Journal: Drug Metabolism and Disposition

Title: The effect of ABCG2 and ABCC4 on the pharmacokinetics of methotrexate in brain

Ramola Sane, Shu-Pei Wu, Rong Zhang, James M. Gallo

Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, New York, NY, USA
Running Title: ABCG2 and ABCC4 influence methotrexate pharmacokinetics

Corresponding author:

James M. Gallo

Department of Pharmacology and Systems Therapeutics, Icahn School of Medicine at Mount Sinai, Box 1603, One Gustave L. Levy Place, New York, NY 10029. Phone: 212-241-7770; Fax: 212-996-7214; E-mail: james.gallo@mssm.edu

Manuscript statistics:

Number of figures: 1
Number of tables: 1
Number of Pages: 20
Number of References: 35
Number of Words in Abstract: 245
Number of words in introduction: 363
Number of words in Results and Discussion: 960

List of abbreviations:

BBB, blood-brain-barrier; CNS, central nervous system; BCRP, breast cancer resistance protein; MRP4, multidrug resistance protein 4; LC-MS/MS, liquid chromatography-tandem mass spectrometry; AUC, area under the curve; MTX, methotrexate; 7-OH MTX, 7-hydroxy methotrexate
Abstract:

Methotrexate [MTX] is the cornerstone of chemotherapy for primary CNS lymphoma [PCNSL] yet how blood-brain barrier [BBB] efflux transporters ABCG2 and ABCC4 influence required high-dose therapy is unknown. To evaluate their role we used four mouse strains, C57/B6 [WT], Abcg2-/-, Abcc4-/-, and Abcg2-/-;Abcc4-/- (double knockout, DKO) and conducted brain microdialysis studies following single IV MTX doses of 50 mg/kg. Based on the AUCplasma to assess systemic exposure to MTX the rank order was Abcc4-/- < WT < Abcg2-/- < Abcg2-/-Abcc4-/-, with only the DKO exposure being significantly higher than the WT group (p<0.01), and a reflection of role of Abcg2 -in biliary excretion and Abcc4 in renal excretion. MTX brain interstitial fluid concentrations obtained by microdialysis were used to calculate the AUCbrain that found the rank order of exposure to be WT < Abcc4-/- < Abcg2-/- < Abcg2-/-Abcc4-/- with the largest difference being 4-fold; 286.13 ± 130 ug*min/ml (DKO) vs. 66.85 ± 26 (WT). Since the transporters affected the systemic disposition of MTX, particularly in the DKO group, the ratio of the AUCbrain/AUCplasma or the brain/plasma partition coefficient Kp was calculated and revealed that the double knockout 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, yet 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 limit MTX distribution in patients.
Introduction:
The blood-brain barrier (BBB) is comprised of capillaries lined by endothelial cells with tight junctions to limit passive diffusion of drugs, as well as protein pumps that when directed towards 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 BBB penetration of several drugs (Schinkel et al., 1996). The consequences of limited brain access for CNS-active drugs are a lower therapeutic index that 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 mechanisms of CNS disposition and to offer a rational means to overcome limited brain distribution.

Methotrexate 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 – 5 % of all primary brain tumors (Gerstner and Batchelor, 2007). Methotrexate (MTX) is a folate antagonist with limited uptake in brain that likely contributes to 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 utilized brain microdialysis to measure brain interstitial fluid MTX concentrations attest to its limited distribution in 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 BCRP (Volk and Schneider, 2003; Li et al., 2013) and 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; Kodaira et al., 2010; Agarwal et al., 2011) 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, and was the subject of this investigation that utilized Abcg2-/-, Abcc4-/- and Abcg2-/-;Abcc4-/- transgenic mice. To assess the role of these transporters in the brain penetration of methotrexate we employed brain microdialysis in conjunction with serial plasma sampling following single intravenous dose administrations.

Materials and Methods:

Methotrexate hydrate (Molecular weight: 454.44), 7-hydroxy methotrexate (molecular weight: 470.44) and aminopterin were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were reagent grade or HPLC grade and purchased from Sigma-Aldrich. Guide cannulas (5 mm shaft) and MetaQuant probes (regenerated cellulose, dialysis tip length 2 mm, MW cut-off 13 kDa) were purchased from Brainlink (Groningen, The Netherlands).

Animals:

C57BL wild-type 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 lastly at the University of Illinois – Chicago - and then transferred and maintained at Charles River Inc (Wilmington, MA) through an agreement with Mount Sinai School of Medicine. Mice were transferred to Mount Sinai approximately 7 days prior to entry into the pharmacokinetic studies and were maintained at 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 (IACUC). All mice used in the experiment were male and 10-12 weeks of age and weighed 25-30g.

**Microdialysis preparation:**

Anesthetized mice underwent a surgical microdialysis and vascular cannula implantation procedure as previously described (Guo et al., 2007) three days prior to the pharmacokinetic study with the modification that a Meta-Quant probe system was used and 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 µL/min) whilst simultaneously using a higher make-up flow rate (0.9 µL/min) to eliminate long sample collection periods (Cremers et al., 2009). Animals were then placed on a heating pad during recovery and then when fully ambulatory provided food and water ad libitum with food being removed 2 hours prior to the pharmacokinetic study. The guide cannulas were replaced by the microdialysis probes and calibrated by a retrodialysis method and the recovery calculated as previously described (Guo et al., 2007). At the completion of the retrodialysis calibration the animals were administered a dose of 50 mg/kg methotrexate hydrate as an IV bolus through a tail vein. Serial dialysate (~20 µL) samples were collected every 20 min up to 8 hours post-dose using a refrigerated sample collector (CMA 470, Harvard Apparatus, Massachusetts) and stored directly at −80°C until analysis. Serial blood samples of 20 µL were collected at 5 min, 15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 6 hr and 8 hours post-dose. Saline (10 µL) 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 analyzed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) according to a previously published method (Guo et al., 2007).
Sample preparation was carried out as described before (Guo et al., 2007). Two µL of plasma and 10 µl of brain dialysate, were precipitated with 20 µL of acetonitrile and used for quantification. The method was modified by changing the flow rate on the HPLC 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 min, followed by 80% acetonitrile from 0.8 to 2 min and then back to 2% from 2.5 min to 7 min at a flow rate of 0.3 ml/min.

**Data analysis:**

Noncompartmental analysis was carried out 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\textsubscript{plasma}) and brain (AUC\textsubscript{brain}), from time 0 to infinity were calculated using the log-linear method with the AUC from 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 (Kp,brain) of methotrexate was calculated as a ratio of AUC\textsubscript{brain}/AUC\textsubscript{plasma}.

**Statistical analysis:**

GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) was used to determine differences between genotypes by non-parametric one-way analysis of variance (ANOVA) using a Kruskal-Wallis test followed by a Dunns post-test to compare between strains such that a p-value less than 0.05 is indicative of significance.

**Results and Discussion:**
Given the extensive use of methotrexate 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 doing so it is important to also evaluate the influence of the transporters on the systemic disposition of methotrexate by assessing changes in drug clearance since clearance – the inverse of the AUC - will determine plasma drug concentrations and influence tissue distribution. Both the $\textit{Abcg2}^{-/-}$ and $\textit{Abcg2}^{-/-}\textit{Abcc4}^{-/-}$ strains had reduced total clearance values (Table 1, Figure 1 A) compared to the wild-type and the $\textit{Abcc4}^{-/-}$ mice; however only the DKO strain reached statistical significance being 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, and indicates 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 methotrexate could be inferred since the total clearance in the DKO was quite a bit lower than 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 methotrexate is dominant.

The AUC for the 7-hydroxy metabolite of methotrexate was higher in $\textit{Abcg2}^{-/-}$ and reached statistical significance in $\textit{Abcg2}^{-/-}\textit{Abcc4}^{-/-}$ groups (59.9 ± 23.7 and 81.5 ± 23.9 µg*min/ml, respectively, p= 0.0022) as compared to the wild-type mice (26.13 ± 8.7 µg*min/ml); consistent with the work of Vlaming et.al. in $\textit{Abcg2}^{-/-}$ mice that concluded that the increased AUC$_{7-OHMTX}$ is likely due to enhanced retention of MTX in the liver (Vlaming et al., 2009) 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 determines CNS efficacy (Liu et al., 2009). Therefore, since 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 that observed in the wild-type and either single knockout mice (Figure 1B) that yielded corresponding changes in the AUC<sub>brain</sub> (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 (Kp) – the ratio AUC<sub>brain</sub>/AUC<sub>plasma</sub> – that accounts for changes in the systemic exposure of MTX. Specifically, the brain partition coefficient was 2-fold greater (Kp = 0.23 ± 0.09, see Table 1) in the DKO compared to the WT strain (Kp = 0.11 ± 0.05), which represented the largest difference amongst all strains (p value = 0.017) (Figure 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 Kp in Abcc4 knockout mice (0.19 ± 0.09), while not statistically different from the WT strain, and analogous to that in the Abcg2 knockout mice suggests a local BBB effect due to deletion of the transporter, since the AUC<sub>plasma</sub> values in Abcc4 knockout and wild-type groups are virtually identical. MTX is a low affinity substrate for human BCRP, having a Km of about 680 μM to 1.3 mM (Chen et al., 2003; Volk and Schneider, 2003), compared to 220 μM for human MRP4 (Chen et al., 2002); as determined by in vitro studies. 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 (Km) 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 Vmax for the respective transporters; the clearance out of the brain (ratio of Vmax to Km, 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 has 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 Kp values of MTX, and supports the cooperative action of these transporters at the BBB. A similar study with topotecan also revealed the cooperative behavior of MRP4 with BCRP and P-gp 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 in a cooperative manner. This is an important effect to be considered, as MTX is a standard of care in primary CNS lymphoma, as well as in metastatic brain tumors (Lassman et al., 2006; Jacot et al., 2010). While breakdown of the BBB in a tumor can result in higher brain concentration of MTX (Blakeley et al., 2009); many brain tumors are invasive and diffuse, and exist 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 methotrexate and that its efficacy could be limited by their presence.
Authorship Contributions

Participated in research design: Wu, Gallo

Conducted experiments: Wu, Zhang

Contributed new reagents or analytic tools: Zhang, Wu

Performed data analysis: Sane, Gallo

Wrote or contributed to the writing of the manuscript: Sane, Gallo
References:


Kodaira H, Kusuhara H, Ushiki J, Fuse E, and Sugiyama Y (2010) Kinetic analysis of the cooperation of P-glycoprotein (P-gp/Abcb1) and breast cancer resistance protein (Bcrp/Abcg2) in limiting the brain and testis penetration of erlotinib, flavopiridol, and mitoxantrone. J Pharmacol Exp Ther 333:788-796.


Footnotes

This work was supported by the National Institute of Health Cancer Institute [Grant CA073728].
Figure Legends:

**Figure 1:** (A) Plasma concentrations of methotrexate in wild-type, Abcg2-/-, Abcc4-/-, and Abcg2-/-Abcc4-/- mice following a 50 mg/kg dose of methotrexate hydrate intravenously. Mean ± SD (n=4 or n=5). Plasma concentrations in the Abcg2-/-Abcc4-/- mice are consistently higher than those observed in wild-type (B) Corresponding brain concentrations of methotrexate in wild-type, Abcg2-/-, Abcc4-/-, and Abcg2-/-Abcc4-/- mice following a 50 mg/kg dose of methotrexate hydrate intravenously. Mean ± SD (n=4 or n=5). Brain concentrations in the Abcg2-/- and Abcg2-/-Abcc4-/- mice are higher than those observed in wild-type

**Figure 2:** The partition coefficient for methotrexate in the brain (Kp,brain) in wild-type, Abcg2-/-, Abcc4-/-, and Abcg2-/-Abcc4-/- mice. Kp,brain in Abcc4-/-, Abcg2-/- mice is two-fold higher than in the wild-type mice(*, p<0.05)
Table 1: Pharmacokinetic parameters in the plasma for MTX and 7-OH MTX and for MTX in the brain determined by noncompartmental analysis after a 50 mg/kg intravenous bolus dose of methotrexate in wild-type, Abcg2-/-, Abcc4-/- and Abcg2-/-Abcc4-/- mice (*p<0.05, ** p<0.01).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>units</th>
<th>Wild-type n=6</th>
<th>Abcg2(-/-) n=5</th>
<th>Abcc4(-/-) n=5</th>
<th>Abcg2(-/-);Abcc4(-/-) n=6</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLASMA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL</td>
<td>ml/min</td>
<td>1.96 ± 0.38</td>
<td>1.58 ± 0.52</td>
<td>2.00 ± 0.40</td>
<td>1.00 ± 0.20*</td>
</tr>
<tr>
<td>Vd</td>
<td>L</td>
<td>0.54 ± 0.40</td>
<td>0.28 ± 0.14</td>
<td>0.73 ± 0.29</td>
<td>0.18 ± 0.09 *</td>
</tr>
<tr>
<td>Half-life_{MTXplasma}</td>
<td>min</td>
<td>195 ± 171</td>
<td>124 ± 28</td>
<td>247 ± 81</td>
<td>116 ± 28*</td>
</tr>
<tr>
<td>AUC_{MTX(0-inf)}</td>
<td>min*ug/ml</td>
<td>633 ± 121</td>
<td>836.9 ± 208</td>
<td>631 ± 185</td>
<td>1259 ± 289**</td>
</tr>
<tr>
<td>AUC_{7-OH-MTX(0-inf)}</td>
<td>min*ug/ml</td>
<td>26.1 ± 8.7</td>
<td>59.9 ± 23.7</td>
<td>22.2 ± 6.2</td>
<td>81.5 ± 23.9**</td>
</tr>
<tr>
<td>Half-life_{7OH-MTX}</td>
<td>min</td>
<td>224 ± 131</td>
<td>237 ± 74.7</td>
<td>242 ± 96</td>
<td>200 ± 74</td>
</tr>
<tr>
<td>AUC_{7OH}/AUC_{MTX}%</td>
<td></td>
<td>4.16 ± 1.35</td>
<td>7.80 ± 1.59</td>
<td>3.55 ± 0.6</td>
<td>6.52 ± 1.10</td>
</tr>
<tr>
<td><strong>BRAIN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC_{brain}</td>
<td>min*ug/ml</td>
<td>66.85 ± 26</td>
<td>134 ± 54.80</td>
<td>118.12 ± 60.50</td>
<td>286.13 ± 130**</td>
</tr>
<tr>
<td>Half-life_{brain}</td>
<td>min</td>
<td>151 ± 69</td>
<td>104 ± 44</td>
<td>62.3 ± 22.7</td>
<td>88 ± 15</td>
</tr>
<tr>
<td>Cmax</td>
<td>ug/ml</td>
<td>1.2 ± 0.5</td>
<td>1.8 ± 0.9</td>
<td>1.5 ± 0.6</td>
<td>3.7 ± 2.2</td>
</tr>
<tr>
<td>Kp,brain</td>
<td></td>
<td>0.11 ± 0.05</td>
<td>0.17 ± 0.08</td>
<td>0.19 ± 0.09</td>
<td>0.23 ± 0.09*</td>
</tr>
</tbody>
</table>
Figure 1

A

Plasma methotrexate (ng/ml)

Time (min)

Wild-type
Abcg2-/-
Abcc4-/-
Abcg2-/-Abcc4-/-

B

Brain methotrexate (ng/ml)

Time (min)

Wild-type
Abcg2-/-
Abcc4-/-
Abcg2-/-Abcc4-/-
Figure 2

This article has not been copyedited and formatted. The final version may differ from this version.

DMD Fast Forward. Published on January 24, 2014 as DOI: 10.1124/dmd.113.055228

at ASPET Journals on January 18, 2018 dmd.aspetjournals.org Downloaded from