

Short Communication

The Effect of ABCG2 and ABCC4 on the Pharmacokinetics of Methotrexate in the Brain

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ABSTRACT

Methotrexate (MTX) is the cornerstone of chemotherapy for primary central nervous system lymphoma, yet how the blood-brain barrier (BBB) efflux transporters ABCG2 and ABCC4 influence the required high-dose therapy is unknown. To evaluate their role, we used four mouse strains, C57BL/6 (wild-type; WT), *Abcg2*^{-/-}, *Abcc4*^{-/-}, and *Abcg2*^{-/-};*Abcc4*^{-/-} (double knockout; DKO) to conduct brain microdialysis studies after single intravenous MTX doses of 50 mg/kg. When the area under the concentration-time curve for plasma (AUC_{plasma}) was used to assess systemic exposure to MTX, the rank order was *Abcc4*^{-/-} < WT < *Abcg2*^{-/-} < *Abcg2*^{-/-};*Abcc4*^{-/-}. Only the DKO exposure was significantly higher than that of the WT group ($P < 0.01$), a reflection of the role of *Abcg2* in biliary excretion and *Abcc4* in renal excretion. MTX brain interstitial fluid concentrations obtained by microdialysis were

used to calculate the area under the concentration-time curve for the brain (AUC_{brain}), which found the rank order of exposure to be WT < *Abcc4*^{-/-} < *Abcg2*^{-/-} < *Abcg2*^{-/-};*Abcc4*^{-/-} with the largest difference being 4-fold: $286.13 \pm 130 \mu\text{g} \cdot \text{min}/\text{ml}$ (DKO) versus 66.85 ± 26 (WT). Because the transporters affected the systemic disposition of MTX, particularly in the DKO group, the ratio of the $AUC_{\text{brain}}/AUC_{\text{plasma}}$ or the brain/plasma partition coefficient K_p was calculated, revealing that the DKO strain had a significantly higher value (0.23 ± 0.09) than the WT strain (0.11 ± 0.05). Both *Abcg2* and *Abcc4* limited BBB penetration of MTX; however, only when both drug efflux pumps were negated did the brain accumulation of MTX significantly increase. These findings indicate a contributory role of both ABCG2 and ABCC4 to limiting MTX distribution in patients.

Introduction

The blood-brain barrier (BBB) is composed of capillaries lined by endothelial cells with tight junctions to limit passive diffusion of drugs, and protein pumps that when directed toward the blood act as efflux transporters that limit the distribution of drugs into the central nervous system (CNS) (Deeken and Loscher, 2007). Primary BBB efflux pumps, notably *Abcb1* and *Abcg2*, are members of the ABC family of transporters and have been implicated in restricting the BBB penetration of several drugs (Schinkel et al., 1996). The consequence of limited brain access for CNS-active drugs is a lower therapeutic index, which could prevent use of the drug. It is essential to determine the role of the BBB and its efflux transporters in mediating the brain distribution of drugs, both to characterize the mechanisms of CNS disposition and to offer a rational means to overcome limited brain distribution.

Methotrexate (MTX) is the standard of care and a component of numerous experimental regimens for primary CNS lymphoma (DeAngelis, 2003; Zhu et al., 2009), a disease that accounts for 2% and 5% of all primary brain tumors (Gerstner and Batchelor, 2007). MTX is a folate antagonist with limited uptake in the brain, which likely contributes to the need for high-dose therapy (Nierenberg et al., 1991; Wolff et al., 2011). Studies in rodents (Devineni et al., 1996) and patients (Blakeley

et al., 2009) that used brain microdialysis to measure the brain interstitial fluid MTX concentrations attest to its limited distribution in the normal brain, with a brain-to-plasma ratio 0.051 ± 0.032 and 0.032 ± 0.094 in rodents and humans, respectively. MTX has been shown to be a substrate for breast cancer resistance protein (BCRP) (Volk and Schneider, 2003; Li et al., 2013) and multidrug resistance protein 4 (MRP4) (Chen et al., 2002) using in vitro membrane vesicle systems. *Abcg2* has been implicated in limiting the brain distribution of several drugs (Agarwal et al., 2010, 2011; Kodaira et al., 2010), and *Abcc4* has been shown to prevent the brain distribution of topotecan and oseltamivir (Leggas et al., 2004; Ose et al., 2009). However, the role of these transporters in limiting the distribution of MTX at the BBB has not been adequately explored, hence our investigation in *Abcg2*^{-/-}, *Abcc4*^{-/-}, and *Abcg2*^{-/-};*Abcc4*^{-/-} transgenic mice. To assess the role of these transporters in the brain penetration of MTX, we employed brain microdialysis in conjunction with serial plasma sampling after single intravenous dose administrations.

Materials and Methods

Methotrexate hydrate (molecular weight: 454.44), 7-hydroxy methotrexate (7-OH MTX; molecular weight: 470.44), and aminopterin were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were reagent grade or high-performance liquid chromatography grade and were purchased from Sigma-Aldrich. Guide cannulas (5-mm shaft) and MetaQuant probes (regenerated cellulose, dialysis tip length 2 mm, mol. wt. cutoff 13) were purchased from Brainlink (Groningen, The Netherlands).

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ABBREVIATIONS: AUC, area under the curve; BBB, blood-brain barrier; BCRP, breast cancer resistance protein; CL, clearance; CNS, central nervous system; DKO, double knockout; K_p , brain partition coefficient; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRP4, multidrug resistance protein 4; MTX, methotrexate; 7-OH MTX, 7-hydroxy methotrexate; WT, wild-type.

Animals. C57BL/6 wild-type (WT) and transgenic mice with genetic deletions of either the *Abcg2* transporter (*Abcg2* knockout, *Abcg2*^{-/-}), *Abcc4* transporter (*Abcc4* knockout, *Abcc4*^{-/-}) or both transporters (double knockout or DKO, *Abcg2*^{-/-};*Abcc4*^{-/-}) were derived in the laboratory of Dr. Gary Kruh (deceased) (Belinsky et al., 2007; Wang et al., 2011), previously at Fox Chase Cancer Center and finally at the University of Illinois at Chicago, and then transferred and maintained at Charles River Laboratories (Wilmington, MA) through an agreement with Mount Sinai School of Medicine. The mice were transferred to Mount Sinai approximately 7 days before entry into the pharmacokinetic studies and were maintained on a 12-hour light/dark cycle with unlimited access to food and water. All animal studies were approved by the Institutional Animal Care and Use Committee at Mount Sinai School of Medicine. All mice used in the experiment were male, were 10 to 12 weeks of age, and weighed 25 to 30 g.

Microdialysis Preparation. Anesthetized mice underwent a surgical microdialysis and vascular cannula implantation procedure, as previously described elsewhere (Guo et al., 2007), 3 days before the pharmacokinetic study with the modification that a MetaQuant probe system was located at 0.74 mm anterior and 2.0 mm lateral to the bregma with the guide cannula lowered to a depth of 2.5 mm. The Meta-Quant microdialysis system is designed to increase analyte recovery by perfusion of the dialysis membrane at a low flow rate (0.1 μ l/min) while simultaneously using a higher makeup flow rate (0.9 μ l/min) to eliminate long sample collection periods (Cremers et al., 2009). The animals were placed on a heating pad during recovery, and when fully ambulatory were provided with food and water ad libitum. The food was removed 2 hours before the pharmacokinetic study.

The guide cannulas were replaced by the microdialysis probes and calibrated by a retrodialysis method, and the recovery was calculated as previously described elsewhere (Guo et al., 2007). At the completion of the retrodialysis calibration, the animals were administered a dose of 50 mg/kg MTX hydrate as an i.v. bolus through a tail vein. Serial dialysate (~20 μ l) samples were collected every 20 minutes up to 8 hours after the dose using a refrigerated sample collector (CMA 470; Harvard Apparatus, Holliston, MA), and they were stored directly at -80°C until analysis. Serial blood samples of 20 μ l were collected at 5, 15, and 30 minutes, and 1, 2, 3, 4, 6, and 8 hours after the dose. Saline (10 μ l) was injected through the carotid artery cannula to avoid volume depletion. The samples were then centrifuged, and the resultant plasma was stored at -80°C until the analysis with liquid chromatography/tandem mass spectrometry (LC-MS/MS) according to a previously published method (Guo et al., 2007).

Sample preparation was performed as described elsewhere (Guo et al., 2007). We precipitated 2 μ l of plasma and 10 μ l of brain dialysate with 20 μ l of acetonitrile, which was then used for quantification. The method was modified by changing the flow rate for high-performance liquid chromatography as follows: a mobile phase of 0.5 mM ammonium formate with 0.1% formic acid and acetonitrile using gradient elution, 2% acetonitrile for the first

0.5 minutes, then 80% acetonitrile from 0.8 to 2.0 minutes, then 2% acetonitrile from 2.5 minutes to 7 minutes at a flow rate of 0.3 ml/min.

Data Analysis. Noncompartmental analysis was performed on the both the plasma and brain concentration-time profiles for each mouse in all four genotypes using Kinectica (version 5.1; Thermo Fisher Scientific, Pittsburgh, PA). The terminal rate constants were calculated using the data points that best described the terminal elimination phase. The areas under the concentration-time curve for plasma (AUC_{plasma}) and brain (AUC_{brain}) from time 0 to infinity were calculated using the log-linear method, with the AUC from the last measured time point to infinity estimated by dividing the last measured concentration by the elimination rate constant. In all cases, the contribution of the extrapolated AUC to the time infinity values was 8% or less. The brain-to-plasma partition coefficient ($K_{p,brain}$) of MTX was calculated as a ratio of AUC_{brain}/AUC_{plasma}.

Statistical Analysis. GraphPad Prism 5 (GraphPad Software, La Jolla, CA) was used to determine differences between genotypes by nonparametric one-way analysis of variance using a Kruskal-Wallis test followed by Dunn's post test to compare between strains. $P < 0.05$ was considered statistically significant.

Results and Discussion

Given the extensive use of MTX in the treatment of primary and secondary CNS tumors, it is essential that the role of BBB in limiting its brain distribution be examined in detail. However, in so doing, it is important to also evaluate the influence of the transporters on the systemic disposition of MTX by assessing changes in the drug clearance, because clearance (CL)—the inverse of the AUC—will determine plasma drug concentrations and influence tissue distribution. Both the *Abcg2*^{-/-} and *Abcg2*^{-/-}*Abcc4*^{-/-} strains had reduced total clearance values (Table 1, Fig. 1 A) compared with the wild-type and the *Abcc4*^{-/-} mice; however, only the DKO strain reached statistical significance, 2-fold lower than the wild-type strain ($P = 0.004$). The absence of *Abcc4* did not alter the clearance of MTX in mice (Kitamura et al., 2008), consistent with our results. This indicates that the reduction in total clearance in the DKO strain is largely due to reduced biliary clearance—a major route of elimination of MTX in rodents (Bremnes et al., 1989)—and the lack of *Abcg2* on bile canaliculi (Zamek-Gliszczynski et al., 2006). Nonetheless, a role for *Abcc4* in the renal clearance of MTX could be inferred, because the total clearance in the DKO was quite a bit lower than in the single *Abcg2* knockout strain. This potential role of MRP4—localized to the renal proximal tubule (van Aubel et al., 2002)—could be more important in humans where renal clearance of MTX is dominant.

TABLE 1

Pharmacokinetic parameters in the plasma for MTX and 7-OH MTX and in the brain for MTX determined by noncompartmental analysis after a 50-mg/kg intravenous bolus dose of MTX in wild-type, *Abcg2*^{-/-}, *Abcc4*^{-/-}, and *Abcg2*^{-/-}*Abcc4*^{-/-} mice

Parameters	Wild Type n = 6	<i>Abcg2</i> ^{-/-} n = 5	<i>Abcc4</i> ^{-/-} n = 5	<i>Abcg2</i> ^{-/-} ; <i>Abcc4</i> ^{-/-} n = 6
Plasma				
CL (ml/min)	1.96 ± 0.38	1.58 ± 0.52	2.00 ± 0.40	1.00 ± 0.20*
V _d (l)	0.54 ± 0.40	0.28 ± 0.14	0.73 ± 0.29	0.18 ± 0.09*
Half-life _(MTX,plasma) (min)	195 ± 171	124 ± 28	247 ± 81	116 ± 28*
AUC _{MTX(0-inf)} (min* μ g/ml)	633 ± 121	836.9 ± 208	631 ± 185	1259 ± 289**
AUC _{7-OH-MTX(0-inf)} (min* μ g/ml)	26.1 ± 8.7	59.9 ± 23.7	22.2 ± 6.2	81.5 ± 23.9**
Half-life _{7OH MTX} (min)	224 ± 131	237 ± 74.7	242 ± 96	200 ± 74
AUC _{7OH} /AUC _{MTX} (%)	4.16 ± 1.35	7.80 ± 1.59	3.55 ± 0.6	6.52 ± 1.10
Brain				
AUC _{brain} (min* μ g/ml)	66.85 ± 26	134 ± 54.80	118.12 ± 60.50	286.13 ± 130**
Half-life _{brain} (min)	151 ± 69	104 ± 44	62.3 ± 22.7	88 ± 15
C _{max} (μ g/ml)	1.2 ± 0.5	1.8 ± 0.9	1.5 ± 0.6	3.7 ± 2.2
K _{p,brain}	0.11 ± 0.05	0.17 ± 0.08	0.19 ± 0.09	0.23 ± 0.09*

* $P < 0.05$; ** $P < 0.01$.

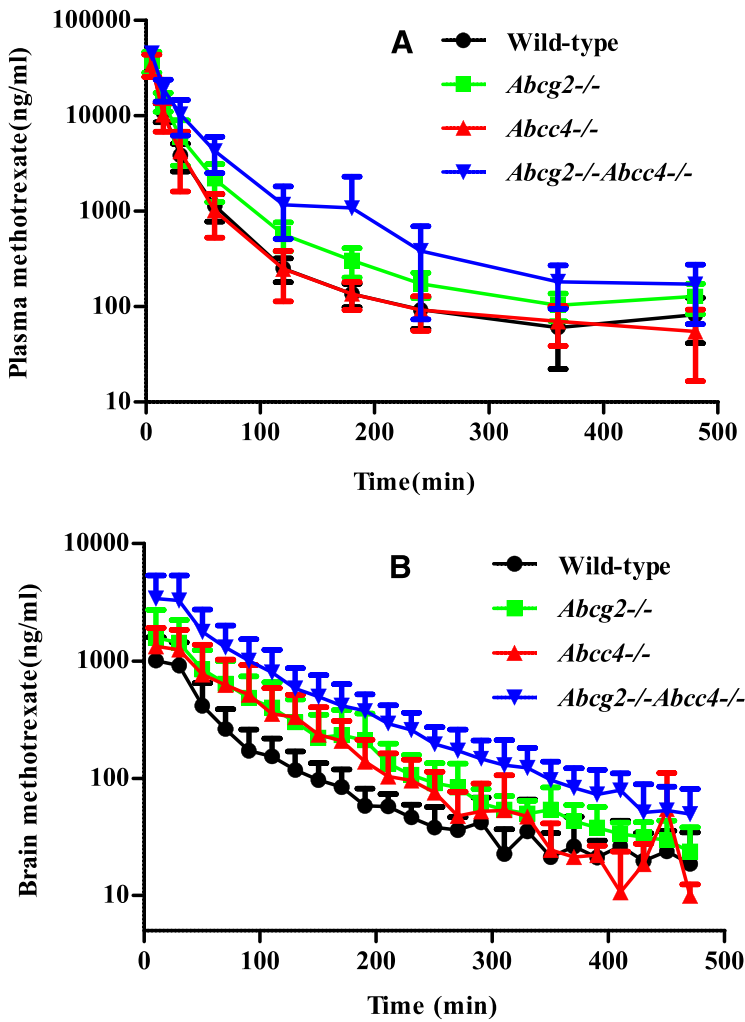


Fig. 1. (A) Plasma concentrations of methotrexate in wild-type, *Abcg2*^{-/-}, *Abcc4*^{-/-}, and *Abcg2*^{-/-}*Abcc4*^{-/-} mice after a 50-mg/kg dose of i.v. methotrexate hydrate. Mean ± S.D. (*n* = 4 or 5). Plasma concentrations in the *Abcg2*^{-/-}*Abcc4*^{-/-} mice are consistently higher than those observed in the wild type. (B) Corresponding brain concentrations of methotrexate in wild-type, *Abcg2*^{-/-}, *Abcc4*^{-/-}, and *Abcg2*^{-/-}*Abcc4*^{-/-} mice after a 50-mg/kg dose of i.v. methotrexate hydrate. Mean ± S.D. (*n* = 4 or 5). Brain concentrations in the *Abcg2*^{-/-} and *Abcg2*^{-/-}*Abcc4*^{-/-} mice are higher than those observed in the wild type.

The AUC for the 7-hydroxy metabolite of methotrexate was higher in *Abcg2*^{-/-} and reached statistical significance in *Abcg2*^{-/-}*Abcc4*^{-/-} groups (59.9 ± 23.7 and $81.5 \pm 23.9 \mu\text{g} \cdot \text{min}/\text{ml}$, respectively, $P = 0.0022$) as compared with the wild-type mice ($26.13 \pm 8.7 \mu\text{g} \cdot \text{min}/\text{ml}$). This was consistent with the work of Vlaming et al. (2009) in *Abcg2*^{-/-} mice; they concluded that the increased AUC_{7-OH} MTX is likely due to enhanced retention of MTX in the liver and its subsequent metabolism rather than any transporter effect on the metabolite.

BCRP and MRP4 are among the most highly expressed transport proteins at the mouse and human BBB (Carl et al., 2010; Agarwal et al., 2012). Unbound brain concentrations and not total brain concentrations are indicative of interactions with target receptors and determine CNS efficacy (Liu et al., 2009). Therefore, because brain microdialysis provides a measure of the unbound drug concentration, it is the ideal technique to indicate the amount of drug available to the intracellular compartment for drug-receptor binding as well as to indicate the influence of drug efflux pumps at the BBB.

The brain concentrations in the DKO mice were higher than those observed in the wild-type mice or either single-knockout mice (Fig. 1B), which yielded corresponding changes in the AUC_{brain} (see Table 1). However, to accurately assess the role of the transporters on regional brain distribution, it is important to use the brain partition coefficient (K_p)—the ratio AUC_{brain}/AUC_{plasma}—which accounts for changes in the systemic exposure of MTX. Specifically, the brain partition coefficient was 2-fold greater ($K_p = 0.23 \pm 0.09$, see Table 1) in the

DKO mice compared with the WT strain ($K_p = 0.11 \pm 0.05$), which represented the largest difference among all strains ($P = 0.017$) (Fig. 2). The brain partition coefficient in *Abcg2* knockouts (0.17 ± 0.08) was not significantly different from that in wild-type mice (0.11 ± 0.05) and partially reflects the increased plasma exposure. On the other hand, the K_p in *Abcc4* knockout mice (0.19 ± 0.09), although not statistically significantly different from the WT strain and analogous to that in the

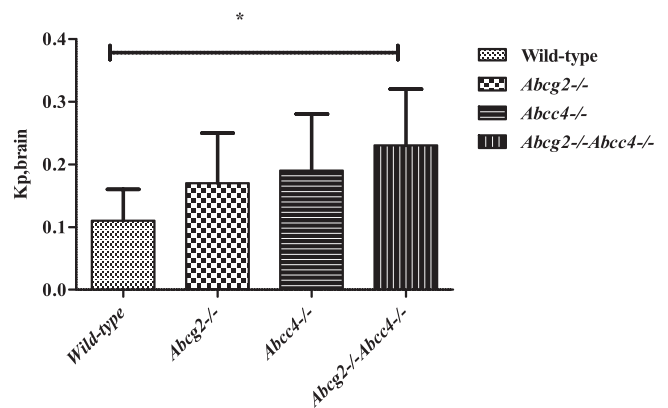


Fig. 2. The partition coefficient for methotrexate in the brain ($K_{p,\text{brain}}$) in wild-type, *Abcg2*^{-/-}, *Abcc4*^{-/-}, and *Abcg2*^{-/-}*Abcc4*^{-/-} mice. The $K_{p,\text{brain}}$ in *Abcc4*^{-/-}, *Abcg2*^{-/-} mice is 2-fold higher than in the wild-type mice (* $P < 0.05$).

Abcg2 knockout mice, suggests a local BBB effect due to deletion of the transporter; that is, the AUC_{plasma} values in the Abcc4 knockout and wild-type groups were virtually identical. MTX is a low-affinity substrate for human BCRP, having a K_m of about 680 μM to 1.3 mM (Chen et al., 2003; Volk and Schneider, 2003), compared with 220 μM for human MRP4 as determined by in vitro studies (Chen et al., 2002). The expression of BCRP is almost 2-fold higher than MRP4 at the mouse BBB (Agarwal et al., 2012) and nearly 40-fold higher in humans (Uchida et al., 2011). If we assume that the transporter affinity (K_m) for MTX in mice is similar to the in vitro values, and that the expression of the transporters at the BBB is reflective of the V_{max} for the respective transporters, then the clearance out of the brain (ratio of V_{max} to K_m , assuming linearity) for both transporters could be comparable. Although this extrapolation is speculative, it could explain why the single deletion of either Bcrp or Mrp4 had nearly similar effects on the brain distribution of MTX. Not unexpectedly, the simultaneous absence of Abcg2 and Abcc4, the DKO strain, caused the greatest increase in the K_p values of MTX, which supports the cooperative action of these transporters at the BBB. A similar study with topotecan also revealed cooperative behavior of MRP4 with BCRP and P-glycoprotein at the mouse BBB (Lin et al., 2013).

In this study, we show conclusively that the brain exposure of MTX is limited by efflux due to BCRP and MRP4 acting in a cooperative manner. This is an important effect to be considered, as MTX is a standard of care in primary CNS lymphoma and in metastatic brain tumors (Lassman et al., 2006; Jacot et al., 2010). Although the breakdown of the BBB in a tumor can result in a higher brain concentration of MTX (Blakeley et al., 2009), many brain tumors are invasive and diffuse behind an intact BBB (DeAngelis, 2003). The results from this study reinforce the idea that the BBB and the transporters at the BBB play an important role in the disposition of MTX and that its efficacy could be limited by their presence.

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