate form—MTX (Wielinga et al., 2005). Recently, ABCC11 (MRP8) was found to be able to deliver MTX as well (Chen, Guo, Belinsky, Kotova, & Kruh, 2005), whose structure closely resembles ABCC5 (Bera, Lee, Salvatore, Lee, & Pastan, 2001; Tammur et al., 2001; Yabuuchi, Shimizu, Takayanagi, & Ishikawa, 2001). Compared to ABCC subfamily members, the first report of ABCG2 implying its capability of transporting MTX was published till 2000. Enhanced ATP-driven efflux of MTX was shown in ABCG2 overexpressing MCF-7/MX cells and it can be reversed by the treatment of an ABCG2 inhibitor GF120918 and fumitremorgin C (Volk et al., 2000). Interestingly, MCF-7/MX vesicles also transport significant amounts of MTX di- and tri-

glutamate conjugates upon a 60-min exposure of 400 μM [^3H]MTX, although such transports decreased gradually with the increase of polyglutamate chain (Volk & Schneider, 2003). The transport pathway of MTX and its polyglutamate conjugates by ABCs and ABCG2 can be vividly summarized by the following figure (see Figure 6). The detailed transport kinetic properties of the different effluxers for MTX are illustrated in the Table 3 (modified from Assaraf, 2006; Chen et al., 2005). With low affinities and high capacities, the ABC transporters established their clinical relevance, as high (1 mM) MTX plasma concentrations could be achieved during certain chemotherapeutic treatments in human (Sczesny, Hempel, Boos, & Blaschke, 1998). Given the 100-fold lower capacity of the RFC (Assaraf & Goldman, 1997), it would be no surprise, if these ABC transporters are likely to have impact on MTX bioavailability and disposition. A typical demonstration of the hypothesis was the aforementioned *in vivo* evidence of loss of biliary excretion of MTX in MRP2-deficient rats (Masuda et al., 1997).

Several other *in vivo* studies using specific Mrp knockout mouse models also indicated that the absence of certain Mrps were associated with the alteration of MTX PK profiles. Yet the transporters approved to deliver MTX *in vitro* may not significantly affect ADME or bioavailability of MTX *in vivo*. For example, Sugiyama's group (Kitamura et al, 2008) observed elevated biliary clearance (from 15 to 23 m/min/kg) and a decreased fraction of absorption in *Abcc3*^{-/-} mice compared to WT mice, after an oral dose (2.2 $\mu\text{mol/kg}$) and steady state infusion (0.22 $\mu\text{mol/h/kg}$), but not in *Abcc4*^{-/-} strain, although both types of effluxers are located at the basolateral side of hepatocytes. On the contrary, *Abcc2* was found as an important determinant for liver disposition and bile flow

(Chu et al., 2006) and further assured as an important player in MTX PKs by different *in vivo* studies with *Abcc2* knockout mouse models (Chu et al., 2006; Vlaming et al., 2006). A demonstration was a 1.8-fold higher AUC (area under the curve) of MTX plasma concentration profile in *Abcc2*^{-/-} mice relative to WT strain after intravenous 50 mg/kg [³H]MTX (Vlaming et al., 2006). Further evaluation of protein and levels revealed 2-fold expression level of hepatic *Abcc3*, a basolateral backup, as well as increased *Abcc4* level in kidney and liver in *Abcc2*^{-/-} strain relative to WT strain (Vlaming et al., 2006), which seemed the underline mechanisms, though controversial from what Chu et al. (2006) found yet with mice of a different genetic background. Recently, detection of other ABC transporters, such as *ABCC3* and *ABCG2*, on MTX PKs were conducted by the same group, using single, double and triple knockout strains after 50 mg/kg intravenous and oral doses of MTX (Vlaming et al., 2008, 2009a&b, 2011), demonstrating *Abcc2* and *Abcg2* may have a more important role in MTX disposition than *Abcc3* of an imperceptible role, which may conflict with the previous observation of reduced AUC and oral bioavailability in *Abcc3*^{-/-} mice at a lower dose (Kitamura et al, 2008). Therefore, further comprehensive investigations on ABC transporters are still needed.

Consistent with *in vivo* studies, *ABCC2* had gained its clinical relevance by the observation of severe impairment of MTX elimination in ALL female patients with a non-functional *ABCC2* variant (a frequent gender-specific -24C > T polymorphism) given a high dose infusion of 5 g/m² of MTX (Rau et al., 2006). Therefore, genetic polymorphism resulting in functional loss of key transporters may subsequently influence

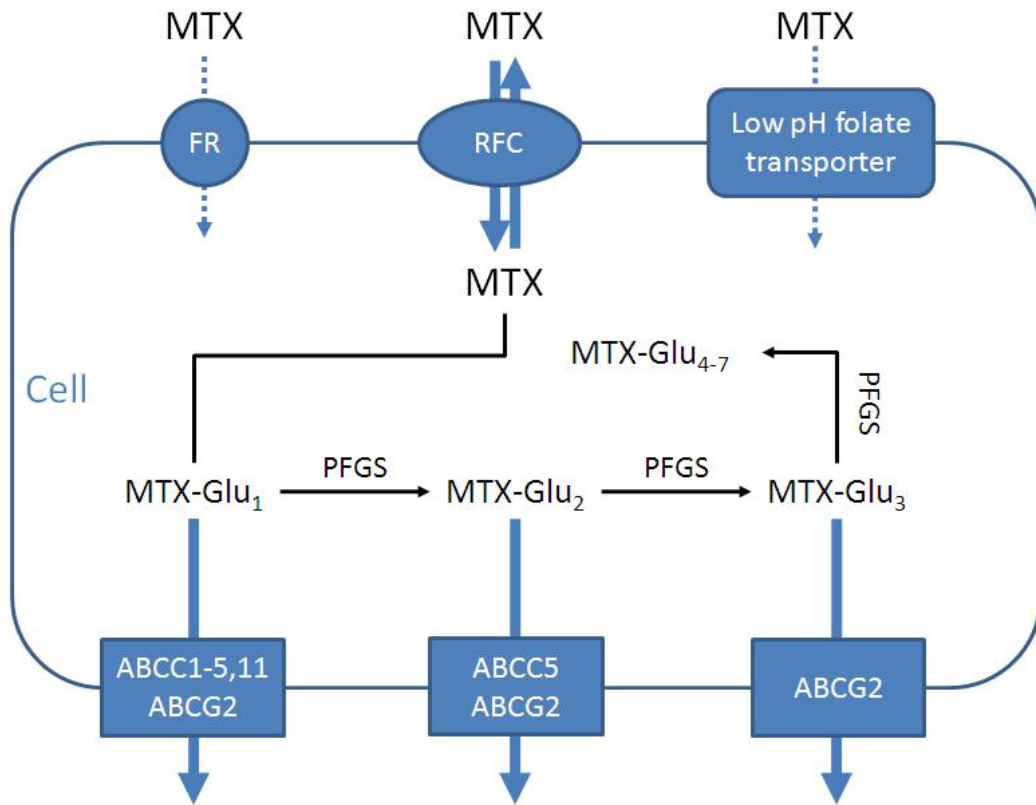


Figure 6. Summarized figure for transport routes for both MTX and its cellular polyglutamate forms (modified from Assaraf, 2006; Kruh et al., 2007)

Table 3. Summarized table of transport kinetic properties of the different efflux ABC transporters for MTX

ABC Transporter	MTX		References
	K_m (mM)	V_{max} (pmol/mg/min)	
MRP1/ABCC1	2.15 ± 0.79	2050 ± 910	Zeng et al., 2001
MRP2/ABCC2	2.5-3	~1000	Bakos et al., 2000
MRP3/ABCC3	0.62 ± 0.23	2930 ± 320	Zeng et al., 2001
MRP4/ABCC4	1.3 ± 0.2	430 ± 30	van Aubel et al., 2002
	0.22 ± 0.01	240 ± 50	Chen et al., 2002
MRP5/ABCC5	1.3 ± 0.3	780 ± 70	Wielinga et al., 2005
MRP8/ABCC11	957 ± 28	317 ± 17	Chen et al., 2005
BCRP (R482)	0.681 ± 0.18	2384 ± 445	Volk & Schneider, 2003
/ABCG2	1.34 ± 0.18	687 ± 87	Chen et al., 2003

MTX disposition and produce toxicity in human, and also may contribute to the inter-patients variability.

In conclusion, multiple influx and efflux transporters are involved in the transport of MTX across the biological membranes in the body. Among those transporters, some may have pharmacokinetic and toxicological implications in clinical, due to the existence of genetic variants that lead to the function loss of those associated transporters and inter-patient variability. Therefore, to elucidate the fate of MTX in the body confronting of different types of transporters is difficult; further investigations and strategies may be needed for better description of drug disposition and prediction in therapeutic treatment.

1.3 Objectives of Study

Characterization of physiological and biochemical determinants of a drug's PK properties is crucial in developing efficacious therapeutic treatment regimens particularly for drugs with narrow therapeutic indexes, such as MTX. A large body of evidence has demonstrated that membrane transporters, especially efflux transporters belonging to the ABC transporter family, are key determinants of anticancer drug disposition. Further it has been shown that MTX is a substrate for ABC transporters; notably ABC2, ABCC3 and ABCG2, involved in gastrointestinal absorption and hepatobiliary disposition, both biliary excretion and extrusion into plasma from hepatocytes. However, complete characterization that includes analysis of dose-dependent effects of the impact of those efflux transports on MTX has not been investigated. The objective of the current project was to characterize the role of both Abcc2 and Abcc3 on the PKs of MTX. In order to fulfill the above objective dose-dependent PK studies of MTX were completed in WT,

single *Abcc2* and *Abcc3* gene knockout mice. Low (10 mg/kg), medium (50 mg/kg) and high (200 mg/kg) doses were given to all the strains intravenously and orally. Prior to the PK studies, each mouse had implanted a carotid artery vascular cannula to facilitate serial collection of small blood volumes and thus, each animal provided a complete MTX concentration-time profile. For the PK study, each mouse was placed in individual metabolism cages that allow urine and feces to be collected separately. The plasma, urine and fecal samples were analyzed for MTX and its 7OH-MTX metabolite by LC/MS/MS and the resultant measurements provided the basis for both noncompartmental and compartmental data analysis. Through these analyses, a clear understanding of the role of *Abcc2* and *Abcc3* on the PKs of MTX was obtained.



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April 16, 2012

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Dear Zhan Wang:

This is to grant you permission to include the following article in your thesis entitled "Impact of Multidrug Resistance-Associated Protein 2 (MRP2/ABCC2) and 3 (MRP3/ABCC3) on the Pharmacokinetics of Methotrexate" for Temple University:

Zhan Wang, Qingyu Zhou, Gary D. Kruh, and James M. Gallo, Dose-Dependent Disposition of Methotrexate in *Abcc2* and *Abcc3* Gene Knockout Murine Models, *Drug Metab Dispos* November 2011 39:2155-2161

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CHAPTER 2
EVALUATION OF ABCC2 AND ABCC3
TRANSPORTERS IN THE PHARMACOKINETICS OF
METHOTREXATE AFTER INTRAVENOUS DOSING

2.1 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 & 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 & 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 & 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 CNS lymphoma (Deangelis & Iwamoto, 2006), acute lymphoblastic leukemia (ALL) (Chessells, Leiper, Tiedemann, Hardisty, & Richards, 1987), and other neoplastic diseases (Rizzoli, Mangoni, Caramatti, Degliantoni, & Costi, 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 & 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.

2.2 Material and Methods

2.2.1 Animals

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 (See Appendix A). 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.

2.2.2 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 °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 °C until analysis.

2.2.3 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 μ L aliquot into the LC/MS/MS system. The chromatographic system consisted of a C18 guard cartridge (4.0 \times 2.0 mm, Phenomenex, Torrance, CA) and analytical column (50 \times 2.0 mm, 3 μ m particle size, Phenomenex) set at an operation temperature of 35 $^{\circ}$ 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 \rightarrow 308.0, 7OH-MTX m/z 471.0 \rightarrow 324.2, and aminopterin 441.1 \rightarrow 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 μ L) that was deproteinized by 4-fold volume of methanol containing the internal standard aminopterin (1 μ g/mL). After centrifugation (14,000 rpm \times 5 min), an aliquot of 10 μ L of supernatant was further diluted 100 times with the mobile phase and a 5 μ L 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 μ L aliquot of the resultant homogenate was deproteinized with 180 μ L of methanol containing the internal standard aminopterin (2 μ g/mL). After centrifugation (14,000 rpm \times 5 min), an aliquot of 2 μ L of supernatant was further diluted 500 times with double distilled water and then mixed with a 5 μ L aliquot injected into the LC/MS/MS system. The limits of quantitation were 2.74 and 2.31 μ g/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.

2.2.4 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_r 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.

2.3 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, $Abcc2^{-/-}$ and $Abcc3^{-/-}$ mouse strains. MTX plasma concentration-time profiles for each strain and dose level are illustrated in Figure 7, in which a number of 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 (Figure 8). 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 (Balis, Holcenberg, & Bleyer, 1983). The PK parameters are summarized in Table 4.

2.3.1 Interstrain Analysis

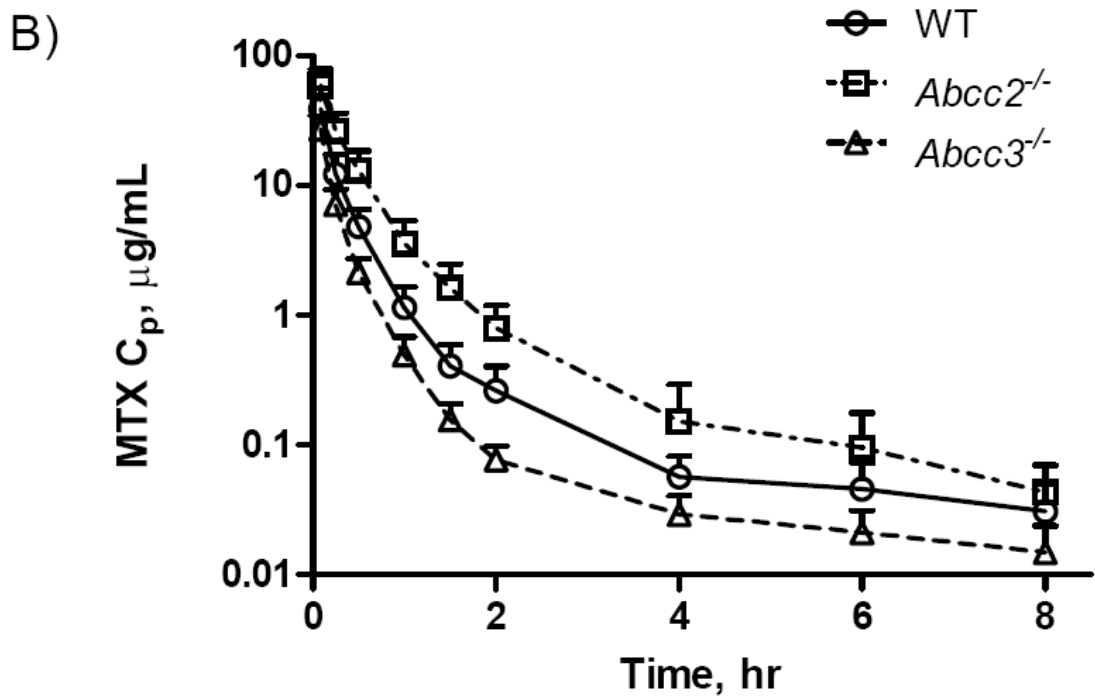
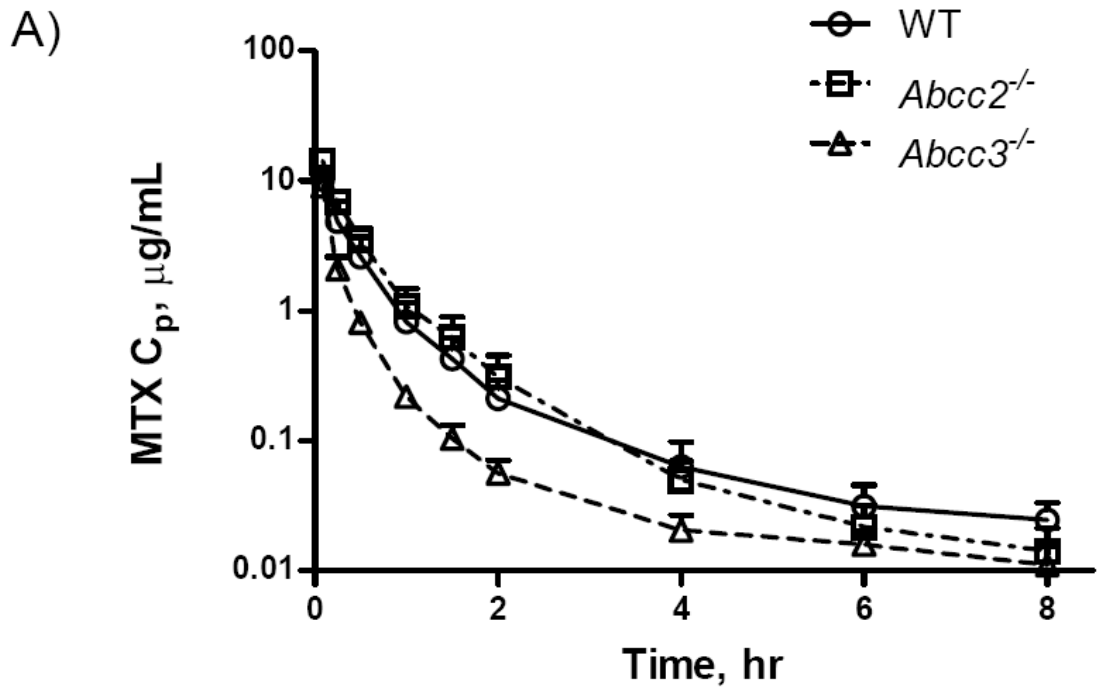
Interstrain analysis indicated a number of differences in the PK parameters. Relative to WT mice, *Abcc2*^{-/-} mice exhibited significantly higher AUC ($P < 0.01$) and reduced CL ($P < 0.05$ for low and high doses; $P < 0.01$ for middle dose) values at all dose levels that are attributed to the inability of *Abcc2*, located on the apical membrane of bile canaliculi as well as renal proximal tubule 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 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

Table 4. Pharmacokinetic parameters (mean \pm SD) of MTX following IV administration of MTX at three dose levels for WT, *Abcc2*^{-/-} and *Abcc3*^{-/-} mice

Parameters	Dose = 10 mg/kg	Dose = 50 mg/kg	Dose = 200 mg/kg
WT mice	n = 8	n = 10	n = 11
CL (mL/min/kg)	33.8 \pm 10.2 [†]	68.1 \pm 31.1 [†]	53.8 \pm 16.4
V_d (L/kg)	11.5 \pm 6.2	21.1 \pm 14.0	58.1 \pm 29.8 [‡]
t_{1/2} (min)	229.2 \pm 92.6	211.7 \pm 97.6	730.2 \pm 101.0 [‡]
AUC_{MTX} (min*μg/mL)	308.4 \pm 79.0	842.9 \pm 298.2	4051.1 \pm 1304.3
AUC_{7OH-MTX} (min*μg/mL)	13.1 \pm 5.1	23.2 \pm 12.1	96.1 \pm 43.9
AUC_{m/p} (%)	4.1 \pm 1.0 [†]	3.0 \pm 1.5	2.0 \pm 0.7 [†]
Renal fraction of dose (f_r, %)	35.3 \pm 15.0	43.4 \pm 3.0	59.7 \pm 6.9 [‡]
Fecal fraction of dose (f_f, %)	1.1 \pm 0.7 [‡]	12.4 \pm 6.8	8.6 \pm 5.9
<i>Abcc2</i>^{-/-} mice	n = 9	n = 14	n = 7
CL (mL/min/kg)	23.0 \pm 5.7 ^{**‡}	35.1 \pm 12.4 ^{**}	35.3 \pm 5.4 [*]
V_d (L/kg)	4.4 \pm 1.5 ^{**}	8.0 \pm 3.1 ^{**}	95.8 \pm 34.7 ^{**‡}
t_{1/2} (min)	131.7 \pm 37.4 ^{**}	166.2 \pm 70.7	1928.8 \pm 738.2 ^{**‡}
AUC_{MTX} (min*μg/mL)	412.5 \pm 62.7 ^{**}	1572.5 \pm 520.3 ^{**}	5607.1 \pm 686.0 ^{**}
AUC_{7OH-MTX} (min*μg/mL)	50.9 \pm 15.7 ^{**}	227.3 \pm 94.1 ^{**}	955.1 \pm 202.4 ^{**}
AUC_{m/p} (%)	11.3 \pm 3.5 ^{**}	15.8 \pm 6.2 ^{**}	17.0 \pm 2.6 ^{**}
Renal fraction of dose (f_r, %)	101.1 \pm 9.9 ^{**‡}	79.8 \pm 19.8 ^{**}	76.8 \pm 12.2
Fecal fraction of dose (f_f, %)	0.7 \pm 0.5 [‡]	6.1 \pm 3.3 [*]	7.8 \pm 4.1
<i>Abcc3</i>^{-/-} mice	n = 7	n = 8	n = 6
CL (mL/min/kg)	55.2 \pm 8.3 ^{**†}	100.0 \pm 22.8 ^{*†}	32.6 \pm 2.5 ^{**†}
V_d (L/kg)	21.4 \pm 8.6 ^{**}	35.4 \pm 14.0 [*]	30.3 \pm 7.5 [*]
t_{1/2} (min)	264.8 \pm 88.1	269.9 \pm 150.7	646.5 \pm 166.0 [‡]
AUC_{MTX} (min*μg/mL)	180.8 \pm 31.8 ^{**}	516.7 \pm 11.4 [*]	6153.8 \pm 472.5 ^{**}
AUC_{7OH-MTX} (min*μg/mL)	3.7 \pm 0.8 ^{**}	2.6 \pm 1.1 ^{**}	43.0 \pm 10.4 [*]
AUC_{m/p} (%)	2.1 \pm 0.6 ^{**‡}	0.5 \pm 0.2 ^{**}	0.7 \pm 0.1 ^{**}
Renal fraction of dose (f_r, %)	33.3 \pm 12.6	34.1 \pm 3.3 ^{**}	46.0 \pm 4.4 ^{**‡}
Fecal fraction of dose (f_f, %)	5.9 \pm 4.9 ^{*†}	24.9 \pm 9.9 ^{**†}	16.0 \pm 9.1

* ** 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* < 0.05), with “†” indicating differences between designated dose levels, and “‡” indicating differences from the other two dose levels.



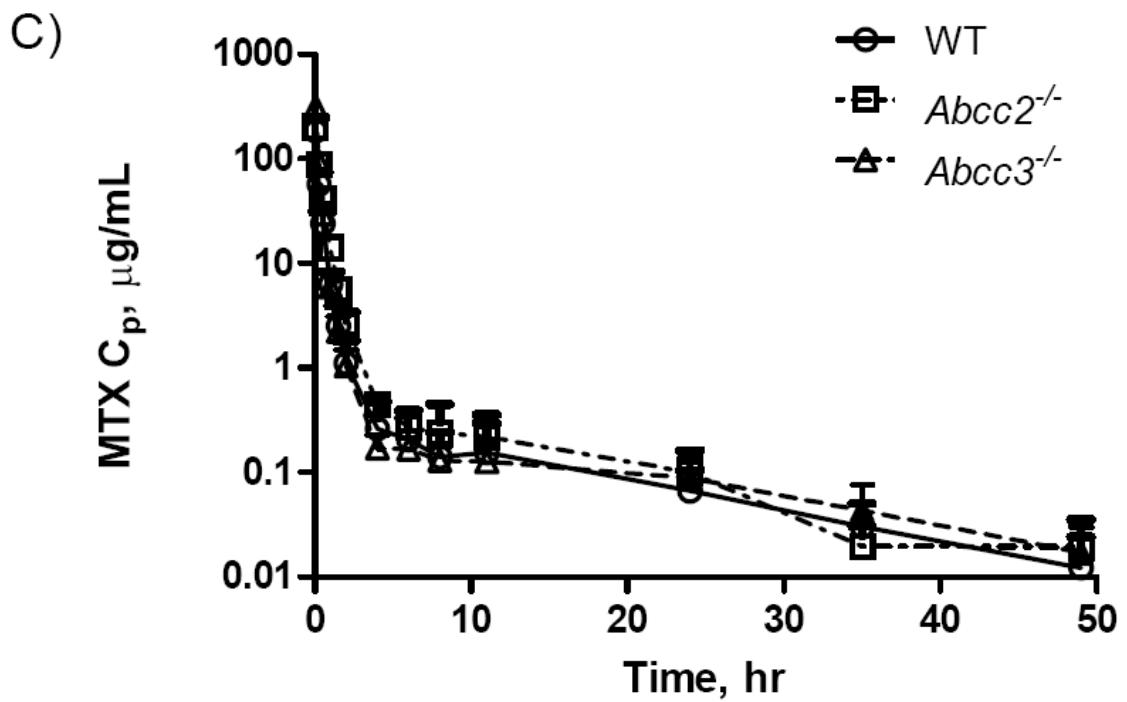


Figure 7. Plasma concentrations (C_p , mean \pm SD; semi-log scale) of MTX as a function of time after IV bolus administration of single doses of MTX at 10 (A), 50 (B) and 200 (C) mg/kg to male WT (diamond), *Abcc2*^{-/-} (square) or *Abcc3*^{-/-} (triangle) mice.

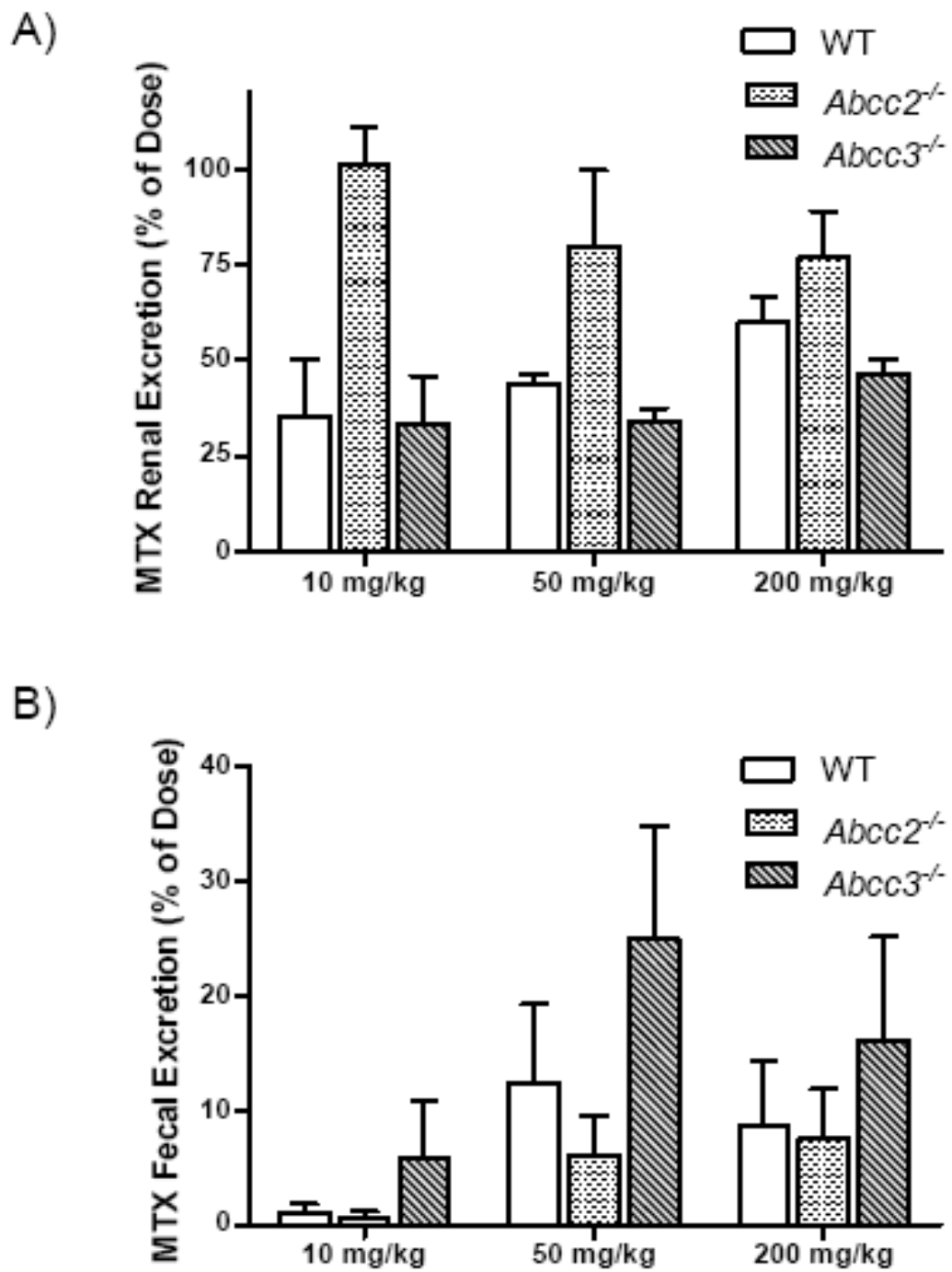


Figure 8. Renal (A) and fecal excretion (B) of MTX (expressed as % of dose) in WT, *Abcc2*^{-/-} and *Abcc3*^{-/-} mice at three different dose levels.

MTX (see % AUC_{m/p}, Table 4), 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 V_d between 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, Lawrence, Stuart, & McNeill, 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 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

4). *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 4). 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 V_d 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 V_d was seen in the *Abcc3*^{-/-} group.

2.3.2 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 4, Figure 9). 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 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 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 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 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

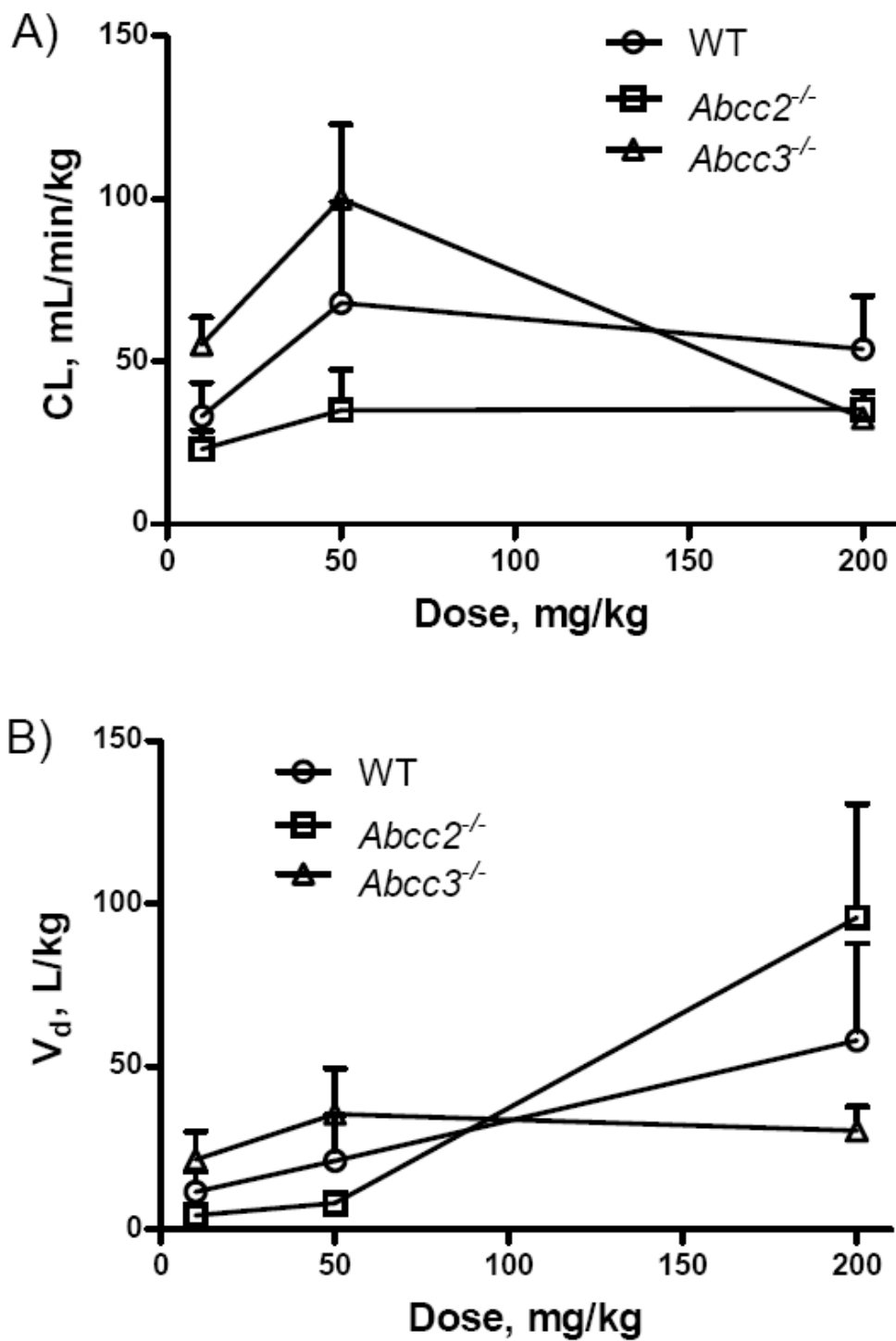


Figure 9. Total clearance (CL) (A) and volume of distribution (V_d) (B) (mean \pm SD) of MTX as a function of dose to different mouse strains.

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.

2.4 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, Liu, Yang, Miwa, & Gan, 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*^{-/-} 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*^{-/-} 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.

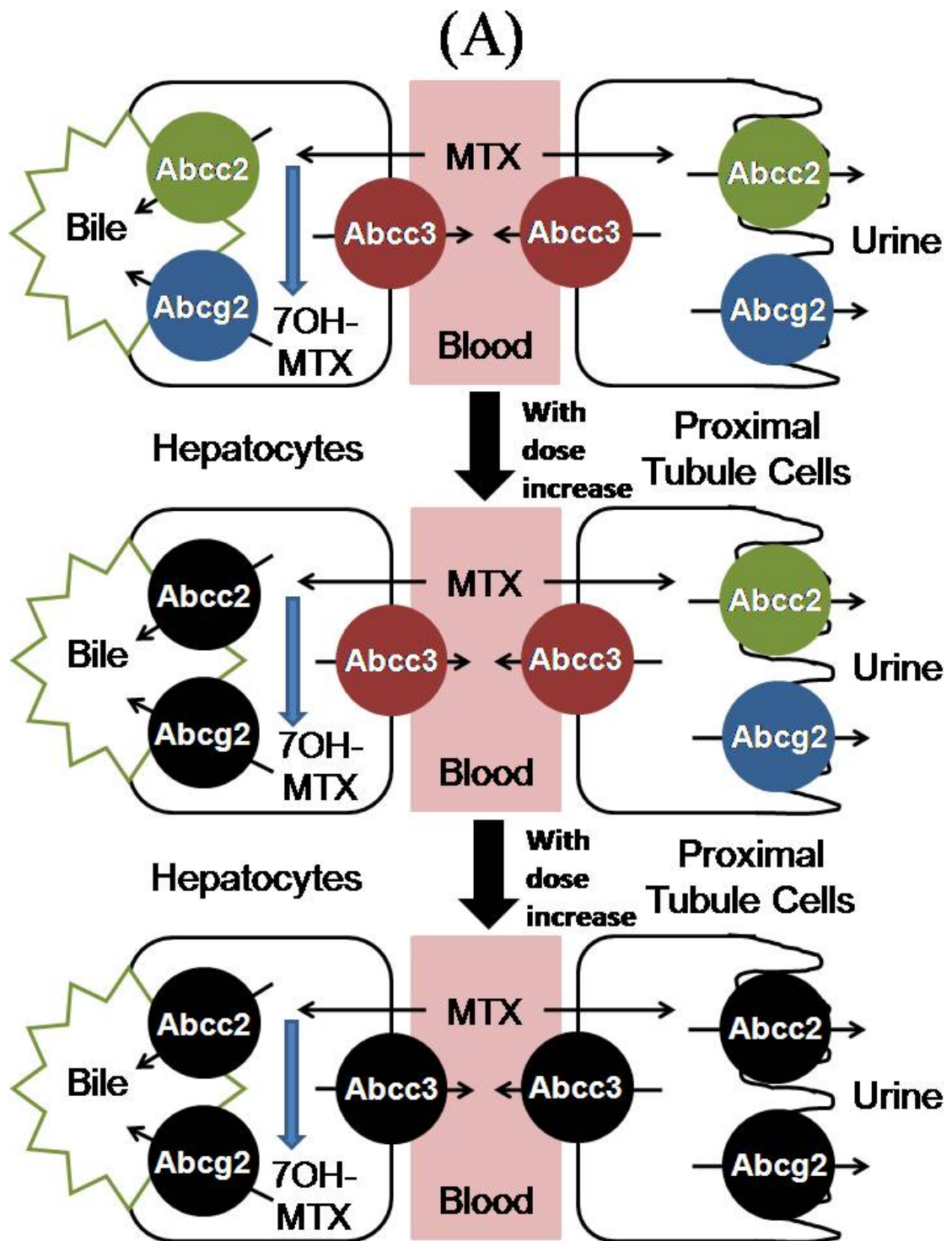
The increased MTX clearance observed at the 50 mg/kg dose is accompanied by increased fecal excretion that implicates *Abcg2* as a biliary excretory pump. The recent studies by Vlaming et al. (2008, 2009a) in both single and double (*Abcc2;Abcg2*^{-/-}) knockout mice have demonstrated the key role *Abcg2* plays in MTX's biliary clearance. We observed additional compensatory elimination methods for MTX in the *Abcc2*^{-/-} 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.

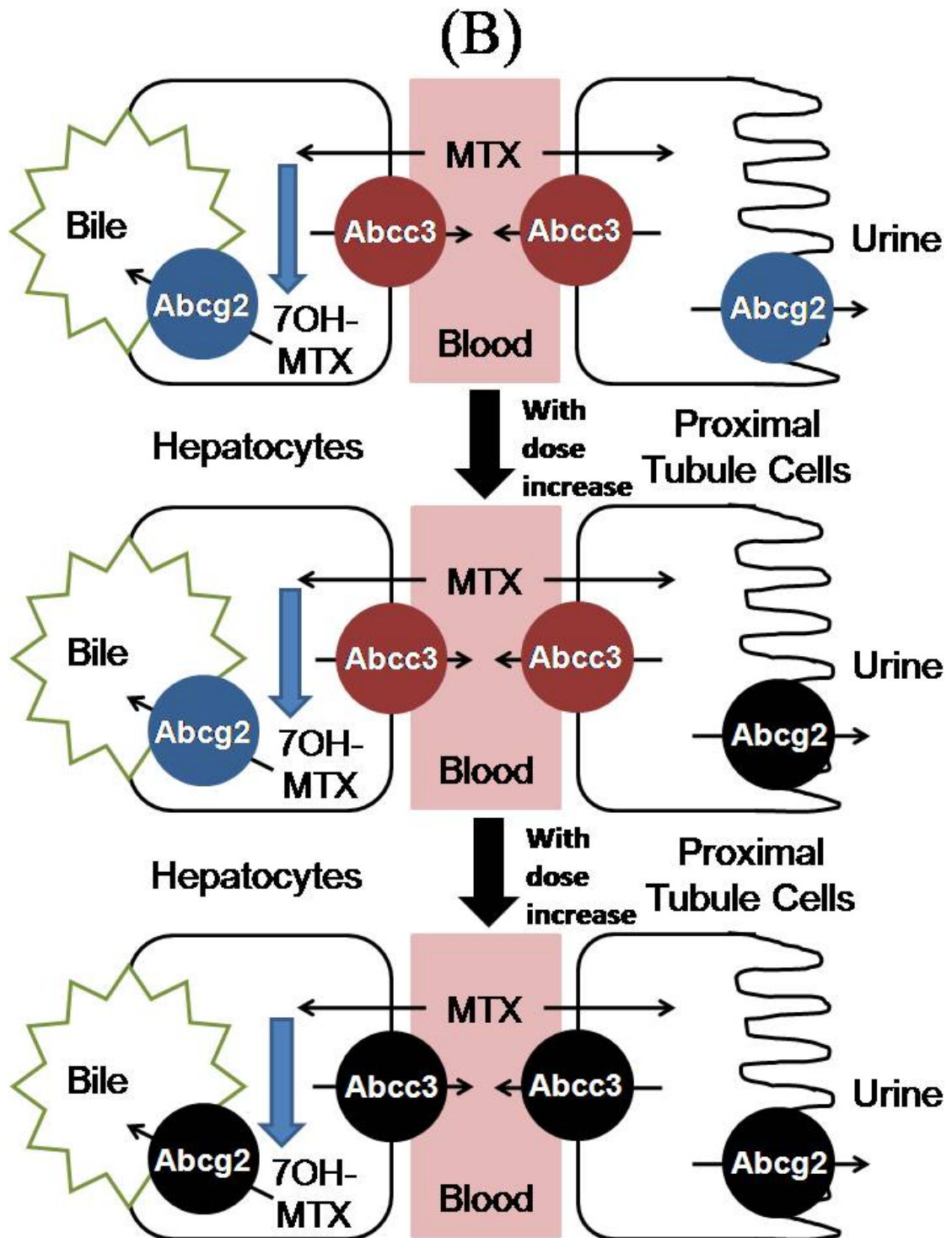
Although there were concordant increases in MTX's CL in going from 10 mg/kg to 50 mg/kg amongst the three mouse strains, there was either a reversal or no change at the high 200 mg/kg MTX dose depending on the strain. At this high dose level in the *Abcc3*^{-/-} strain active transport of MTX into bile 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*^{-/-} 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*^{-/-} 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*^{-/-} 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 than what was observed in the *Abcc2*^{-/-} strain where substantial conversion to 7OH-MTX was seen (~20-fold increase in the AUC_{7OH-MTX} compared to *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, Kristiansen, Olsen, Aarbakke, & Lindal, 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 & Jacobsen, 1981; Pratt, Ruddon, Ensminger, & Maybaum, 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 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 (Ranganathan 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 Figure 10). The





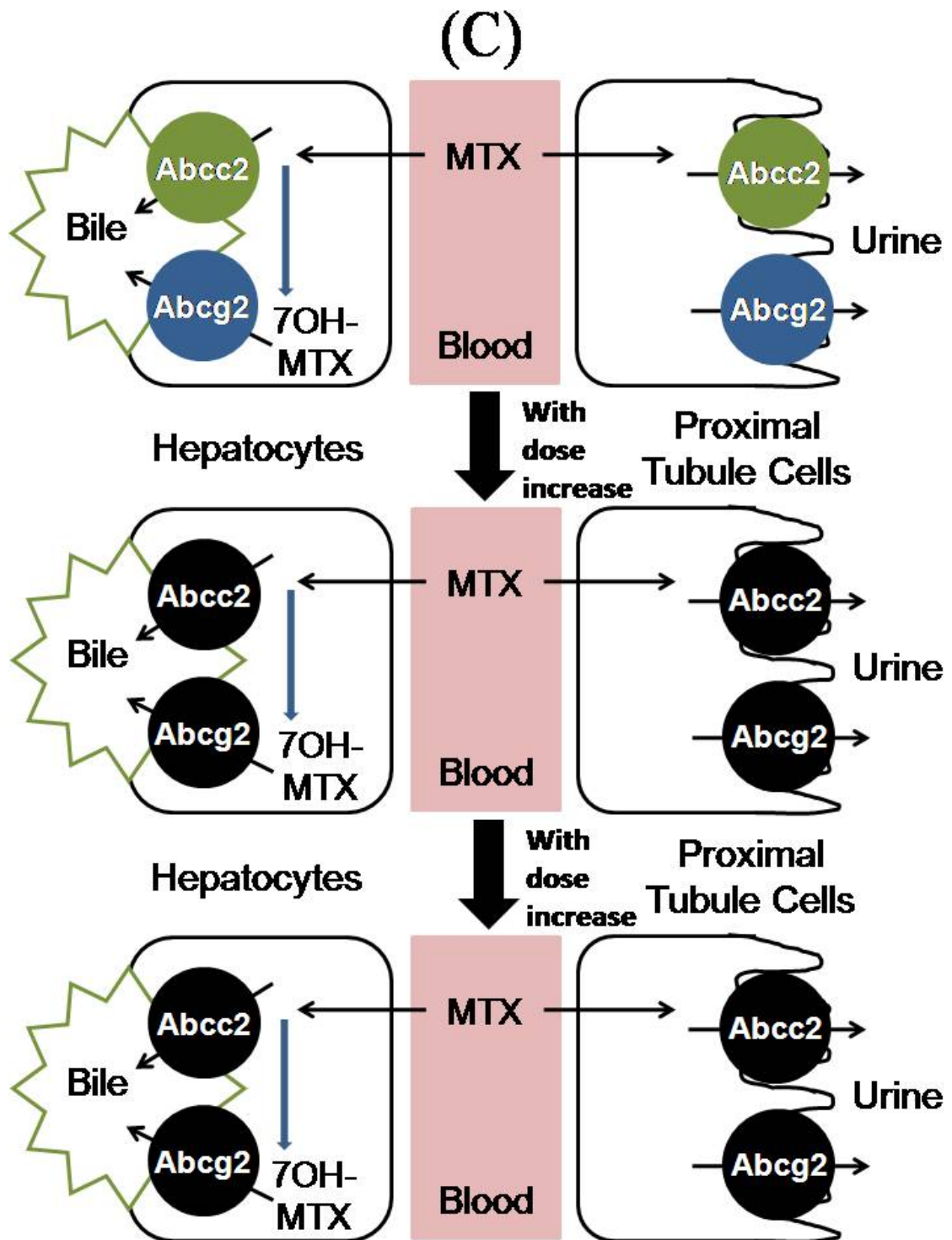


Figure 10. Proposed action of key ABC transporters involved in the pharmacokinetics of MTX in liver hepatocytes and renal proximal tubules as a function of dose in WT (A), *Abcc2*^{-/-} (B) and *Abcc3*^{-/-} (C) strains after IV doses. For each strain three states of transporter function are shown that correspond to our low, medium and high IV 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&b). The extent of conversion of MTX to 7OH-MTX in hepatocytes is indicated by the thickness of the arrow.

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.

2.5 Footnote and Acknowledgement

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The above work has been published in the following publication with other co-authors [Qingyu Zhou (University of Southern Florida), Gary D. Kruh (deceased) and James M. Gallo (Mount Sinai School of Medicine)] with authorship contributions clearly indicated: Wang Z., Zhou Q., Kruh G.D., Gallo J.M. Dose-dependent disposition of methotrexate in Abcc2 and Abcc3 gene knockout murine models. *Drug Metab Dispos.* 39(11):2155-2161, 2011. PMID: 21841039.

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

The publication was also honored to be featured on the November issue (2011) of the peer-reviewed journal: *Drug Metabolism and Disposition*.

CHAPTER 3

**EVALUATION OF ABCC2 AND ABCC3 TRANSPORTERS IN THE
PHARMACOKINETICS OF METHOTREXATE AFTER ORAL DOSING**

3.1 Introduction

The multidrug resistance proteins (MRP) belong to the C subfamily of ATP-binding cassette (ABC) transporters, which are involved in absorption and disposition of variety of structurally diverse endogenous (such as organic anion conjugates) and exogenous (such as chemotherapeutic agents and their metabolites) substrates (Borst & Oude Elferink, 2002; Bardelmeijer et al., 2000; Fromm, 2003; Chan et al., 2004). Numerous *in vitro* and *in vivo* studies have examined the role of these transporters in the PK properties of various drugs, as well as their impact on efficacy and toxicity (Mizuno, Niwa, Yotsumoto, & Sugiyama, 2003). Defining the impact of membrane transporters on PK processes is now an important step in preclinical drug development. The FDA's BCS (Amidon et al., 2005) and BDDCS (Wu & Benet, 2005) provides a rational means to identify drugs that potentially interact with certain transporters, particularly when administered orally. Given that many drugs have to be given orally and in so doing are subject to numerous barriers prior to entrance into the systemic circulation, it is important to characterize how each transporter can impact drug absorption. Improved knowledge about the functional properties of drug transporters can be used to modify drug design

and dosing regimens that may overcome poor bioavailability, as well as avoid potential drug-drug interactions associated with drug absorption.

The ABC transporters are located on epithelial cells of key organs that determine both drug absorption and elimination and include the small intestine, liver and kidney (Borst & Oude Elferink, 2002; Fromm, 2003; Chan et al., 2004). In addition to P-glycoprotein, the first identified ABC transporter, ABCC2/MRP2 and ABCC3/MRP3 share similar drug substrate profiles; however, due to their different cell orientations in the hepatobiliary and intestinal axes, ABCC2 being apical and ABCC3 being basolateral, they have opposing effects on systemic absorption (Borst et al., 2006; Kruh et al., 2007). ABCC2 acts as a drug efflux pump on gastrointestinal enterocytes and can limit drug absorption, whereas its location in bile canaliculi aids in biliary excretion of numerous drugs (Assaraf, 2006; Dietrich, Geier, & Oude Elferink, 2003). On the other hand, the basolateral orientation of ABCC3 on enterocytes and hepatocytes could enhance drug absorption although this role is likely amplified during disease, such as due to cholestasis with up-regulated expression of ABCC3 (Soroka et al., 2001). For example, induced cholestasis via bile duct cannulation in rats caused the blood concentration of M3G (morphine-3-glucuronide) to be elevated rapidly after IV administration of morphine instead of being excreted into bile that was indicative of increased expression of Abcc3 (Hasegawa et al., 2009).

Oral MTX is clinically used for the treatment of the rheumatoid arthritis and psoriasis, and recent interest has shifted towards investigating the oral route for MTX in

cancer chemotherapy (Gebbia, Serretta, Borsellino, Valerio, & GSTU Foundation, 2011; Zulian et al., 2011). Previous studies in rodents have shown that in the absence of *Abcc2* the systemic exposure of MTX can increase (Chen et al., 2003; Naba, Kuwayama, Kakinuma, Ohnishi, & Ogihara, 2004). The action of *Abcc3* is less clear. Reduced MTX systemic exposure yet without a significant difference between WT and *Abcc3*^{-/-} strains in oral bioavailability was observed after 50 mg/kg oral dose (Vlaming et al., 2011). However, another *in vivo* study showed decreased systemic exposure as well as oral bioavailability following 1 mg/kg oral dose in *Abcc3*^{-/-} mice compared to those in WT strain (Kitamura et al., 2008). Although certain features of the action of *Abcc2* and *Abcc3* on the oral absorption of MTX have been established, their action over a wide range of doses has not. Recently, we highlighted how dose-dependent studies revealed an interesting set of compensatory elimination pathways following IV administration of MTX (Wang, Zhou, Kruh, & Gallo, 2011), and by using the same mouse models a comprehensive assessment of the roles of *Abcc2* and *Abcc3* on the absorption of MTX was sought.

3.2 Material and Methods

3.2.1 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*^{-/-} and *Abcc3*^{-/-} mice are described elsewhere (Belinsky et al., 2005). All animals were maintained on an alternating 12 hr light/dark cycle with free access to water and rodent chow *ad libitum*.

The Institutional Animal Care and Use Committee at Temple University approved all animal procedures.

3.2.2 Pharmacokinetic Studies of MTX

One day prior to MTX administration, while 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 at designated different time points after oral administration. For a small subset of mice, MTX was given via the portal vein and had an additional cannula placed there as described below. Pharmacokinetic experiments were conducted the day following the surgical cannulation procedures in conscious, freely-mobile mice placed in metabolic cages (Nalgene®, Braintree Scientific Inc., Braintree, MA) that allowed for the separate collection of urine and feces. Oral doses of MTX (10, 50, and 200 mg/kg) in saline, pH adjusted to 7, were administered by oral gavage using a curved, blunt-ended needle (size 19 g × 2 in), whereas for portal vein administration doses of only 50 and 200 mg/kg were given through cannulated portal vein (see the next section for detailed procedures for portal vein cannulation). Serial (n = 10 to 14) blood samples of 20 µL were collected through the right carotid artery cannula for up to 8 hours at the low dose, for 12 hours at the 50 mg/kg dose, and approximately for 2 days at the high dose level. The blood samples were centrifuged and the resultant plasma was stored at -80 °C until analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) as described previously (Guo et al., 2007). To avoid blood volume depletion, 20 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 were stored at -80 °C until analysis also by LC/MS/MS. The method for quantitation of MTX and its major metabolite, 7OH-MTX, by LC/MS/MS was validated with intra- and inter-day precision and accuracy of less than 15% (Guo et al., 2007; Wang et al., 2011).

3.2.3 Portal Vein Cannulation in Mice

The portal vein cannulation method was modified from Shen et al. (2008). Briefly, mice were sedated in an isoflurane induction chamber at a flow rate of 1 L/min of oxygen and 3% isoflurane, and then positioned with a nose cone at a flow rate of 2% isoflurane. After shaving and disinfecting, the abdominal cavity was exposed with a midline incision and the portal vein was identified and dissected exposing approximately 5 mm for cannulation. The distal side of portal vein was temporarily ligated to block the blood flow. Within a very short time (~ 1 min), the elevated portal vein was punctured with a self-made catheter made from a ~ 10 cm vinyl tube (0.011 in ID × 0.025 in OD) connected to another 3 mm silastic tube (0.012 in ID × 0.025 in OD) with the help of a beveled 5 mm-length gel-loaded pipet tip. The tip of the catheter was advanced approximately 3 mm into the portal vein to ensure the bevel was covered by the vessel. A very small amount of Vetbond (3M Animal Care Products, St. Paul, MN) was applied at the insertion site to affix the catheter to the portal vein. The ligature was then loosened to retain blood flow. The catheter was tunneled subcutaneously from the abdomen to intrascapular area and secured using a purse-string suture. The catheter was checked for

its patency by withdrawing the blood and was flushed with saline before MTX administration.

3.2.4 Data Analysis

Each individual mouse MTX plasma concentrations were analyzed by noncompartmental analysis using WinNonlin Professional Version 5.3 (Pharsight Co, Mountain View, CA) to obtain estimates of areas under the MTX and 7OH-MTX plasma concentration-time curve (AUC). The renal (f_r) and fecal (f_f) fractions of the MTX dose eliminated unchanged was calculated as the total amount of MTX obtained in the urine and feces, respectively, divided by the total amount MTX eliminated that was set equal to the sum of urine and feces amounts.

Comparisons of relative bioavailability (RF) between knockout and WT strains at each dose level were calculated according to the following equation, $RF^{ko/wt}_{po} = AUC^{ko}_{po} / AUC^{wt}_{po}$. In addition, interstrain and intrastrain analyses were conducted for renal and fecal excretion of MTX.

Since it was not possible to perform standard bioavailability cross-over studies in mice, estimates of absolute bioavailability could be made only by using the data from the IV studies presented in Chapter 2. In this case, absolute bioavailability of MTX in each strain was calculated using the following equation, $F_{po} = (AUC_{po}) / (AUC_{iv})$, where the mean values of AUC_{po} were obtained from the current study and AUC_{iv} from the previously published IV study (Wang et al., 2011). MTX AUCs obtained from

preliminary portal vein studies were further used for the calculation of AUC ratios of f_{ec} [$f_{ec} = (AUC_{po}) / (AUC_{pv})$] and f_{pv} [$f_{pv} = (AUC_{pv}) / (AUC_{iv})$], the fraction of the dose absorbed via enterocyte (ec) and the fraction of the dose absorbed via hepatocytes, respectively.

Comparisons in other PK variables, such as Cmax, AUCs and AUC ratio of metabolite and parent drug, renal and fecal excretion, between knockout and WT strains were conducted based on the mean and standard deviation values from two independent groups using unpaired *t* tests, where $P < 0.05$ 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 $P < 0.05$.

3.3 Results

Previously we reported the role of key ABC transporters, Abcc2 and Abcc3, in altering the PK properties of MTX following intravenous doses of MTX from 10 to 200 mg/kg by using gene knockout mice (Wang et al., 2011). We extended that approach in the present study to determine the oral bioavailability of MTX. MTX plasma concentration-time profiles for each strain after all oral dose levels are illustrated in Figure 11, in which many differences are apparent. Following oral administration, plasma MTX concentrations were generally highest in the *Abcc2*^{-/-} strain followed by the WT and *Abcc3*^{-/-} strain. Regardless of the strain, fecal excretion was the primary elimination pathway at the two highest dose levels that was in contrast to the IV route where renal excretion was dominant (Figure 12). In addition, shoulder peaks in MTX plasma

concentration-time profiles are observed in strains at all doses (Figure 11), especially in *Abcc2*^{-/-} groups it is more apparent, possibly because of enterohepatic recirculation (EHC) (Bleyer, 1978) or pulsatile absorption due to re-dissolution of precipitated MTX in GI tract at low pH. Oral bioavailability of MTX was low in all the strains and is dose-dependent (see Table 5), with comparable values at 50 mg/kg doses as previously reported (Vlaming et al., 2011). All the PK parameters are summarized in Table 5.

3.3.1 Interstrain Analysis of Oral MTX Pharmacokinetics

Interstrain analysis highlighted several differences in the PK parameters of MTX (Table 5, Figure 11-13). Relative to WT mice, *Abcc2*^{-/-} mice exhibited significantly higher AUC ($P < 0.01$ for low and medium dose, $P < 0.05$ for high dose) and C_{\max} ($P < 0.01$) values at corresponding dose levels (Table 5). As can be seen from the relative bioavailability values (Table 5) that are 2.1 (for the low and medium doses) and 1.7 (for the high dose), the AUC are about doubled and can be attributed to the loss of *Abcc2* efflux action on enterocytes and its excretory function in bile canaliculi and renal proximal tubules. Examination of the renal and fecal contributions to MTX elimination between WT and *Abcc2*^{-/-} strains does not indicate a consistent pattern across all doses. Interestingly renal excretion is elevated ($P < 0.5$ for low dose, $P < 0.01$ for medium dose, 1.4 fold increase at high dose though $P > 0.5$) in the *Abcc2*^{-/-} strain relative to the WT strain that suggests a compensatory renal elimination mechanism that further supports enhanced intestinal absorption and reduced biliary excretion (obvious at low dose yet without significant difference) to attain greater systemic exposures of MTX in the *Abcc2*^{-/-} strain. It is also feasible that the *Abcc2*^{-/-} strain utilize *Abcc3*'s function on the

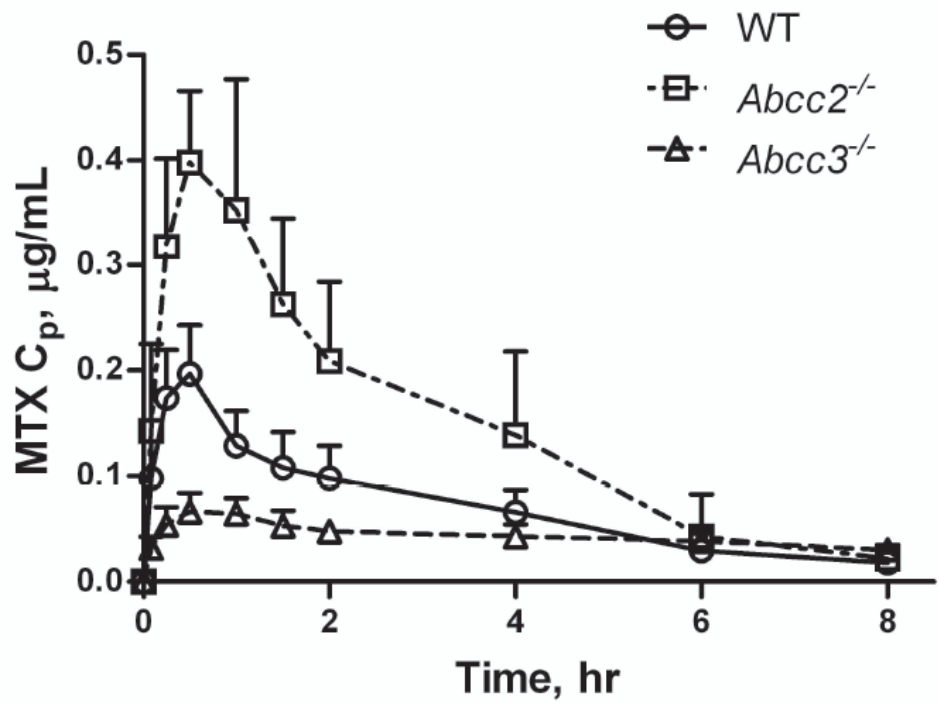
Table 5. Pharmacokinetic parameters (mean \pm SD) of MTX following oral administration of MTX at three dose levels in WT, *Abcc2*^{-/-} and *Abcc3*^{-/-} strains

Parameters	Dose=10 mg/kg	Dose=50 mg/kg	Dose=200 mg/kg
WT mice	n = 8	n = 12	n = 8
F (%)	11.2	6.9	11.0
C_{max} (µg/mL)	0.197 \pm 0.046	0.191 \pm 0.059	0.604 \pm 0.146 [‡]
AUC_{MTX} (min*µg/mL)	34.4 \pm 8.9	58.4 \pm 17.0	445.6 \pm 174.6
AUC_{7OH-MTX} (min*µg/mL)	2.7 \pm 1.1	5.7 \pm 3.0	16.0 \pm 11.4
AUC_{m/p} (%)	7.8 \pm 2.5	8.8 \pm 3.6	3.3 \pm 1.2 [‡]
Renal fraction of dose (f_r, %)	10.7 \pm 4.4 [‡]	3.5 \pm 1.0	4.5 \pm 1.1
Fecal fraction of dose (f_f, %)	15.6 \pm 14.6 [‡]	43.8 \pm 14.6	44.3 \pm 18.8
<i>Abcc2</i>^{-/-} mice	n = 8	n = 12	n = 7
F (%)	17.3	7.9	13.8
C_{max} (µg/mL)	0.433 \pm 0.087 ^{**}	0.317 \pm 0.048 ^{**}	1.090 \pm 0.280 ^{**‡}
AUC_{MTX} (min*µg/mL)	71.3 \pm 20.4 ^{**}	121.3 \pm 30.1 ^{**}	775.1 \pm 264.7 [*]
AUC_{7OH-MTX} (min*µg/mL)	11.1 \pm 3.2 ^{**}	32.9 \pm 22.0 ^{**}	164.8 \pm 95.5 ^{**}
AUC_{m/p} (%)	16.0 \pm 3.9 ^{**}	25.7 \pm 13.6 ^{**}	16.5 \pm 5.4 ^{**}
Renal fraction of dose (f_r, %)	22.5 \pm 3.4 ^{**‡}	9.4 \pm 2.4 ^{**}	6.5 \pm 2.8
Fecal fraction of dose (f_f, %)	7.0 \pm 8.2 [‡]	45.3 \pm 14.2	38.3 \pm 15.7
RF^{ko/wt}_{po}	2.1	2.1	1.7
<i>Abcc3</i>^{-/-} mice	n = 6	n = 8	n = 6
F (%)	11.7	6.7	2.1
C_{max} (µg/mL)	0.071 \pm 0.013 ^{**}	0.079 \pm 0.010 ^{**}	0.260 \pm 0.150 ^{**‡}
AUC_{MTX} (min*µg/mL)	21.2 \pm 4.8 ^{**}	34.6 \pm 14.7 ^{**}	128.0 \pm 65.5 ^{**}
AUC_{7OH-MTX} (min*µg/mL)	0.8 \pm 0.4 ^{**}	1.3 \pm 1.0 ^{**}	1.6 \pm 1.2 ^{**}
AUC_{m/p} (%)	3.8 \pm 0.8 ^{**}	3.4 \pm 1.5 ^{**}	1.2 \pm 1.0 ^{**‡}
Renal fraction of dose (f_r, %)	7.8 \pm 8.3	2.1 \pm 1.4 [*]	3.9 \pm 3.7
Fecal fraction of dose (f_f, %)	12.9 \pm 13.1 [‡]	44.9 \pm 22.5	44.7 \pm 10.9
RF^{ko/wt}_{po}	0.6	0.6	0.3

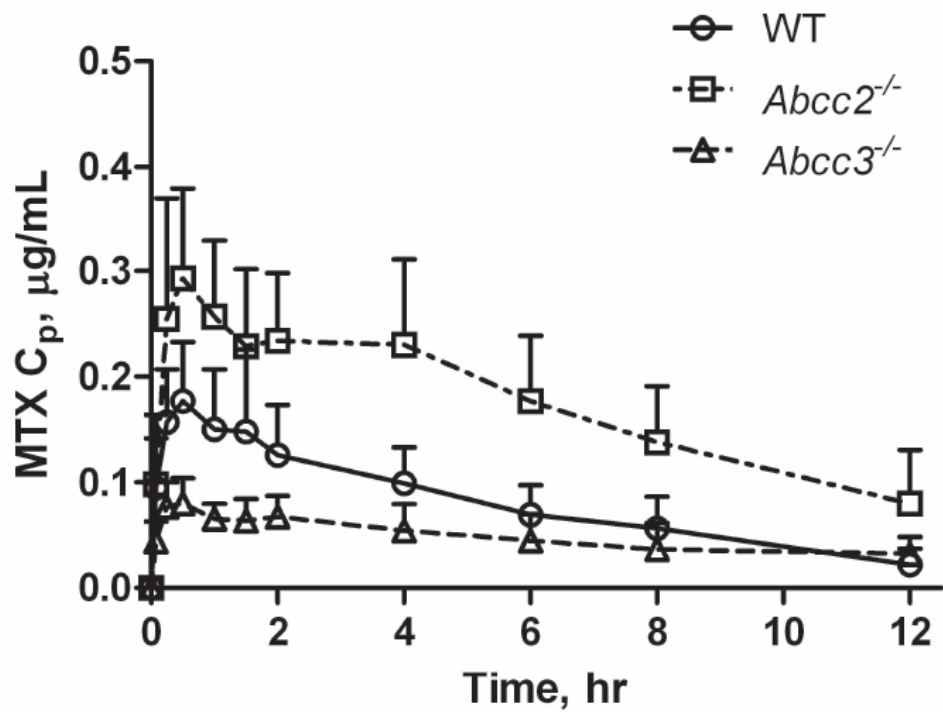
^{*}/^{**} significant difference between WT and knockout strains at that dose level (^{*}*P* < 0.05, ^{**}*P* < 0.01).

[†]/[‡] significant difference among dose levels within the same strain (*P* < 0.05), with “[†]” indicating differences between designated dose levels, and “[‡]” indicating differences from the other two dose levels.

A)



B)



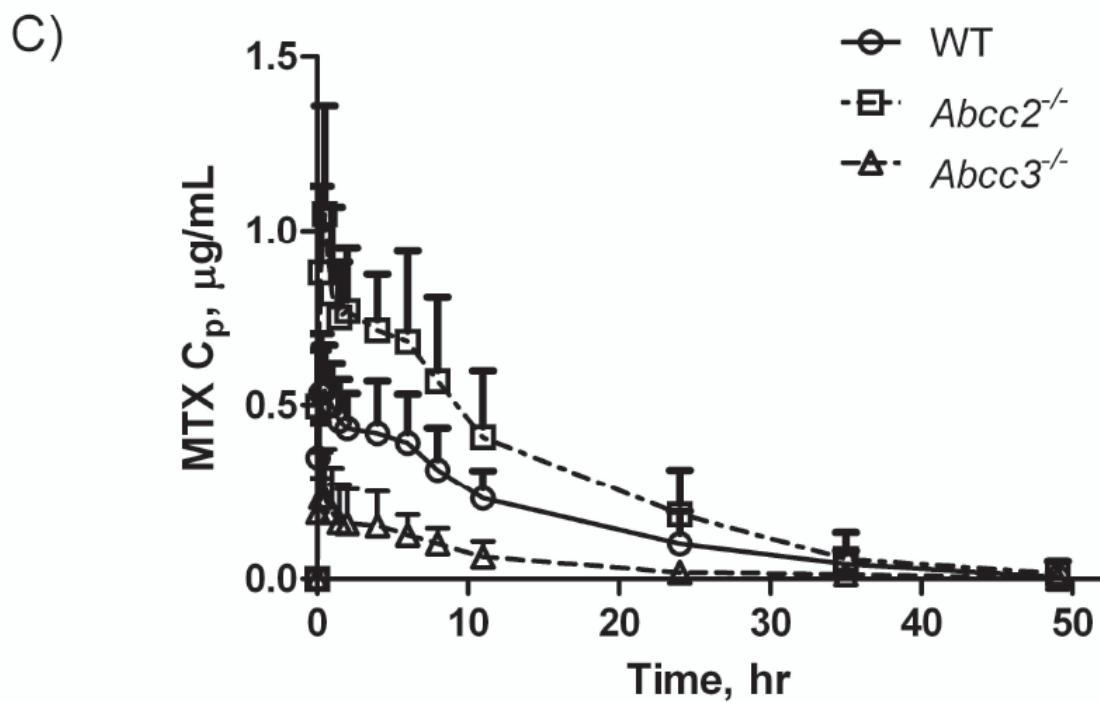


Figure 11. Plasma concentrations (C_p , mean \pm SD; semi-log scale) of MTX as a function of time after oral administration of single doses of MTX at 10 (A), 50 (B) and 200 (C) mg/kg to male WT (diamond), *Abcc2*^{-/-} (square) or *Abcc3*^{-/-} (triangle) mice.

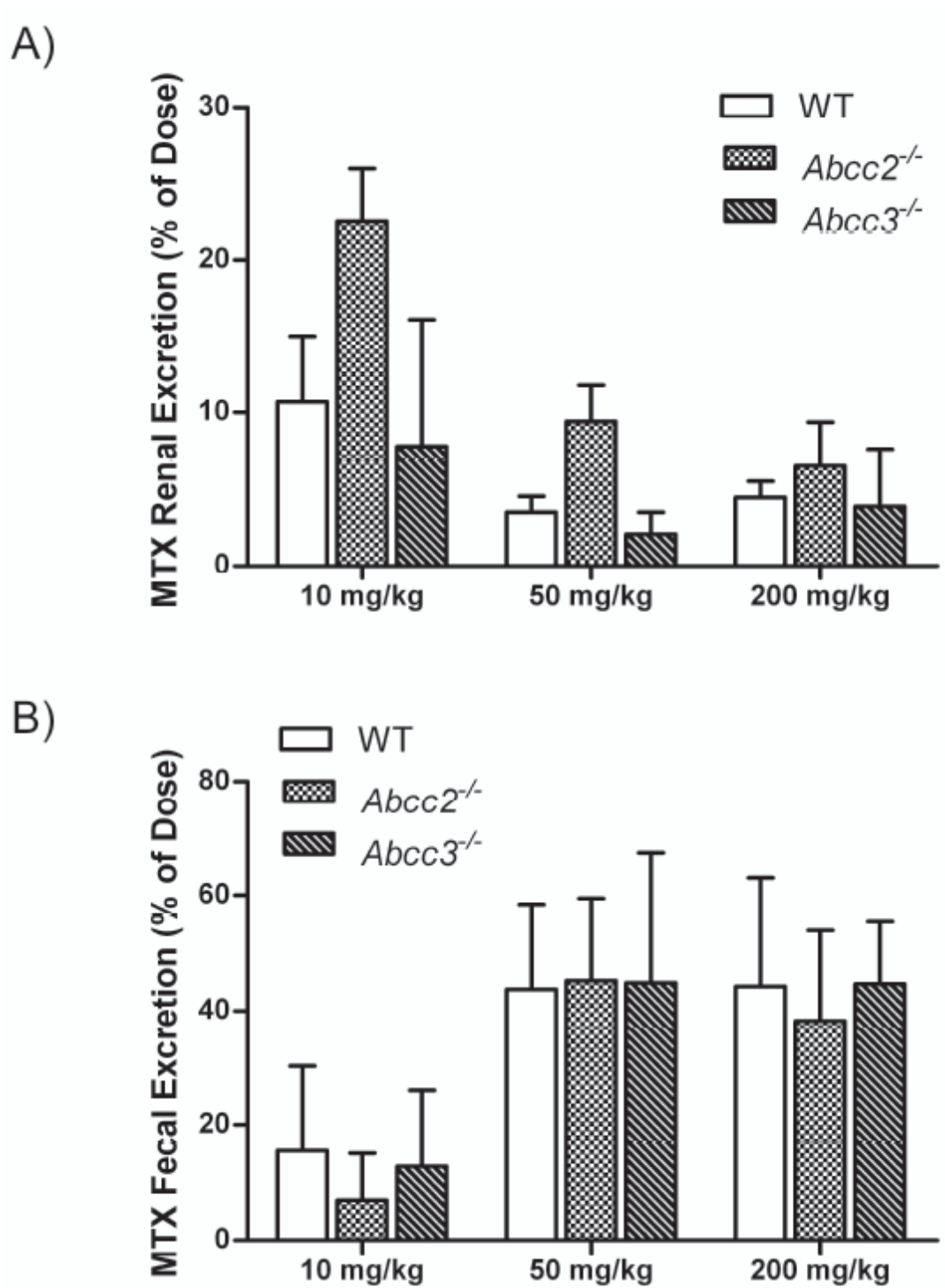


Figure 12. Renal (A) and fecal excretion (B) of MTX (expressed as % of dose) in WT, *Abcc2*^{-/-} and *Abcc3*^{-/-} mice at three different oral dose levels.

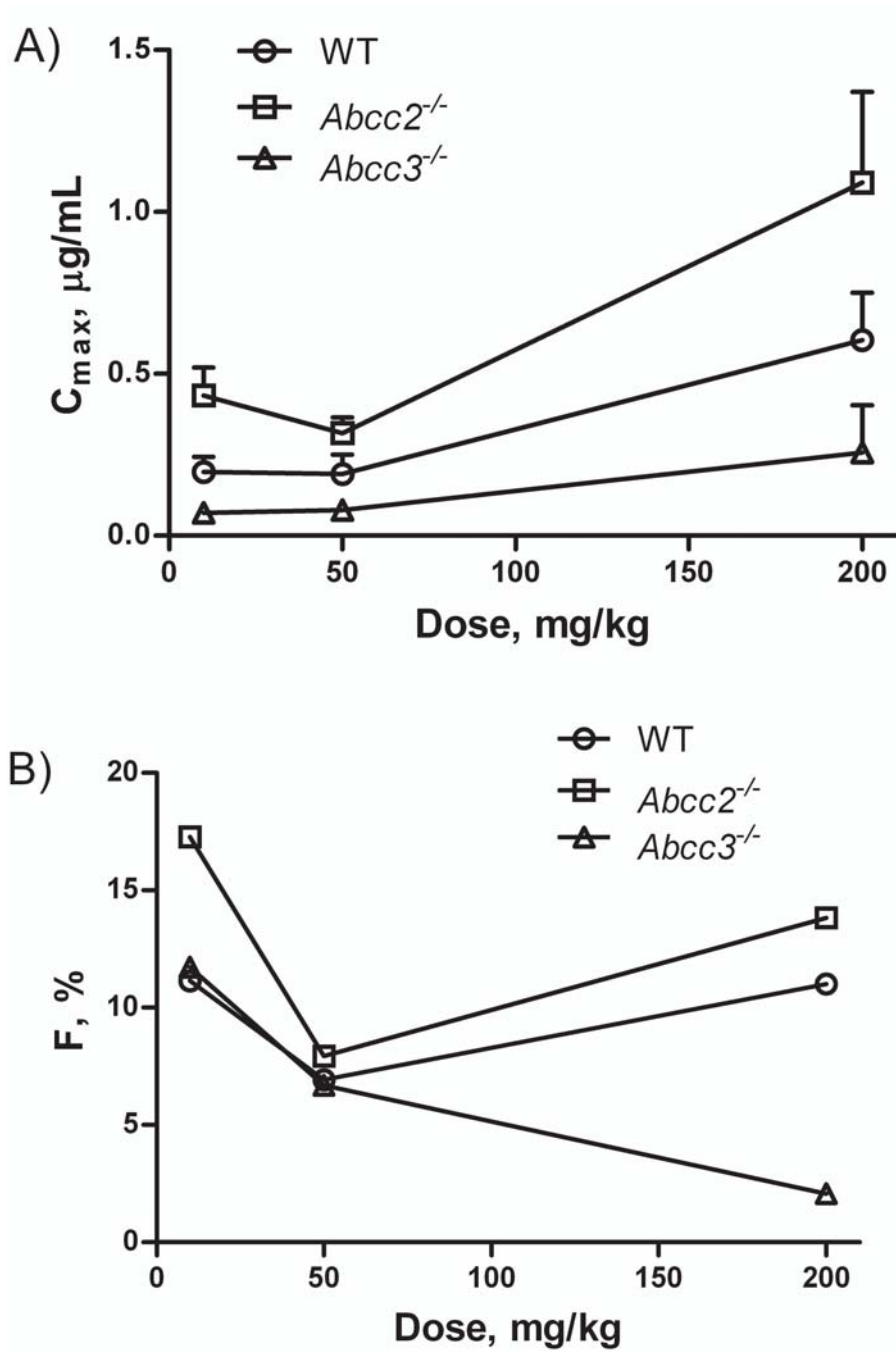


Figure 13. C_{max} (mean \pm SD) (A) and Oral bioavailability (F) (B) of MTX as a function of dose to different mouse strains.

basolateral membranes of enterocytes and hepatocytes to pump MTX into plasma to compensate for the loss of Abcc2's action as an efflux pump. Another compensatory mechanism exhibited by *Abcc2*^{-/-} strain relative to the WT strain was increased metabolism of MTX to 7OH-MTX with the fractional conversion, % AUC_{m/p} (see Table 5), values being 2 to 5 fold greater depending on the dose. Given that this metabolic change would hasten the elimination of MTX in the *Abcc2*^{-/-} strain the higher MTX AUC values attest to the role of Abcc2 in absorption and biliary excretion. This was not surprising as similar results were obtained at IV doses in *Abcc2*^{-/-} mice (Wang et al., 2011), presumably because 7OH-MTX is a substrate of Abcc2 (Vlaming et al, 2008). In addition, the oral bioavailability in *Abcc2*^{-/-} strain was increased at low 10 mg/kg doses (~55%), whereas it only slightly increased at 200 mg/kg (~25%) comparison to WT mice (see Table 5), this is consistent with our previous IV studies suggesting an important role of Abcc2 in excretion of MTX at low doses (Wang et al., 2011). However, the compensatory increase in renal and metabolic pathways in the *Abcc2*^{-/-} strain may not sufficiently account for the reduced AUC or relative bioavailability particularly at 200 mg/kg.

WT and *Abcc2*^{-/-} groups were also given 50 mg/kg and 200 mg/kg doses as portal vein injections to distinguish transport action in enterocytes and hepatocytes. The enterocyte-based fractions of MTX absorbed (f_{ec}) observed in *Abcc2*^{-/-} mice (7.23 % and 7.20 %) and WT mice (6.78 % and 5.17 %), were comparable after 50 and 200 mg/kg and indicate that the contribution of Abcc2 on enterocytes to oral absorption is minimal (Table 6). It was interesting to find the fractions of MTX absorbed via hepatocytes (f_{pv})

Table 6. Methotrexate exposures following portalvein administration in WT and *Abcc2*^{-/-} strains

Doses (mg/kg)	WT mice		<i>Abcc2</i> ^{-/-} mice	
	50	200	50	200
$f_{ec} (\%) = 100\% \times (AUC_{po}) / (AUC_{pv})$	6.78	5.17	7.23	7.20
$f_{pv} (\%) = 100\% \times (AUC_{pv}) / (AUC_{iv})$	102.12	212.76	106.64	191.90
$F_{po} (\%) = 100\% \times f_{ec} \times f_{pv}$	6.93	11.00	7.94	13.82
$AUC_{m/p} (\%)$	4.88 ± 0.99	2.84	26.70 ± 10.20 ^{**}	17.60
Renal fraction of dose ($f_r, \%$)	58.90 ± 3.10	55.00	91.50 ± 19.80 ^{**}	79.50
Feces fraction of dose ($f_f, \%$)	14.20 ± 3.00	10.10	3.05 ± 0.80 [*]	9.58

*/**: significant difference between WT and KOs (*: $P < 0.05$, **: $P < 0.01$)

were close to 1 at 50 mg/kg in WT (102.12 %) and *Abcc2*^{-/-} strains (106.64 %), suggesting that the overall oral bioavailability in both strains is primarily determined by gastrointestinal absorption, yet not appreciably influenced by *Abcc2*. However, at 200 mg/kg dose, those fractions were much greater than 1 in both strains (212.76 % and 191.90 % for WT and *Abcc2*^{-/-} strains, respectively), indicative of rapid saturation of all hepatic transporters and possibly accumulation of MTX in the hepatocytes as conversion to 7OH-MTX and renal excretion did not increase compared to lower doses.

The PK parameters of MTX following oral dosing in the *Abcc3*^{-/-} and WT strains revealed a number of significant differences (Table 5, Figure 11-13). MTX plasma AUC and C_{max} values were significantly decreased ($P < 0.01$) in the *Abcc3*^{-/-} strain compared to those in the WT strain at all three oral dose levels with the relative bioavailability values being 0.6 at the two lower doses and 0.3 at the high dose. The decrease in MTX AUC values in the *Abcc3*^{-/-} strain reflect the absence of normal protective function of *Abcc3* in basolateral hepatocytes and enterocytes that pumps substrates into the systemic circulation. Although the renal excretion at all three doses were reduced (27%, 41% and 13% for low, medium and high dose, respectively) with the only significance at 50 mg/kg dose, it was surprising that compensatory mechanisms, neither fecal, renal or metabolic, were not observed in the *Abcc3*^{-/-} strain. Alternate elimination mechanisms in the *Abcc3*^{-/-} strain might account for this disparity, and could include an altered elimination pathway, such as increased polyglutamylation, yet it could also reflect an incomplete renal or fecal collection. Additional studies would be needed to address these possibilities.

3.3.2 Intrastrain Analysis Of Oral MTX Pharmacokinetics

Intrastrain analysis revealed dose-dependent changes in each strain over a dose range of MTX from 10 – 200 mg/kg (Table 5, Figure 12-13). The WT mice exhibited similar MTX plasma C_{max} values and less than proportional increase of AUC in going from 10 to 50 mg/kg dose level, which is consistent with Michaelis-Menten kinetics and was further supported by increased fecal elimination with dose increase. There was not a compensatory increase in renal excretion of MTX in the WT strain as dose increased that seems compatible with the elevated fecal excretion at both the 50 mg/kg and 200 mg/kg dose levels.

The *Abcc2*^{-/-} group exhibited less than proportional increases in MTX's C_{max} , and AUC values as dose increased from 10 mg/kg to 200 mg/kg. Increased fecal elimination was seen at 50 mg/kg and 200 mg/kg dose levels, which more likely indicates MTX is not absorbed and passes through the gastrointestinal tract. Without *Abcc2* biliary elimination function, *Abcg2* could still serve to pump MTX into bile, yet the large increase in fecal excretion in going from 10 mg/kg to 50 mg/kg seems more consistent with limited drug absorption. There were also no compensatory mechanisms of increased metabolism to 7OH-MTX or increased renal excretion at both the 50 mg/kg and 200 mg/kg dose levels.

Finally, for the *Abcc3*^{-/-} group, although similar trend of MTX's C_{max} , renal and fecal excretions were observed throughout the 10 to 200 mg/kg dose range, a retarded oral bioavailability and AUC increase without more than proportional at 200 mg/kg dose

were observed. The decrease of oral bioavailability in going from 10 to 50 mg/kg could be again attributed to the less intestinal absorption due to the absence of Abcc3 in enterocytes and incomplete dissolution in intestinal lumen, as evidenced by increased fecal excretion. Whereas, the loss of Abcc3 in hepatocytes coupled with potential saturation of biliary transporters as well as incomplete absorption may cause further dropping of the bioavailability in going from 50 to 200 mg/kg.

3.4 Discussion

In the past half century, we have learned newer uses of MTX in autoimmune diseases, severe psoriasis, adult rheumatoid arthritis (RA), and graft-versus-host diseases (Khan, Tripathi, & Mishra, 2012; Vigouroux et al., 2011). In addition, MTX has shown significant efficacy alone or in combination as an anti-neoplastic drug in a variety of cancers, such as head and neck squamous cell carcinoma, gestational choriocarcinoma, small cell and squamous cell type lung cancer, breast cancer, advanced stage non-Hodgkin's lymphoma and osteosarcoma (Walling, 2006). The oral administration of MTX although seems more desirable and convenient to patient, is limited due to relatively poor oral bioavailability coupled with its wide inter-patient variability, especially in cancer patients where IV doses higher than 15 mg/m^2 are usually administered (Gorlick & Bertino, 1999). With other reasons, the impact of ABC transporters on the oral bioavailability of MTX has been extensively investigated in *in vitro* and *in vivo* models (Xia et al., 2005; Kitamura et al., 2008; Vlaming et al., 2008, 2009a&b, 2011; Wang et al., 2011). Previously, through multi-dose IV study on MTX, we have shown the impact of overlapping functions of Abcc2 and Abcc3 transporters in

MTX disposition, in WT, *Abcc2*^{-/-} and *Abcc3*^{-/-} mice strains. Here, we further confirm the role of these transporters on MTX oral pharmacokinetics, which suggests that the absence of *Abcc2* or *Abcc3* will cause activation of alternate elimination pathways. Although, similar findings have been reported in recent past, they were limited to a single dose (Kitamura et al., 2008; Vlaming et al., 2011). Our dose-dependent oral studies on MTX disposition, reassure previous findings, and extend these past analyses to completely profile the overlapping function of these transporters in saturable oral MTX kinetics, including the role of individual transporter in altered hepatic metabolism of MTX. In addition, a preliminary comprehensive dose-dependent bioavailability is performed, which further explains the impacts of these transporters on the MTX absorption processes.

Methotrexate is a substrate for ABCC1-5, ABCC11 and ABCG2 transporters. Many of these transporters could transport MTX with high capacity, yet have low affinity towards it (Assaraf, 2006). Previously, we suggested that when these transporters have overlapping functions, the excretion of a drug is expected to be engaged primarily by transporters with relatively higher affinities, followed by ones with lower affinities; however, both contributing to the overall transport rate (Wang et al., 2011). In the current investigation, regardless of strains, following an oral dose of 10 to 50 mg/kg of MTX, an increase in the fecal excretion of MTX was observed, consistent with Michaelis-Menten kinetics. A similar plateau in fecal fraction values in all the strains following oral administration of MTX suggests that MTX is being directly excreted into the feces and

not absorbed, perhaps due to potential precipitation of MTX at low pH in the intestinal lumen.

In the current oral studies, we observed compensatory intrahepatic conversion of MTX to toxic 7OH-MTX in the *Abcc2*^{-/-} strain, which is consistent with the observations seen in the previous dose-dependent IV studies in the same type of mice (Wang et al., 2011). Such compensatory metabolism may primarily account for the elevated total excretion of MTX in *Abcc2*^{-/-} strain. Among the total excretion, the fecal excretions were elevated to a plateau at medium and high doses, which was in agreement with the observations seen in IV studies (Wang et al., 2011) as well, suggesting Abcg2 also plays a role at biliary canaliculi. Previous single dose oral study, using the single and double (*Abcc2;Abcg2*^{-/-}) knockout mice by Vlaming et al. (2011), also showed that Abcg2 could mediate substantial elimination of MTX through hepatobiliary pathway, even in the absence of *Abcc2* transporters.

In our dose-dependent oral studies, we observed reduced C_{max} and AUC in *Abcc3*^{-/-} strain relative to WT strain at all dose levels, suggesting a role of *Abcc3* in oral PKs of MTX. However, a recent study reported insignificant AUC difference of in *Abcc3*^{-/-} strain relative to WT strain at 50 mg/kg (Vlaming et al. 2011), one of the dose levels applied in our study. Nonetheless, when an unsaturated lower (1 mg/kg) dose was used in a separate study by Kitamura et al. (2008), a relatively smaller F_aF_g (fraction of dose reaching into the liver, after escaping the intestinal barrier and gut wall metabolism) was obtained in *Abcc3*^{-/-} mice, indicating *Abcc3* may have a role in MTX absorption at

very low doses. At low and medium doses, the current study conveyed comparable oral bioavailabilities in *Abcc3*^{-/-} and WT strains, which suggests a possibility of other efflux or uptake transporters may facilitate the transport of MTX across intestine. In this regard, reduced folate carriers (Rfc) were shown to efflux MTX based on anionic-exchange (Ifergan & Assaraf, 2008). In addition, proton-coupled folate transporter/haem carrier protein 1 (Pcft) located on the apical side of intestine was also shown to be involved in site specific absorption of MTX from intestinal lumen into enterocytes, yet with low capacity (Yokooji et al., 2007, 2009). Yet at high dose we observed a retarded oral bioavailability in *Abcc3*^{-/-} strain instead of a rebound as seen in both WT and *Abcc2*^{-/-} strains. It should be noted that in the WT and *Abcc2*^{-/-} strains, there was no problem of seeing the increase in C_{max} and the rebound of bioavailability of MTX (Table 5), because intestinal *Abcc3* though saturated, was still functioning for the transport of MTX into portal vein following oral administration; whereas, as the biliary excretory pumps were saturated, the hepatic *Abcc3* may be responsible for its compensatory increase in its ability to export MTX. However, the surprising continuous reduction of oral bioavailability in *Abcc3*^{-/-} strain, though presumably attributed to the incomplete absorption because of loss of *Abcc3* coupled with possible saturation of canalicular effluxers, might warrant further investigation for clarification, particularly at high dose. Yet the low oral bioavailability without rebound at high dose in the *Abcc3*^{-/-} strain may indicate the protective function of *Abcc3* at intestinal membrane also, as we have previously suggested its protective function at hepatocytes in our IV studies. Though the damage of intestinal epithelium could bring more paracellular absorption of MTX following oral administration of high-dose MTX in all strains (Keefe et al., 1997), such

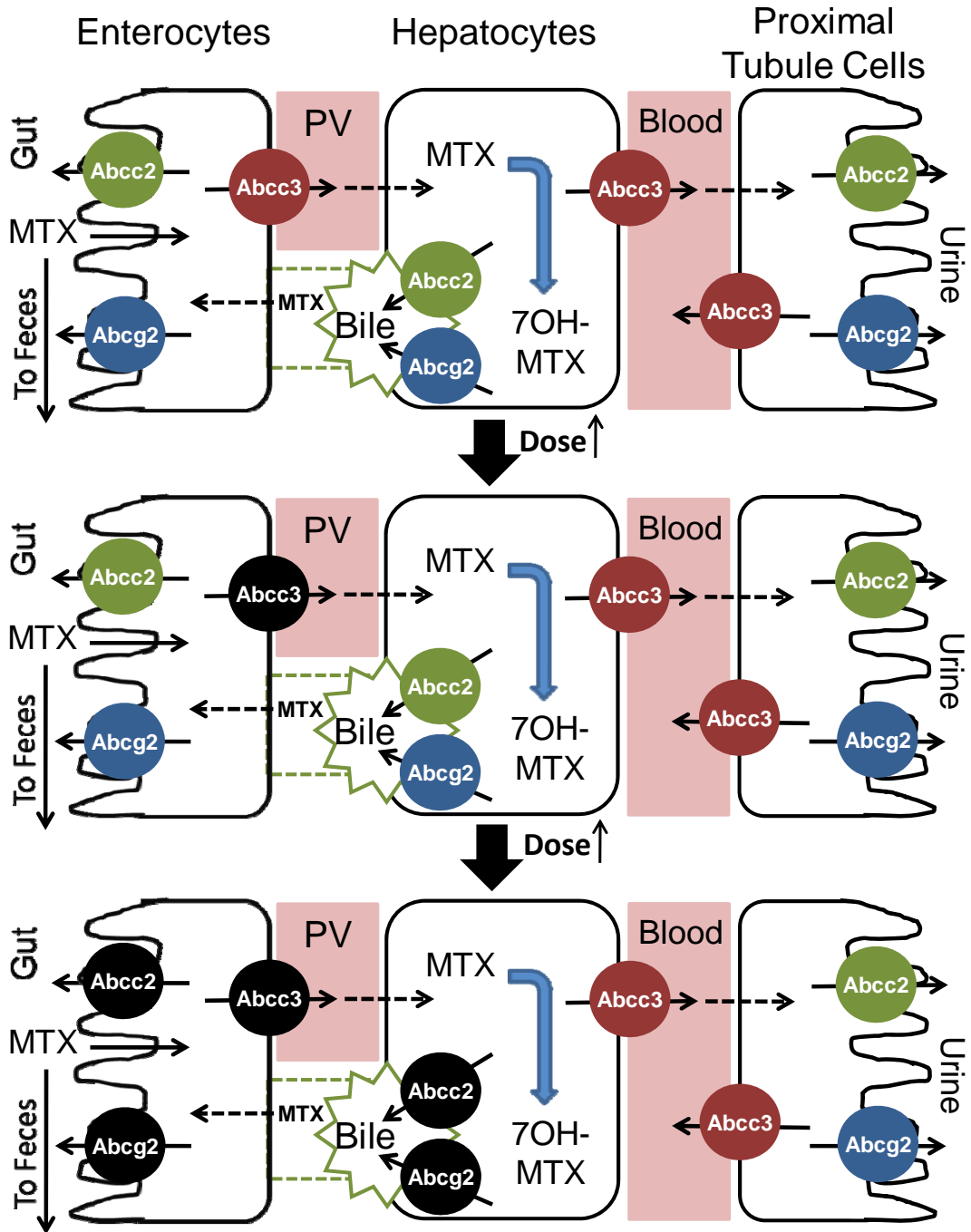
damage may take time and hence is not evaluated in our current analysis of the MTX oral PK.

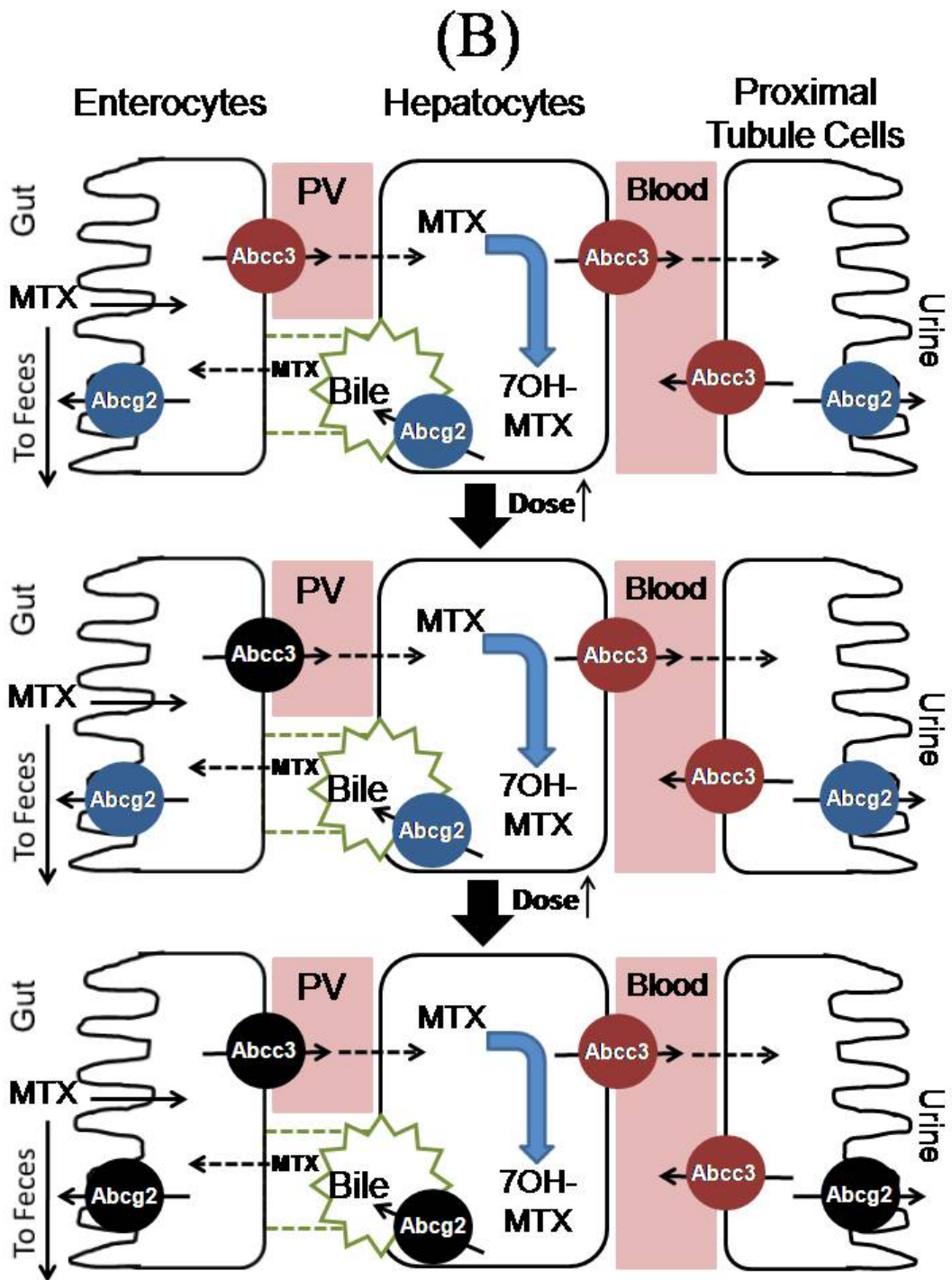
To gain greater insight into the oral bioavailability of MTX we employed portal vein administrations that could differentiate enterocyte-based and hepatocyte-based mechanisms. Portal vein cannulation in rodents has been reported previously for serial portal venous blood collection, to compare oral AUC with that of IV route (Shen et al., 2008). However, combined portal vein drug administration (Vrancken Peeters, Perkins, & Kay et al., 1996) and carotid artery cannulation for serial blood collection has not been often completed. Our preliminary dose-dependent portal venous studies revealed interesting findings that the mechanism of the rebound oral bioavailability at 200 mg/kg could dwell in the compensatory hepatic basolateral absorption of MTX upon the saturated transporters on the canaliculi, while the reduction of oral bioavailability at medium dose could be merely limited to the saturation of basolateral effluxers in enterocytes. More related experiment may be warranted to verify such unique findings.

In summary, a mechanistic scheme (Figure 14) is proposed for describing the complexity of MTX disposition by the overlapping functions of the key ABC transporters over a large dose range. The current study confirmed our previous finding of compensatory elimination pathways into bile and urine upon the saturation of associated ABC transporters after MTX oral doses over a large range and revealed dose-dependent oral bioavailability. The systemic exposure of MTX seems to be elevated by Abcc2 expression, which has a slightly positive impact on MTX oral bioavailability. Whereas,

Abcc3 has less influence on the oral bioavailability of MTX at low doses, nonetheless it does not affect the pivotal role of Abcc3 as a protectant for hepatocytes, enterocytes and kidney cells, particularly at high doses. In one word, functional loss of Abcc2 and Abcc3 is able to affect MTX oral PKs and the extent of the impact depends on the dose.

(A)





(C)

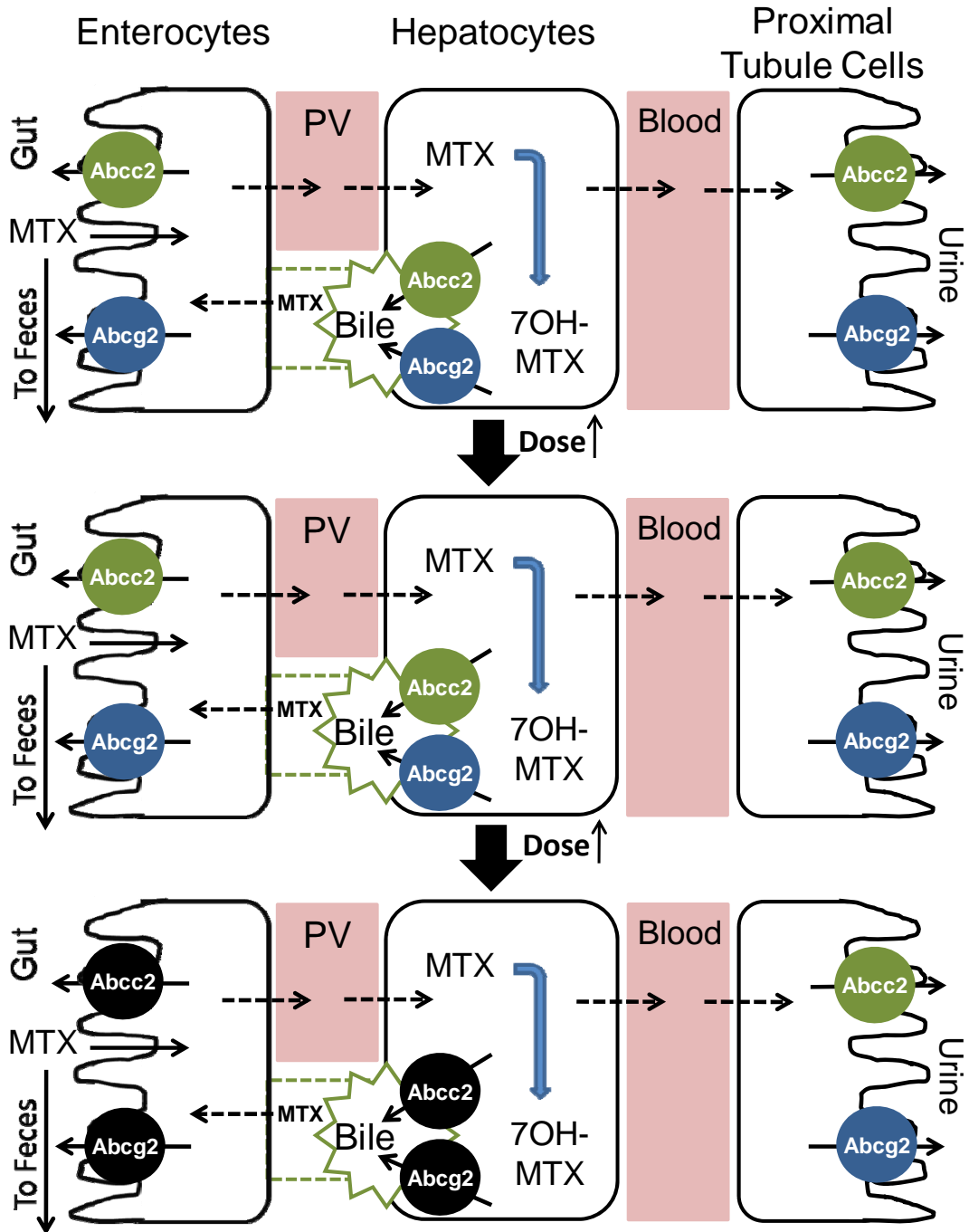


Figure 14. Proposed action of key ABC transporters involved in the pharmacokinetics of MTX in enterocytes, liver hepatocytes and renal proximal tubules as a function of oral doses in WT (A), *Abcc2*^{-/-} (B), and *Abcc3*^{-/-} (C) strains. For each strain, three states of transporter function are shown corresponding to our low-, medium- and high-dose oral MTX groups. Non-black colored transporters are operating below saturation whereas black colored indicates saturation. Dashed arrows indicate EHC or possible transport through passive diffusion. Other transporters may be involved, such as Pcf1 and Rfc, and are not shown, but the current illustration is most consistent with our data and others highlighting the key transporters (Vlaming et al., 2011). The extent of conversion of MTX to 7OH-MTX in hepatocytes is indicated by the thickness of the arrows.

CHAPTER 4
A PK MODEL OF MTX IN MICE:
A SEMI-MECHANISTIC COMPARTMENTAL CONDITIONAL MODEL
INCORPORATING KEY ABC TRANSPORTERS

4.1 Introduction

MTX is a classic folate antagonist used for the treatment of different autoimmune and neoplastic diseases that require a wide dose range (Braun & Rau, 2009). In addition, MTX is also a known substrate for numerous transporters and its PKs was reported to be influenced by key transporters, such as Abcc2 and Abcc3. Since 1970's, MTX PKs was investigated and several models were developed to describe MTX's plasma profiles, yet no model has directly incorporated key transporters into it for a wide dose range. Previous attempts to account for the observed nonlinearity of MTX PKs, such as "S-shaped" biliary efflux curve, were described by a finite series of discrete compartments (Bischoff, Dedrick, & Zaharko, 1970) yet not transporter activities. A recent report delineated an intracellular biochemical model of MTX, its polyglutmylated metabolites and targets after high dose infusion (Panetta, Sparreboom, Pui, Relling, & Evans, 2010), yet efflux transporters significant for MTX systemic disposition were neglected. Overall, the impact of efflux transporters on drug disposition has been recognized and has been considered in both compartmental and physiologically-based PK modeling (Pang & Durk, 2010).

In an effort to better understand key transporters properties and the extent of their impact on MTX PKs, a semi-mechanistic PK model was developed to characterize the nonlinear PK characteristics of MTX in different wild-type and gene-disrupted mouse strains following IV doses over a wide range. This model allowed us to relate the change of MTX plasma concentration profiles to the loss of certain transporters, thereby verifying the function of the transporters in MTX disposition.

4.2 Methods

4.2.1 MTX PK Studies In WT and Knockout Mice Strains

Single dose PK studies were conducted at three IV dose levels (10, 50, 200 mg/kg) on WT, *Abcc2*^{-/-} and *Abcc3*^{-/-} mice strains. Plasma concentrations of MTX at different time points were obtained for each mouse via serial blood sampling and determined by LC/MS/MS. The experimental design and conduction of the studies were described in detail in the methods sections of Chapter 2.

4.2.2 MTX Nonlinear Semi-Mechanistic

Conditional Compartmental Model Development

Based on the nonlinear observations in MTX plasma profiles over a large dose range as well as the noncompartmental analysis performed in Chapter 2, nonlinear semi-mechanistic 3-compartmental model incorporating key transporters (*Abcc2*, *Abcc3* and *Abcg2*) was constructed for WT mice (Figure 15, Part A), with one central compartment representing fast distributing organs and two peripheral compartments representing fast and slow equilibrium tissue compartments. Thus, nonlinear semi-mechanistic

compartmental models for the knockouts (*Abcc2*^{-/-} and *Abcc3*^{-/-} mice) were subsequently obtained by deleting associated transporters according to the knockout types (Figure 15, Part B&C). To avoid overparameterization, transporters *Abcc2* and *Abcg2* in WT and *Abcc3*^{-/-} mice were further lumped into a hybrid efflux transporter (Hyb), by assuming the hybrid efflux transporters is another type of efflux transporter with properties similar to other real transporters (i.e. *Abcc2* and *Abcg2*). Combining those models for WT and knockouts, a nonlinear semi-mechanistic conditional 3-compartmental model was shown in Figure 16 to describe MTX plasma profile, with relevant transporters or hybrid transporters activated depending on the strain. The differential equations (equation (1) – (3)) were:

$$\begin{aligned} \frac{dX_1}{dt} = & -(k_{10} + k_{12} + k_{13}) \times X_1 + k_{21} \times X_2 + k_{31} \times X_3 \\ & - \frac{\alpha \times V_{mH} \times X_1}{k_{mH} \times V_1 + X_1} + \frac{\beta \times V_{m3} \times X_1}{k_{m3} \times V_1 + X_1} - \frac{\gamma \times V_{mg} \times X_1}{k_{mg} \times V_1 + X_1} \end{aligned} \quad (1)$$

$$\frac{dX_2}{dt} = k_{12} \times X_1 - k_{21} \times X_2 \quad (2)$$

$$\frac{dX_3}{dt} = k_{13} \times X_1 - k_{31} \times X_3 \quad (3)$$

where for WT mice, $\alpha = 1$, $\beta = 1$, $\gamma = 0$, while for *Abcc2*^{-/-} mice, $\alpha = 0$, $\beta = 1$, $\gamma = 1$ and for *Abcc3*^{-/-} mice, $\alpha = 1$, $\beta = 0$, $\gamma = 0$. In the above equations, abbreviations represent MTX amounts in compartment 1 (X_1), 2 (X_2), 3 (X_3), volume of central compartment (V_1), intercompartment rate constants (k_{12} , k_{21} , k_{13} , k_{31}), elimination rate constant from compartment 1 (k_{10}), capacities of hybrid efflux transporter (V_{mH}), *Abcc3* (V_{m3}) and *Abcg2* (V_{mg}), respectively, and affinities of hybrid efflux transporter (k_{mH}), *Abcc3* (k_{m3}) and *Abcg2* (k_{mg}), respectively.

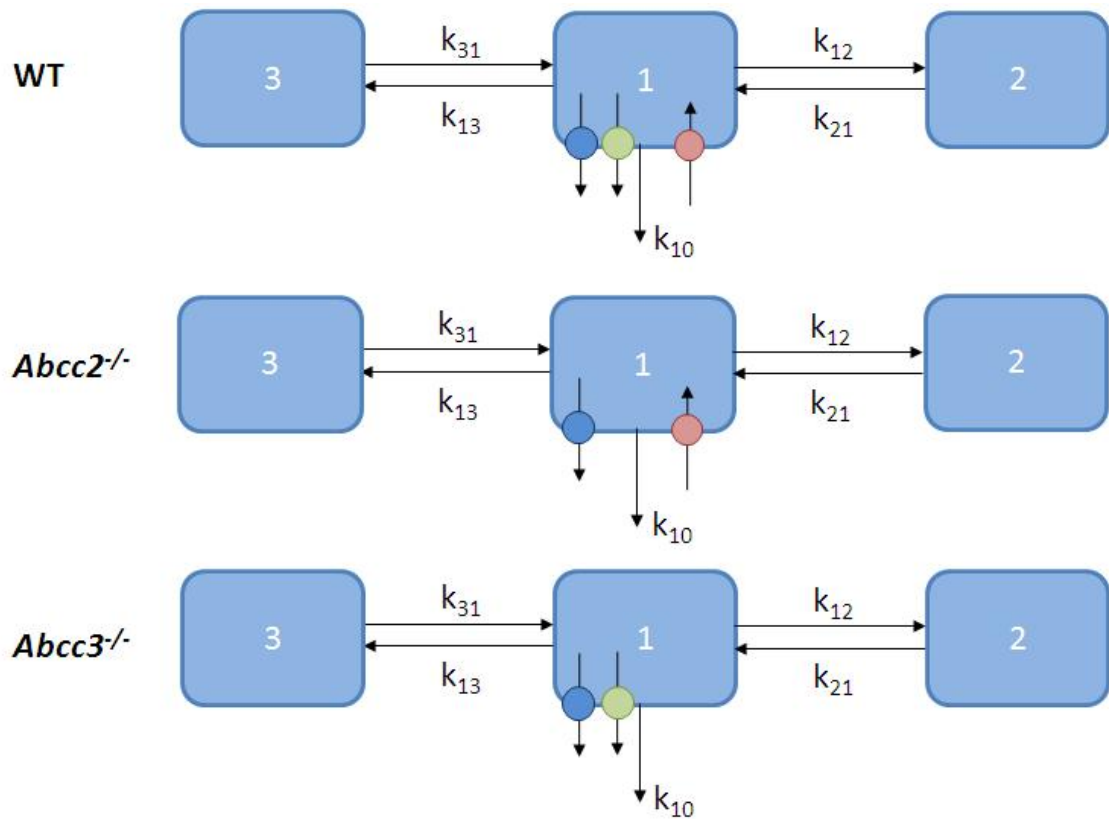


Figure 15. Nonlinear semi-mechanistic 3-compartmental conditional model for WT, *Abcc2*^{-/-} and *Abcc3*^{-/-} mice. This model incorporates three key transporters with colorful dots indicating different transporter types: 1) *Abcc2* (green), 2) *Abcc3* (red) and *Abcg2* (blue). The k s are either elimination rate constant (k_{10}) or inter-compartmental connections (the rest).

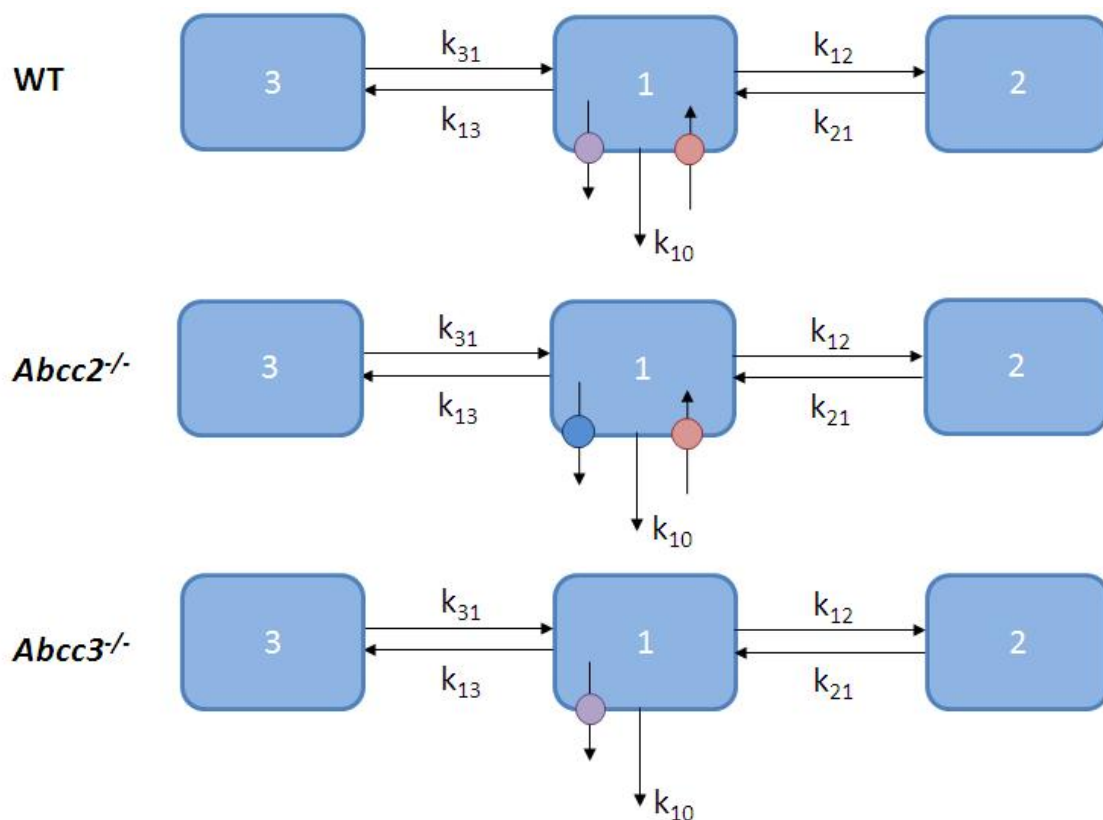


Figure 16. Nonlinear semi-mechanistic 3-compartmental conditional model employing hybrid efflux transporter for WT, *Abcc2*^{-/-} and *Abcc3*^{-/-} mice. This model incorporates three transporter types with colorful dots indicating different ones: 1) Hybrid efflux transporter (purple, a combined form of *Abcc2* and *Abcg2*), 2) *Abcc3* (red) and 3) *Abcg2* (blue). The k s are either elimination rate constant (k_{10}) or inter-compartmental connections (the rest).

4.2.3 Data Analysis

The nonlinear semi-mechanistic conditional model fittings for MTX plasma profiles in all strains at three dose levels were performed in ADAPT 5 (version 5, Biomedical Simulations Resource, Los Angeles, CA) using the Iterative Two Stage (ITS) method. The error variance model was:

$$V_i = (\sigma_{inter} + \sigma_{slope} \times Y_i)^2$$

where V_i is the variance of the i th data point, σ_{inter} and σ_{slope} are the parameters in the variance model, and Y_i represents the i th model predicted value. According to the NCA, the data of high dose in $Abcc3^{-/-}$ mice possibly requires additional mechanism and therefore not included in the model fitting. The hybrid efflux transporter terms were first estimated from separated $Abcc3^{-/-}$ mice data, and then fixed to obtain the other parameters in the combined all strains data.

Various proposed models for describing MTX plasma profiles in different strains were fitted and compared. The final model was selected based on the goodness-of-fit criteria including visual inspection of the fitted curves, Akaike information criterion (AIC), Bayesian information criterion (BIC), negative 2 loglikelihood (NLL), and coefficients of variation (CV) of the estimated parameters. Only results of the final model fitting are presented in this Chapter.

4.3 Results

MTX pharmacokinetics was investigated in WT, $Abcc2^{-/-}$ and $Abcc3^{-/-}$ mice following single IV doses of 10, 50 and 200 mg/kg, as $Abcc2$ and $Abcc3$ have

overlapping function to transport MTX in vivo. Besides Abcc2 and Abcc3, Abcg2 is also reported to affect MTX pharmacokinetics (Vlaming et al., 2009a&b). Therefore, in the current study, we proposed a nonlinear semi-mechanistic conditional 3-compartmental PK model, which incorporates those key transporters as nonlinear terms to describe MTX plasma profile in all strains. The model parameters estimated by ADAPT 5 were listed in the following Table 7. The hybrid efflux transporter can be seen as a combined one of Abcc2 and Abcg2.

As shown in Figure 17 for typical individual mice at each dose level and strain, the proposed 3-compartmental PK model was able to capture the MTX concentration-time profiles. This model was improved over alternate models such as a 2-compartmental model incorporating the same types of transporters, based on objective goodness of fit criteria that included NLL (-1212.75), AIC (-1078.75) and BIC (-924.382). In addition, the individual standardized residual plots vs model predicted concentrations and time exhibited an even distribution of residuals about the line of $y = 0$ and were of low values (Figure 18).

Based on the estimated Abcc3's capacity (1.16 $\mu\text{g}/\text{min}$) being larger than the capacities of Abcg2 (0.246 $\mu\text{g}/\text{min}$) and the hybrid efflux transporter (0.232 $\mu\text{g}/\text{min}$), the model supported our conclusion from previous NCA that basolateral Abcc3 exerts its alternate or compensatory protective role for exporting MTX, especially when those apically expressed efflux transporters (Abcc2 and Abcg2) may be overwhelmed first with less capacities and comparable or higher affinities.

Table 7. MTX PK parameter estimation and CV% in the nonlinear semi-mechanistic conditional 3-compartmental model by ADAPT 5 using ITS for algorithm method

Parameters (Algorithm method: ITS)	Mean	CV%
V_1 , volume of central compartment (ml)	14.0	29.4
k_{12} , rate constant from compartment 1 to 2 (min^{-1})	0.0315	62.0
k_{21} , rate constant from compartment 2 and 1 (min^{-1})	0.0685	45.7
k_{13} , rate constant from compartment 1 to 3 (min^{-1})	0.00294	94.1
k_{31} , rate constant from compartment 3 and 1 (min^{-1})	0.00165	66.7
k_{10} , elimination rate constant from compartment 1 (min^{-1})	0.0930	37.6
V_{mH} , capacity of hybrid efflux transporters ($\mu\text{g}/\text{min}$)	0.232	77.8
k_{mH} , affinity of hybrid efflux transporters ($\mu\text{g}/\text{ml}$)	1.00	0.00301
V_{m3} , capacity of Abcc3 transporters ($\mu\text{g}/\text{min}$)	1.16	29.6
k_{m3} , affinity of Abcc3 transporters ($\mu\text{g}/\text{ml}$)	0.983	26.3
V_{mg} , capacity of Abcg2 transporters ($\mu\text{g}/\text{min}$)	0.246	47.6
k_{mg} , affinity of Abcg2 transporters ($\mu\text{g}/\text{ml}$)	0.408	46.2
σ_{inter} , parameter of intercept in the variance model	0.00287	
σ_{slope} , parameter of slope in the variance model	0.134	

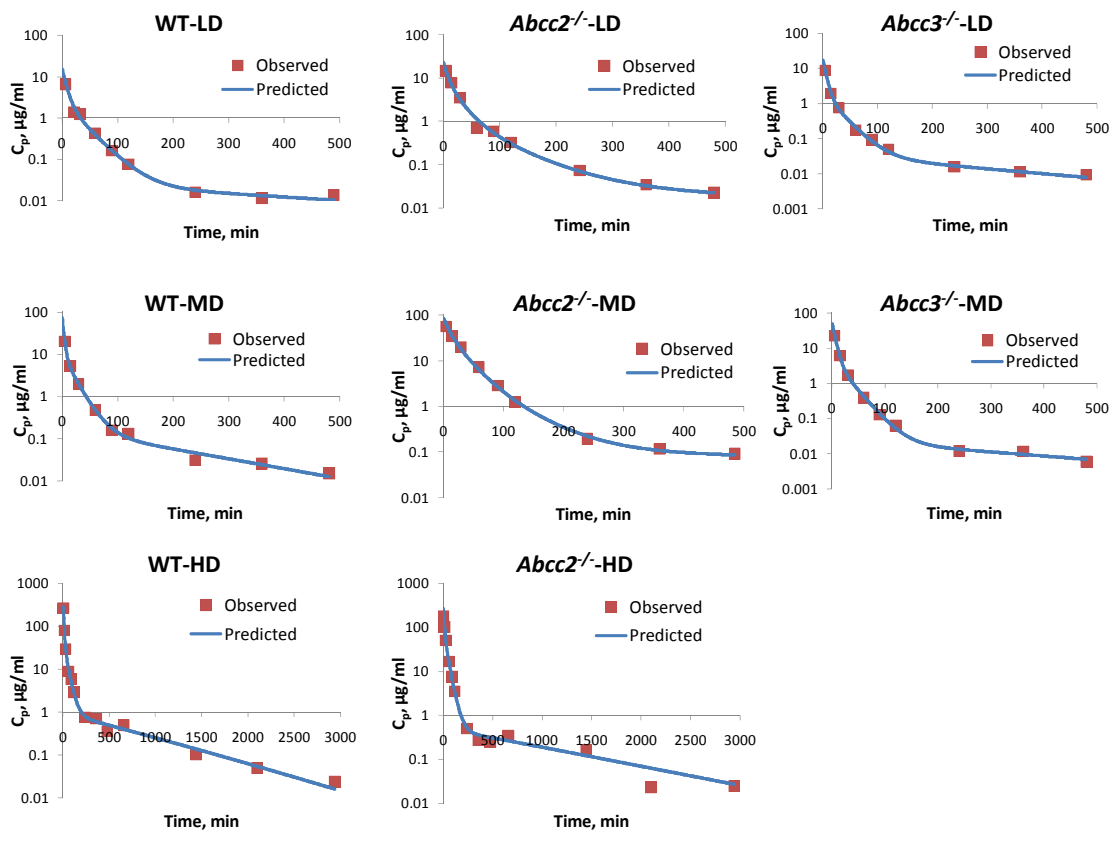
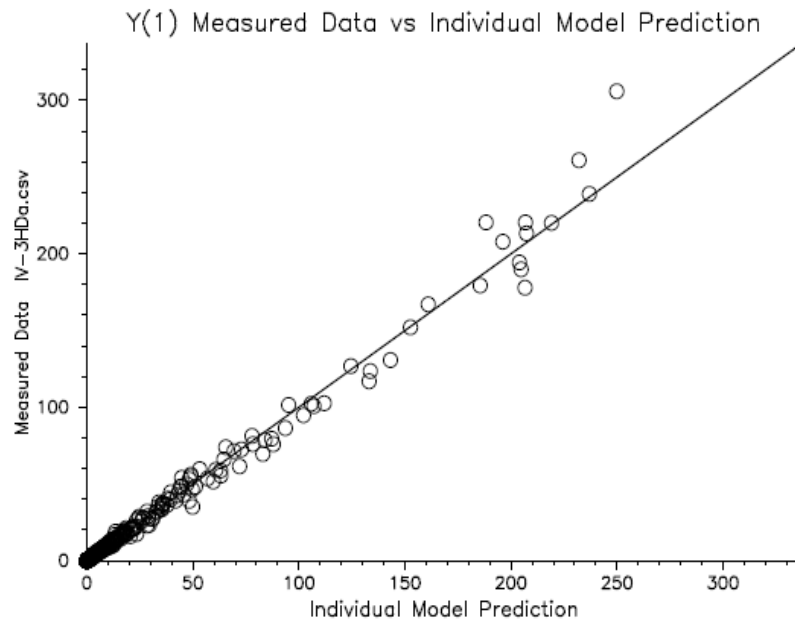
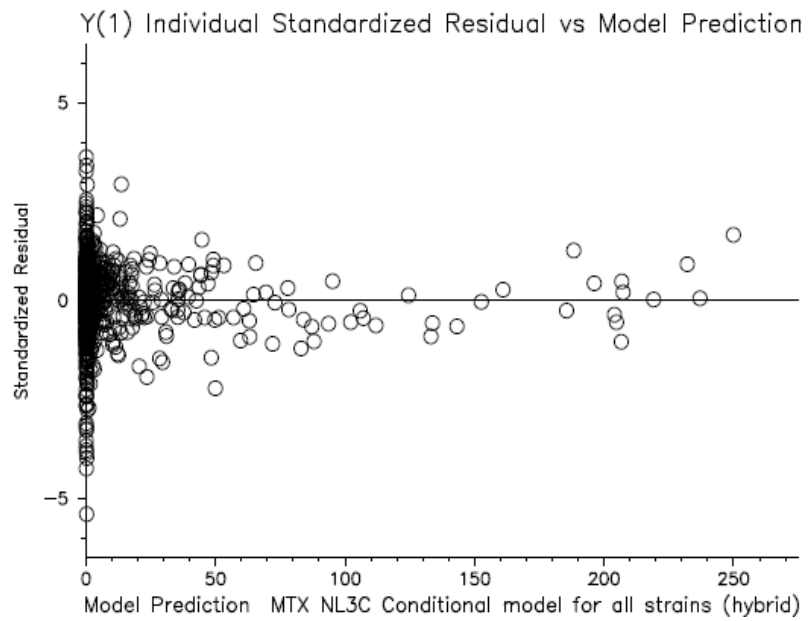


Figure 17. Observed vs model predicted for typical individual mice. Typical individual mouse observed data (red squares) of different dose levels in each strain vs the predicted (blue line) by the reduced semi-mechanistic 3-compartmental conditional model.

A)



B)



C)

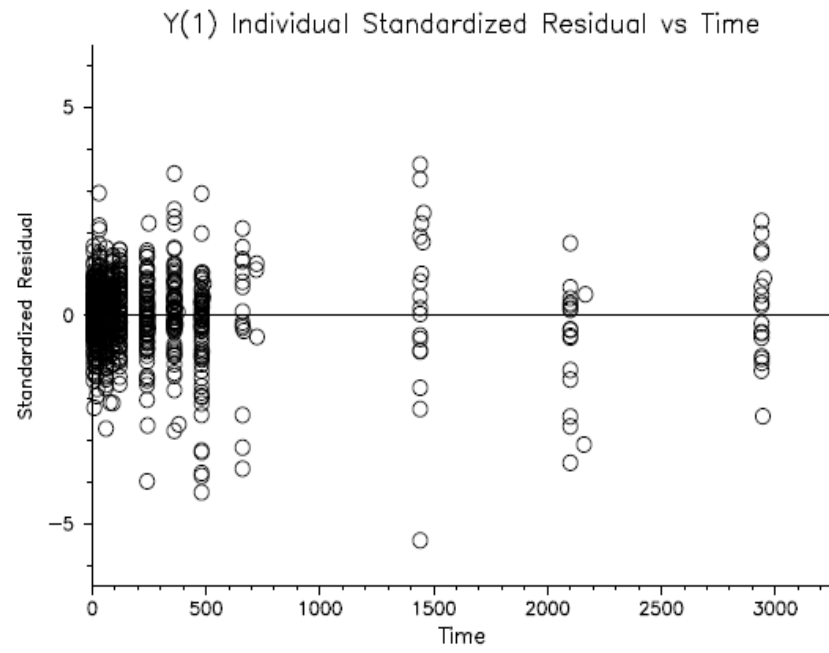


Figure 18. Resulting plots after running the model fitting based on population analysis via ADAPT 5. A) Measured data vs model prediction; B) Individual standardized residual vs model prediction; C) Individual standardized residual vs time.

4.4 Discussion

Interest to construct a PK model for MTX was motivated by the fact that the NCA indicated it possessed nonlinear kinetics that could be attributed to saturable transport. Such a model not only can provide mechanistic insight but also be used as a tool to design therapeutic dosing regimens in preclinical studies as well as offer the possibility of being scale to predict MTX's PK in patients. Based on our study design of using the gene knockout animal models, we were able to consider three key transporters: Abcc2, Abcc3 and Abcg2 to be incorporated during the development of a PK model. The modeling effort resulted in a nonlinear semi-mechanistic 3-compartmental model.

The strategy of model development was to combine MTX plasma concentrations from all strains and dose levels in a population modeling format to increase the number of mice and observations for common features between strains. This approach was implemented using conditional terms for the various transporters that were "active" or "inactive" depending on the particular mouse strain. In this manner, common drug distribution terms (intercompartmental rate constants) and first-order elimination terms could be estimated from all the strains. It is appreciated that these common terms may not be of equally value indicating altered function in particular strains; however in the final model all parameters were uniquely identified to avoid significant strain-bias. Nonetheless, depending on the magnitude of interstrain differences in MTX's PK related to these common processes the final model may still have certain biases. These could be related to MTX's metabolism and tissue distribution. With regard to metabolism of MTX to 7OH-MTX there were strain differences that have now been lumped into a single first-

order elimination rate constant. Attempts to apply a conditional elimination rate constant did not improve the model fit, which is not too surprising given that only a small percentage of the dose is metabolized. Strain differences in the volume of distribution could be attributed to the role of Abcc2 or Abcc3 altering tissue distribution, yet here again the magnitude of such changes seems to be small. Although additional studies could be completed, the final model reflects the most salient features of the nonlinear disposition of MTX. One final consideration for the model was to employ a hybrid efflux transporter to account for the well-established function of Abcg2 on MTX's biliary elimination that was necessitated, since we did not study this knockout strain. Given these limitations the current model and model development approach provide a foundation that can be further explored in the future.

In summary, a nonlinear semi-mechanistic 3-compartmental model for MTX incorporating key transporters was developed to capture MTX plasma concentration-time profiles over a large dose range. A unique feature of model building was to employ a population-based approach and conditional transport terms to make maximum use of the large data set. The potential limitations of the model can best be addressed by studying the Abcg2 knockout strain, and possibly the Abcc2;Abcg2 double knockout strain.

CHAPTER 5

SUMMARY OF RESEARCH FINDINGS

My research project was designed to characterize the role of *Abcc2* and *Abcc3* on pharmacokinetics of MTX using single *Abcc2* and *Abcc3* gene knockout murine models.

The studies were conducted in the different mouse strains (WT, *Abcc2*^{-/-}, and *Abcc3*^{-/-}) over a large dose range (10, 50, 200 mg/kg) following both IV and oral drug administrations. For each mouse entered into the studies, serial plasma, and cumulative urine and feces were analyzed for MTX and its 7OH-MTX metabolite. The resultant plasma concentration time profiles analyzed by noncompartmental analysis indicated a number of significant changes in the PK parameters both as a function of dose [intrastrain differences] as well as due to strain [interstrain differences].

For IV administrations, the primary findings were: i) *Abcc2*^{-/-} mice exhibited significant decreases in total clearance of MTX relative to WT strain at all dose levels, and such decreases were accompanied by compensatory renal excretion and metabolic conversion to 7OH-MTX; ii) *Abcc3*^{-/-} mice exhibited a significant increase in total clearance of MTX at lower dose levels accompanied by compensatory fecal excretion, yet by a precipitous drop at a saturable high dose.

For oral MTX administrations, the primary findings were: i) regardless of the mouse strain, a dose increase of MTX was not accompanied by proportional increases in AUC_{MTX} ; ii) The loss of Abcc2 resulted significant increase in MTX systemic exposure as well as slight increase in oral bioavailability relative to WT mice at all dose levels, accompanied with compensatory increases in renal excretion and conversion to 7OH-MTX; iii) The lack of Abcc3 caused significant decreases in C_{max} and AUC_{MTX} relative to WT mice at all dose levels yet without obvious compensatory pathways suggesting further investigation.

Both the IV and oral results indicated that 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.

In addition to the NCA analysis, a PK modeling strategy was also applied to the data obtained by the IV route. The method employed conditional programming, dependent on the strain, to allow the maximum number of mice to be used within the framework of a population-based approach, and specifically represented the saturable transport mechanisms due to Abcc2 and Abcc3. This resulted in an optimized semi-mechanistic 3-compartmental PK model that captured the MTX plasma concentration-time course profiles based on goodness-of-fit criteria, such as NLL, and individual standardized residuals vs model and time.

The results of the study indicate the importance of using a large dose range to understand compensatory mechanisms of elimination and provide a rationale of using similar study designs for other drugs that are subject to saturable transport clearance. Although not specifically evaluated in the current project, the study also provides a basis to design combination drug regimens with MTX that either are analogous or have overlapping transport specificity. In such cases, combinations that maximize drug activity without toxicity may be achieved.

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APPENDICES

APPENDIX A

VERIFICATION OF ANIMAL GENOTYPES

For verifying the genotypes of the animals used in the pharmacokinetic studies, regular PCR experiment was applied for each in-bred mouse initially and later the breeding pairs for each strain. Before the PCR verification, tail tip about 0.75 cm length of the animal was cut for purification of the DNA segments. The DNA purification procedures was referred to the section of solid tissue protocols in the manual of PUREGENE[®] DNA purification Kit, including cell lysis, RNase treatment, protein precipitation, DNA precipitation and DNA hydration. Briefly, each cut tail tip was added into a 1.5 ml microfuge tube containing 500 μ l Cell Lysis Solution and manually homogenized. The tail tip lysate was then treated with 1.5 μ l RNase A Solution (4 mg/ml) for well mixing by inverting the mixture 25 times and incubation at 37 °C for 1 hr. The lysate was cooled to room temperature by placing on ice for 1 min added 100 μ l Protein Precipitation Solution. Again for uniform mixture, vigorous vortex was applied at high speed for 20 sec and protein was precipitated after 3-min centrifugation (at 13,000 – 16,000 \times g for 3 min) into a tight pellet. The supernatant containing the DNA was saved into a clean 1.5 ml microfuge tube containing 300 μ l 100 % Isopropanol and was gently mixed by inverting 50 times. DNA was yielded into a white pellet after centrifugation at 13,000 – 16,000 \times g for 1 min. Supernatant was poured off and tube was drained on clean

absorbent paper. The left DNA pellet was washed by adding 300 μ l 70% Ethanol into the tube and inverting several times. The washed DNA was centrifugated again at 13,000 – 16,000 \times g for 1 min and the supernatant was carefully poured off to watch the loose pellet. The tube was drained and allowed to air dry for 5-10 min. The dried pellet was added 50 μ l DNA Hydration Solution and rehydrated overnight at room temperature. The purified DNA was finally stored at 4 $^{\circ}$ C for short term storage and -20 $^{\circ}$ C for long term storage.

Each purified DNA sample was processed for regular PCR test on an associated machine (Gene Amp PCR System 9700, Applied Biosystems) after its purity was checked, which could be determined by the ratio of spectrophotometric readings at 260 nm and 280 nm [A260/A280] on a spectrophotometer (Spectra MaxTM M2, Molecular Devices) with a passing value falling into 1.8 – 2.0. Briefly, each DNA sample was added into a PCR tube containing specific quantities of necessary components (Table 8), including DNase, RNase free water (H₂O), 10 \times buffer, magnesium chloride (MgCl₂, 25 mM), dNTPs (10 mM), primer pairs for specific transporter (detailed information referring to Table 9) and Taq polymerase. Along with a negative control (replacing DNA sample with H₂O), DNA samples were loaded onto the PCR machine following the pre-set PCR programs for specific transporter (Table 10), where Ta stands for annealing temperature for specific strains and each cycle of PCR briefly contains denaturation, annealing and extension period. The primers sequences used for identification of WT, *Abcc2*^{-/-} and *Abcc3*^{-/-} mice were summarized in Table 9.

Table 8. Listed components for one PCR sample or negative control for PCR experiment

Component	Control (μL)	Sample × 1 (μL)
H ₂ O	12.0 + 2.0	12.0
10 × Buffer	2.0	2.0
MgCl ₂ , 25 mM	1.6	1.6
dNTPs, 10 mM	0.5	0.5
Forward Primer, 10 μM	0.8	0.8
Reverse Primer, 10 μM	0.8	0.8
Taq Polymerase	0.3	0.3
DNA	0	2.0
Total	20	20

Table 9. Sequences and sizes of primer pairs for identification of different transporters

Transporter	Primer pairs (starting with 5')	Annealing Temperature (°C)	PCR Product size (bp)
<i>Abcc2</i> ^{+/+} (<i>Mrp2</i> WT)	2F (forward primer): ACCTACAATGTAGCCTTGACTGAC 2400R (reverse primer): TGACTCAAGATCCTTGCCCTTG	67	~400
<i>Abcc2</i> ^{-/-} (<i>Mrp2</i> KO)	2F (forward primer): ACCTACAATGTAGCCTTGACTGAC 2200R (reverse primer): CTGCCTTGGGAAAAGCGCCTC	67	200
<i>Abcc3</i> ^{+/+} (<i>Mrp3</i> WT)	3N004F (forward primer): TAGGAGAGAGAAGGCAACAA 3N537R (reverse primer): TGAATTTTCATGTCAGCACAAT	53	~500
<i>Abcc3</i> ^{-/-} (<i>Mrp3</i> KO)	3F (forward primer): GTTCTGTGCCCTCATCCTGTCC 3300R (reverse primer): AATTGACCTGCAGGGGCCCTCG	53	300

Table 10. PCR steps programmed in the PCR machine

Steps No.	Temperature (°C) or Programmed Commands	Associated Time Duration of the Step (If Applied)
1	94	2 min
2	94	30 sec
3	T _a	30 sec
4	72	1 min
5	Go to Step 2, 34 more times	-
6	72	7 min
7	72	24 h
8	End	-

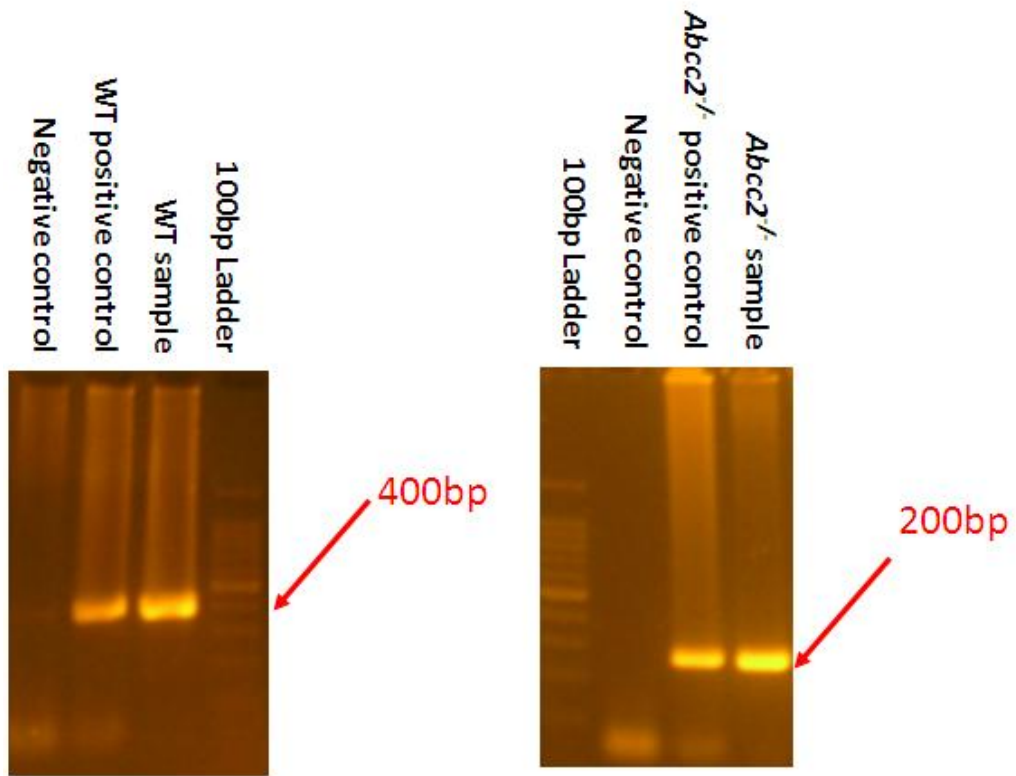


Figure 19. Typical genotype verification of *Abcc2*^{+/+} (*Mrp2* WT, left) and *Abcc2*^{-/-} (*Mrp2* KO, right) mice by regular PCR.

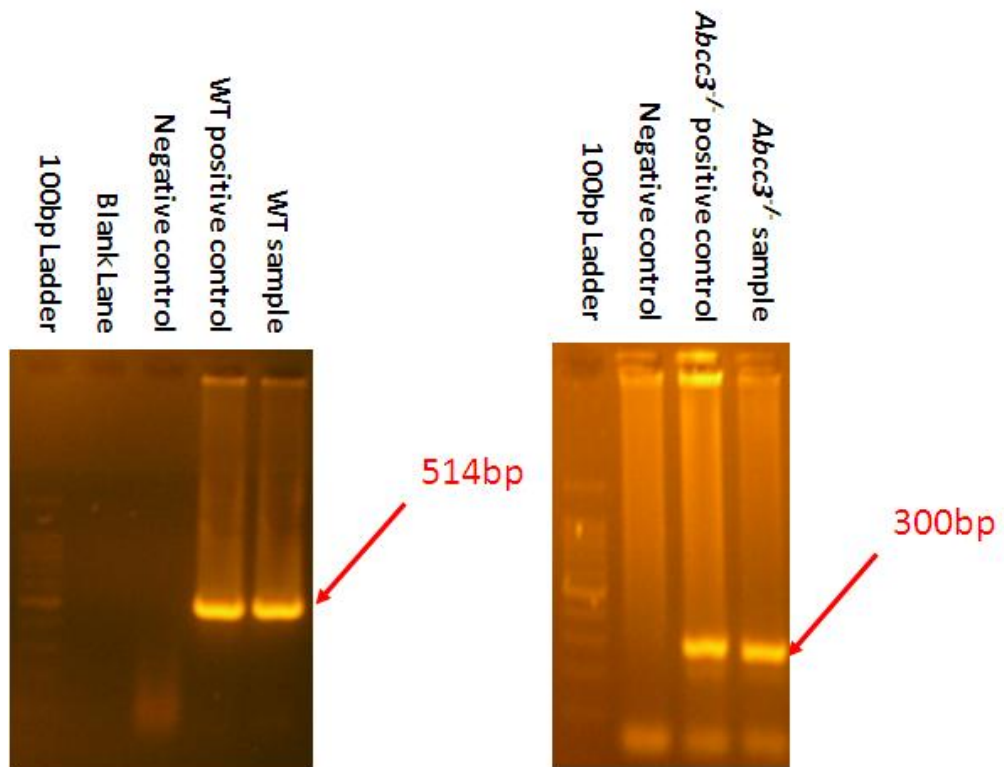


Figure 20. Typical genotype verification of *Abcc3*^{+/+} (*Mrp3* WT, left) and *Abcc3*^{-/-} (*Mrp3* KO, right) mice by regular PCR.

Each PCR product was loaded with 10× die into a well on a 0.5% Agarose gel (containing 0.003% (v/w) Ethidium Bromide) casted on the electrophoresis apparatus containing 1× TAE buffer at the negative electrode side. With negative charges due to all the phosphate groups in the backbone and subsequent different sizes, DNA was differentiated under an electric current applied to the gel for 40 min according to the 100 bpladder as reference. The gel was then visualized under UV visualizer (Hoefer Scientific Instruments, San Francisco) and picture was taken for reference. The genotypes of the mice for each strain were successfully confirmed by the regular PCR studies with typical verification of WT (*Abcc2*^{+/+} and *Abcc3*^{+/+}), *Abcc2*^{-/-} (*Mrp2* KO) and *Abcc3*^{-/-} (*Mrp3* KO) as follows (Figure 19-20).

APPENDIX B

**INTER-DAY AND INTRA-DAY VALIDATION OF BIO-ANALYTICAL
METHOD FOR MTX AND/OR 7OH-MTX IN BIOLOGICAL**

The precision and accuracy of the assay was based on analyses of plasma, urine and feces containing known concentrations of drugs. Quality control samples for plasma, urine and feces were included within the ranges of all calibration curves and processed in triplicate. The intra-day and inter-day means, standard deviations, % biases, and percentage coefficients of variations (% CVs) were calculated by standard methods. The LLOQ in plasma, urine and feces samples was defined as the lowest concentration at which the signal-to-noise peak height ratio was greater than 5:1, and both intra-day and inter-day % CVs and % biases less than 20% were accepted.

Upon validation, 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 (see Table 11) and 2.6 – 2062.9 ng/mL for 7OH-MTX (see Table 12); while both intra- and inter-day precision were all less than 15% over a range of 2.744 – 2000 µg/mL for urine concentrations (see Table 13) and of 2.31 – 300 µg/mL for feces (see Table 14) concentrations of MTX.

Table 11. Precision and accuracy of LC/MS/MS method for determination of MTX in plasma samples

MTX in Plasma (ng/ml)	Intra-day (n = 6)			
	HQ	MQ	LQ	LLQ
Quality of Controls	HQ	MQ	LQ	LLQ
Concentration added	994.9	58.1	10.6	3.5
Concentration measured	923.8	60.5	11.4	3.8
S.D.	35.9	4.7	0.3	0.3
CV (%)	3.9	7.5	2.4	9.0
Bias (%)	-2.2	4.1	7.5	8.6
	Inter-day (n = 5)			
Quality of Controls	HQ	MQ	LQ	LLQ
Concentration added	994.9	58.1	10.6	3.5
Concentration measured	890.8	63.8	11.3	3.8
S.D.	31.7	0.6	0.3	0.3
CV (%)	3.6	1.0	2.5	6.8
Bias (%)	-5.7	9.7	6.5	8.7

Table 12. Precision and accuracy of LC/MS/MS method for determination of 7OH-MTX in plasma samples

7OH-MTX in Plasma (ng/ml)	Intra-day (n = 6)			
Quality of Controls	HQ	MQ	LQ	LLQ
Concentration added	1984.3	122.1	22.2	7.4
Concentration measured	2150.0	137.2	25.3	8.3
S.D.	108.8	10.3	1.0	1.0
CV (%)	5.1	6.9	3.3	11.9
Bias (%)	8.4	12.4	14.0	12.3
	Inter-day (n = 5)			
Quality of Controls	HQ	MQ	LQ	LLQ
Concentration added	1984.3	122.1	22.2	7.4
Concentration measured	1913.0	121.4	22.9	7.5
S.D.	74.3	7.4	1.3	0.6
CV (%)	3.9	6.1	5.7	8.2
Bias (%)	-3.6	-0.6	3.0	1.9

Table 13. Precision and accuracy of LC/MS/MS method for determination of MTX in urine samples

MTX in Urine ($\mu\text{g/ml}$)	Intra-day (n = 6)			
	HQ	MQ	LQ	LLQ
Quality of Controls	HQ	MQ	LQ	LLQ
Concentration added	2000.0	74.1	8.2	2.7
Concentration measured	1990.0	75.0	8.5	2.6
S.D.	64.5	4.1	0.5	0.2
CV (%)	3.2	5.5	5.9	7.4
Bias (%)	-0.5	1.2	3.7	-4.3
	Inter-day (n = 5)			
Quality of Controls	HQ	MQ	LQ	LLQ
Concentration added	2000.0	74.1	8.2	2.7
Concentration measured	1956.3	71.6	8.2	2.7
S.D.	108.5	2.3	0.4	0.1
CV (%)	5.5	3.3	4.6	5.0
Bias (%)	-2.2	-3.4	0.2	-2.0

Table 14. Precision and accuracy of LC/MS/MS method for determination of MTX in feces samples

MTX in Feces ($\mu\text{g/ml}$)	Intra-day (n = 6)			
Quality of Controls	HQ	MQ	LQ	LLQ
Concentration added	300.0	26.3	5.2	2.3
Concentration measured	318.3	25.8	5.0	2.2
S.D.	25.2	1.3	0.4	0.1
CV (%)	7.9	4.9	7.4	5.1
Bias (%)	6.1	-1.8	-4.3	-5.0
	Inter-day (n = 5)			
Quality of Controls	HQ	MQ	LQ	LLQ
Concentration added	300.0	26.3	5.2	2.3
Concentration measured	329.1	25.6	5.2	2.3
S.D.	15.5	1.3	0.2	0.2
CV (%)	4.7	5.1	4.7	7.9
Bias (%)	9.7	-2.7	-0.2	-1.6

APPENDIX C
ABBREVIATION

5-CHO-THF, leucovorin;

6-MP, 6-mercaptopurine;

6-TG, 6-thioguanine;

ABC, ATP-binding cassette;

ABCC, ABC subfamily C;

ADME, absorption, distribution, metabolism and elimination;

ALL, acute lymphoblastic leukemia;

ANOVA, one-way analysis of variance;

Ara-C, arabinofuranosyl cytidine;

ATP, adenosine triphosphate;

AUC, area under the curve;

AZT, azathioprine;

BBB, blood-brain barrier;

BCRP, breast cancer resistant protein;

BCS, Biopharmaceutics Classification System;

BCSFB, blood-cerebrospinal fluid barrier;

BDDCS, Biopharmaceutics Drug Disposition Classification System;

BSA, body surface area;
CL, clearance;
CNS, central nervous system;
CYP, cytochrome P-450;
dAMPA, pterate/4-amino-4-deoxy-N¹⁰-methyl pteric acid;
ddC, 2'-3'-dideoxycytidine;
DHEAS, dehydroepiandrosterone sulfate;
DHFR, dihydrofolate reductase;
DNP-SG, S-(2,4-dinitrophenyl)glutathione;
DOX, doxorubicin;
E217bG, estradiol 17 β -D-glucuronide;
EHC, enterohepatic recirculation;
FDA, Food and Drug Administration;
 f_f , fecal fraction of the dose;
FPGH, polyglutamylate hydrolase;
FPGS, polyglutamylate synthase;
 f_r , renal fraction of the dose;
GSH, glutathione;
HDAC, histone deacetylase;
ITC, International Transporter Consortium;
 K_m , affinity;
LT, leukotriene;
MRP, multidrug resistance protein;

MSD, membrane-spanning domains;
MTX, methotrexate;
MTX-PG, MTX polyglutamylate conjugations;
NBD, nucleotide-binding domain;
NLL, negative 2 log likelihood
OAT/ SLC22A, organic anion transporter;
OATP/ SLCO, organic anion-transporting polypeptide;
OCT/SLC22A, organic cation transporter;
OCTN/ SLC22A, organic cation/carnitine transporter;
PBPK, physiologically-based pharmacokinetics;
PCFT/HCP1, folate transporter/haem carrier protein; NCA, noncompartmental analysis;
PD, pharmacodynamic;
PEPT, SLC15A, peptide transporter;
P-gp, p-glycoprotein;
PK, pharmacokinetics;
PMEA, 9-((2-phosphonylmethoxy)ethyl)-adenine;
RA, rheumatoid arthritis;
SLC, solute carrier;
SNP, single nucleotide polymorphism;
 $t_{1/2}$, half-life of elimination phase;
 V_d , volume of distribution;
 V_{max} , maximum capacity;
WT, wild-type.