BRAIN EFFLUX INDEX TO INVESTIGATE THE INFLUENCE OF ACTIVE EFFLUX ON BRAIN DISTRIBUTION OF PEMETREXED AND METHOTREXATE

Li Li, Sagar Agarwal, William F Elmquist

Department of Pharmaceutics, Brain Barriers Research Center, University of Minnesota, MN, USA (L.L., S.A., W.F.E.).
Running Title: Active efflux of pemetrexed and methotrexate from brain

Corresponding Author: William F. Elmquist, Department of Pharmaceutics, University of Minnesota, 9-177 Weaver Densford Hall, 308 Harvard Street SE, Minneapolis, MN 55455, USA. Phone: +001-612-625-0097; Fax: +001-612-626-2125; e-mail: elmqu011@umn.edu

Number of text pages: 16
Number of tables: 3
Number of figures: 7
Number of references: 41
Number of words in Abstract: 249
Number of words in Introduction: 680
Number of words in Discussion: 1142

Abbreviations: BBB- blood-brain barrier; CNS- central nervous system; BEI- brain efflux index; BCRP - breast cancer resistance protein; ABC - ATP-binding cassette; MRP- multidrug resistance-associated proteins; OAT- organic anion transporter; PMX - pemetrexed; MTX - methotrexate
Abstract

Antifolates, in particular methotrexate (MTX), have been widely used for the treatment in the primary and secondary tumors of the central nervous system (CNS). Pemetrexed (PMX) is a novel antifolate that also exhibits potent anti-tumor activity against CNS malignancies. Studies have shown that brain distribution of both antifolates is significantly restricted, possible due to active efflux transport at the blood-brain barrier (BBB). The objective of this study was to characterize the brain-to-blood transport of pemetrexed and methotrexate and to examine the role of several efflux transporters in brain distribution of the antifolates using the intracerebral microinjection technique (brain efflux index). Results from the current study show that both pemetrexed and methotrexate undergo saturable efflux transport across the BBB, with elimination half-lives of approximately 39 minutes and 29 minutes for pemetrexed and methotrexate, respectively. Of the various efflux transporters investigated in the present study, Mrp2 does not play an important role in the brain distribution of the two antifolate drugs. Interestingly, Bcrp makes a significant contribution to the brain elimination of methotrexate, but not for pemetrexed. In addition, the brain-to-blood transport of both antifolates was inhibited by probenecid and benzylpenicillin, suggesting the involvement of organic anion transporters in the efflux of these compounds from the brain, with Oat3 being a possibility. The results of this study suggest that one of the underlying mechanisms behind the limited brain distribution of pemetrexed and methotrexate is active efflux transport processes at the BBB, including a benzylpenicillin sensitive transport system and/or the active transporter Bcrp.
Introduction

Despite scientific advances in understanding the causes and treatment of human malignancies, the treatment of brain tumors, both primary and metastatic, remains a persistent challenge. Many chemotherapeutic agents that are potent and efficacious against peripheral tumors have been found to be ineffective in treating tumors in the brain. In general, the poor response of CNS tumors to chemotherapy drugs is multifactorial, but the inability to effectively deliver therapeutic agents to the CNS across the blood-brain-barrier (BBB) is certainly a well-known mechanism (Pardridge, 2001; Motl et al., 2006). The BBB is a highly developed defense mechanism that separates the brain from the peripheral circulation, thereby protecting it from circulating toxins and potentially harmful chemicals. The barrier is formed by a dense network of brain capillaries where the endothelial cells contain tight junctions, limiting paracellular transport. In addition, the barrier function is further strengthened by the expression of active efflux transporters in the endothelium. These efflux pumps mediate the brain-to-blood efflux of substrate drugs and thus limit their CNS exposure (Pardridge, 1999; Allen and Smith, 2001; Golden and Pollack, 2003). Drug efflux pumps present at the BBB include ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp; MDR1/ABCB1), breast cancer resistance protein (BCRP/ABCG2) and multidrug resistance-associated proteins (MRP2/ABCC2, MRP4/ABCC4), as well as transporters of the solute carrier family such as organic anion-transporting polypeptide 1A2 (OATP1A2, SLCO1A2) and organic anion transporter 3 (OAT3, SLC22A8) (Miller et al., 2000; Sugiyama et al., 2001; Urquhart and Kim, 2009). Efflux transport mediated by these pumps has been shown to be an important determinant of drug partitioning into the brain.

Primary central nervous system lymphoma (PCNSL) is a rare form of extranodal lymphoma and accounts for approximately 3 to 4 % of brain tumors diagnosed every year in the United States.
Despite high rates of response after whole-brain radiotherapy, rapid recurrence is common and long-term survival is rare (Norden et al., 2011). The classic antifolate agent, methotrexate (MTX), is widely used for the treatment of primary CNS lymphoma and chemotherapy with methotrexate in combination with whole-brain radiotherapy has recently become the treatment of choice. A recent study has reported that high-dose methotrexate alone or in combination with other therapies is the most effective treatment available for PCNSL (Gerstner et al., 2008). Although efficacious, high-dose methotrexate therapy is associated with severe systemic and neurologic toxicities, with studies reporting complications leading to patient death in some cases. Most of the systemic toxicities can be attributed to greater exposure of tissues following treatment with methotrexate at high doses, a need arising from the inability of methotrexate to penetrate the BBB. Preclinical studies have shown that brain distribution of methotrexate is severely restricted with only 5% of free drug in plasma crossing the BBB to reach the brain (Devineni et al., 1996; Dai et al., 2005). In human, Blakeley and coworkers also reported a poor cerebral penetration of methotrexate (3.2% to 9.4%) in patients with recurrent high grade gliomas using microdialysis technique (Blakeley et al., 2009). Pemetrexed (PMX, Alimta®) is a novel antifolate that was developed to overcome some of the problems associated with methotrexate therapy. It has been reported that it has potent anti-tumor activity against a variety of tumors including CNS malignancies (Kuo and Recht, 2006). However, similar to methotrexate, distribution of pemetrexed across the BBB is also very limited (Dai et al., 2005). The mechanisms behind the poor brain penetration of pemetrexed and methotrexate are poorly understood with one plausible explanation being the involvement of efflux transport systems at the BBB.
In the current study, we hypothesize that efflux transporters at the BBB, such as Mrp2, Bcrp and organic anion transporters, mediate the efflux of pemetrexed and methotrexate from brain, thereby restricting their brain penetration. The objective of this study was to investigate the role of efflux transporters in the brain elimination of antifolates using the brain efflux index (BEI, intracerebral microinjection) method. We used transgenic mice lacking the transporters Mrp2 or Bcrp to characterize the involvement of the two transporters in efflux of pemetrexed and methotrexate from the brain. In addition, the involvement of organic anion transporters was studied by examining the effect of OAT inhibitors, probenecid and benzylpenicillin, on brain-to-blood transport of methotrexate and pemetrexed.

**Materials and Methods**

**Chemicals**

[^H]-pemetrexed was a kind gift from Eli Lilly and Company.[^H]-methotrexate,[^H]-valproic acid and [14C]-carboxyl-inulin were obtained from Moravek Biochemicals (Brea, CA). Probenecid and benzylpenicillin were purchased from Sigma-Aldrich (St.Louis, MO).

**Animals**

Male wild-type (C57BL/6) mice were purchased from Taconic Farms. Inc. (Germantown, NY). *Bcrp1(-/-) and Mrp2(-/-) mice of a C57 background were a generous gift from Eli Lilly and Company. All animals were maintained under temperature-controlled conditions with a 12-hour light/dark cycle and were allowed food and water ad libitum. The mice were allowed to acclimatize for a minimum of one week upon arrival. All studies were approved by the Institutional Animal Care and Use Committee at the University of Minnesota (IACUC).
Brain Efflux Index (BEI) Method

The brain efflux study was performed using the intracerebral microinjection technique, as previously reported (Kakee et al., 1996). Wild-type, Mrp2 (-/-) and Bcrp1 (-/-) mice were anesthetized with an intraperitoneal dose of 100 mg/kg ketamine and 10 mg/kg xylazine (Boynton Health Service Pharmacy, Minneapolis, MN), and then mounted on a stereotaxic device. A borehole was made 3.8 mm lateral to the bregma and an injection needle was advanced to a depth of 2.5 mm from the surface of the scalp, i.e., into the secondary somatosensory cortex (S2) region. Then, 0.2 µL of a mixture of [3H]-pemetrexed (10 nCi/ml) or [3H]-methotrexate (10 nCi/ml) and [14C]-carboxyl-inulin (10 nCi/ml) dissolved in extracellular fluid (ECF) buffer (122 mM NaCl, 25 mM NaHCO3, 3mM KCl, 1.4 mM CaCl2, 1.2 mM MgSO4, 0.4 mM K2HPO4, 10 mM D-glucose, and 10 mM HEPES, pH 7.4) was injected over 2 min using a 2.5-µL microsyringe (Hamilton, Reno, NE) fitted with a fine needle (32 gauge, Hamilton, Reno, NE). The injection process was controlled by a Quintessential® stereotaxic injector (Stoelting Co., IL, USA). Time zero was defined as the time when the injection was completed. After injection, the needle was left in place for additional 4 minutes to minimize the backflow of injected solution along the injection track. At designated time points post injection, mice were euthanized and decapitated. The right (ipsilateral), left (contralateral) cerebrum and cerebellum were harvested, weighed and homogenized in 3 volumes of 5% bovine serum albumin solution. A 100-µL sample of brain homogenate from the right, left cerebrum and cerebellum was mixed with 4 ml of scintillation fluid (ScintiSafe Econo cocktail; Thermo Fisher Scientific, Waltham, MA) and the associated radioactivity was measured in a liquid scintillation counter (LS-6500 instrument; Beckman Coulter, Fullerton, CA).
The concentration dependent efflux of [³H]-pemetrexed across the BBB was examined by injecting a solution containing 1 mM and 50 mM unlabelled pemetrexed in addition to trace amount of [³H]-pemetrexed and [¹⁴C]-carboxyl-inulin. To examine the inhibitory effect of probenecid and benzylpenicillin on brain efflux of [³H]-pemetrexed, 100 mM probenecid or benzylpenicillin was dissolved in the injection solution. The pH of the solution was adjusted to 7.4. The inhibitory effect was evaluated by comparing the percentage of [³H]-pemetrexed remaining in the brain in the presence and absence of the inhibitor, at 30 minutes post injection.

The BEI is defined as the percentage of test drug transported from the ipsilateral cerebrum to the circulating blood across the BBB, compared to the amount of test drug injected into the cerebrum (Kakee et al., 1996), and is given by Equation 1.

\[
\text{BEI} \, (\%) = \left( \frac{\text{amount of drug effluxed at the BBB}}{\text{amount of drug injected in the brain}} \right) \times 100 \quad \text{........................................(1)}
\]

100-BEI (%) is defined as the percentage of the amount of test drug remaining in the ipsilateral cerebrum compared to the amount of drug injected and is calculated as the amount of test drug retained in the ipsilateral cerebrum divided by the amount of test drug injected, normalized by the corresponding ratio of the BBB impermeable marker (Equation 2).

\[
100 - \text{BEI} \, (\%) = \left( \frac{\text{amount of drug in the brain}/\text{amount of reference in the brain}}{\text{amount of drug injected}/\text{amount of reference injected}} \right) \times 100 \quad \text{......(2)}
\]

The apparent BBB efflux rate constant, \(k_{eff}\), was obtained from the slope of the semilogarithmic plot of the value of (100-BEI) versus time using the linear regression function in SigmaPlot (version 9.0.1, SYSTAT software).

**Determination of Brain Volume of Distribution by the Brain Slice Uptake Method**
The volume of distribution of pemetrexed and methotrexate in the brain was determined by the
*in vitro* brain slice uptake technique. Brain slices were prepared according to the method
reported by Kakee et al. (Kakee et al., 1996). In brief, coronal brain slices of the cortex (400 μm)
were prepared using a Vibratome™ (series 1000, The Vibratome Company, Bannockburn, IL).
The slices were preincubated at 37°C for 5 minutes in ECF buffer oxygenated with 95% O₂ and
5% CO₂. After preincubation, the slices were transferred into 5 ml oxygenated ECF buffer
containing [³H]-pemetrexed or [³H]-methotrexate (0.3 μCi/mL) and [¹⁴C]-sucrose (0.06 μCi/mL)
at 37°C. At designated time points, the slices were removed from the drug solution, dried on
filter paper and weighed. The radioactivity associated with the slice was determined by liquid
scintillation counting (LS-6500, Beckman Coulter, Inc., Fullerton, CA). The brain volume of
distribution (V<sub>d,brain</sub>) was calculated as the amount of test drug in the brain slice divided by the
medium concentration (S/M ratio). The volume of distribution was corrected for the volume of
adhering fluid, which was determined as the zero-time intercept of the [¹⁴C]-inulin uptake
profile, i.e., 0.096 mL/g brain. As shown in Equation 3, the apparent brain efflux clearance
(C<sub>app,efflux</sub>) was calculated as the product of elimination rate constant (k<sub>eff</sub>) obtained from the BEI
study and brain distribution volume (V<sub>d,brain</sub>) obtained from brain slice uptake study.
\[
C_{app,efflux} = k_{eff} \times V_{d,brain}
\]

**Steady-State Brain Distribution of Pemetrexed**

In addition to the BEI study, steady-state brain distribution of pemetrexed was examined in wild-
type and *Bcrp1(-/-)* mice. Alzet® osmotic minipumps (Durect Corporation, Cupertino, CA) were
used to continuously infuse drug into the peritoneal cavity for 72 hours. The pump operated at a
flow rate of 1 μL/hr, resulting in a constant rate infusion of 25 mg/kg/day. Wild-type mice (n=5)
and Bcrp1(-/-) mice (n=5) were anesthetized with an intraperitoneal dose of 100 mg/kg ketamine and 10 mg/kg xylazine (Boynton Health Service Pharmacy, Minneapolis, MN). A small midline incision was made in the abdominal wall and the pumps were surgically implanted into the peritoneal cavity. The animals were allowed to recover on a heated pad and were administered ibuprofen in their drinking water for pain relief. The animals were euthanized after 72 hours by using a CO2 chamber. Blood was immediately harvested via cardiac puncture and collected in tubes preloaded with potassium EDTA (BD, Franklin Lakes, NJ). Whole brain was immediately harvested, rinsed with ice-cold saline to remove extraneous blood and flash frozen using liquid nitrogen. Plasma was isolated from blood by centrifugation at 3000 rpm for 10 min at 4°C. All samples were stored at -80°C until further analysis.

Quantification of Pemetrexed by Liquid Chromatography-Mass Spectometry (LC-MS/MS)
Mouse plasma and brain samples were analyzed by a rapid and sensitive LC-MS/MS. Whole brain samples were first homogenized in solution comprised of water and ethanol (20:80) with a volume ratio of 1:3. Acetonitrile containing the internal standard (20 ng/mL) was then added to the plasma or brain homogenate, followed by centrifugation to remove precipitated proteins. The supernatant was diluted with two volumes of water and chromatographed under reverse-phase conditions on a Betasil C18 analytical column (2.1 x 20 mm, 5 µm particle size, Thermo Electron Corp., Edina, MN). A gradient mobile phase was used with solvent A containing water, trifluoroacetic acid and 1 mM ammonium bicarbonate (2000:8:2, v:v:v) and solvent B containing acetonitrile, trifluoroacetic acid and 1 mM ammonium bicarbonate (2000:8:2, v:v:v). The injection volume was 10 µL. Pemetrexed detection was accomplished by tandem mass
spectrometry using the parent ion mass-to-charge ratio of 428.1 and the daughter ion m/z of 281.1. The lower limit of quantification was 1 ng/mL.

**Statistical analysis**

Statistical analysis was conducted using SigmaStat (version 3.1, SYSTAT software). Statistical comparisons between two groups were made by using two-sample t-test at p < 0.05 significance level. Multiple groups were compared by one-way analysis of variance with the Holm-Sidak post hoc test for multiple comparisons at a significance level of p < 0.05.

**Results**

**BEI method to characterize the brain-to-blood transport of pemetrexed and methotrexate**

The brain efflux index (BEI) method, also referred to as the intracerebral microinjection technique, is a novel technique to study mechanisms of brain-to-blood efflux transport at the BBB (Kakee et al., 1996; Kusuhara et al., 2003). Because it isolates efflux processes directly, rather than considering efflux as modulator of brain distribution, BEI is an excellent method for determining efflux clearance and studying the mechanisms behind active transport of compounds from the brain. This *in vivo* method has been extensively used to study the brain efflux clearance of various compounds, including steroid conjugates, valproic acid, nucleoside analogs, buprenorphine, human amyloid-β peptide, benzylpenicillin and quinidine (Kusuhara et al., 1997; Takasawa et al., 1997; Sugiyama et al., 2001; Kakee et al., 2002; Ohtsuki et al., 2002; Kikuchi et al., 2003; Ito et al., 2006; Suzuki et al., 2007). In the present study, we first validated the technique by studying brain efflux index of valproic acid. The brain elimination half-life of valproic acid was determined to be 2 minutes (range: 1.6 - 2.3 minutes, N=3), a value that is consistent with the reported value of 3.7 minutes from previous studies using the same technique.
The close agreement of our valproic acid results with literature values supports our brain microinjection technique for the examination of mechanisms responsible for brain efflux of pemetrexed and methotrexate (Kakee et al., 2002). In addition, throughout the course of the efflux studies, less than 0.5% of injected \([^3\text{H}]\)-pemetrexed or \([^3\text{H}]\)-methotrexate was found in the contralateral cerebrum and cerebellum, suggesting that diffusion into the rest of CNS from the injection site was very limited. The percentage of \([^1\text{C}]\)-carboxyl-inulin (BBB impermeable marker) remaining in the brain did not change significantly for over 90 minutes post injection, indicating little damage to the BBB.

**Pemetrexed and Methotrexate Elimination from Mouse Brain**

The efflux of methotrexate and pemetrexed from mouse brain was investigated using the BEI method. Figure 1A shows the time profile of the percentage of \([^3\text{H}]\)-pemetrexed remaining in the brain after intra-cerebral microinjection. Approximately 80% of the administered dose of \([^3\text{H}]\)-pemetrexed was eliminated from the brain within 90 minutes. The apparent elimination rate constant (k_{eff}) of pemetrexed was estimated to be 1.06 ± 0.18 hr\(^{-1}\) corresponding to a half-life of 0.65 hours. The k_{eff} value for methotrexate was 1.42 ± 0.16 hr\(^{-1}\), resulting in a half-life of 0.48 hours (Figure 1B).

**Concentration-Dependent Efflux of Pemetrexed at the BBB**

Figure 2 shows that the percentage of \([^3\text{H}]\)-pemetrexed remaining in the brain significantly increased after the injection of higher doses of total pemetrexed (unlabeled and \([^3\text{H}]\)-pemetrexed). Specifically, 51.1 ± 5.1% of \([^3\text{H}]\)-pemetrexed was retained in the ipsilateral cerebrum 30 minutes after injection of trace amount of \([^3\text{H}]\)-pemetrexed (0.1µM). However, in
the presence of 1 mM and 50 mM unlabeled pemetrexed in the injectate, the percentage of $[^3\text{H}]$-

pemetrexed remaining in the brain increased significantly to $71.7 \pm 7.1\%$ and $95.7 \pm 4.8\%$, respectively ($p < 0.001$, Table 1). This shows that pemetrexed elimination from brain is saturable, indicating the involvement of a carrier-mediated process.

**Role of Mrp2 in Transport of Pemetrexed and Methotrexate at the BBB**

The role of Mrp2 in elimination of pemetrexed and methotrexate from mouse brain was investigated using $Mrp2$ (-/-) mice. At 60 minute postdose, the percentage of $[^3\text{H}]$-pemetrexed remaining in the ipsilateral cerebrum of $Mrp2$ (-/-) mice was not statistically different than that in the wild-type mice (Figure 3). This indicates that absence of Mrp2 alone does not affect the brain-to-blood transport of pemetrexed. A similar result was found for methotrexate where elimination of methotrexate from brain was not different between the wild-type and $Mrp2$ (-/-) mice (Figure 3).

**Role of Bcrp in Transport of Pemetrexed and Methotrexate at the BBB**

The role of Bcrp in brain-to-blood transport of pemetrexed and methotrexate at the BBB was studied using the BEI method as well as by determining the steady-state brain partitioning in wild-type and $Bcrp1$(-/-) mice. The results from the BEI study show that the percentage of $[^3\text{H}]$-pemetrexed remaining in the brain of $Bcrp1$(-/-) mice was not significantly different from that in wild-type mice, suggesting a minor role of Bcrp in the brain-to-blood transport of pemetrexed across the BBB (Figure 4). This was further confirmed in the 72-hour infusion study, where no statistically significant difference was found in steady-state brain-to-plasma ratios of pemetrexed between the wild-type mice and $Bcrp1$(-/-) mice (Figure 5). The achievement of steady-state in
this study was confirmed by the statistical comparison of pemetrexed concentrations in both brain and plasma after 48 hour and 72 hour infusions in the wild-type mice. In contrast to pemetrexed, the results from BEI study suggest the involvement of Bcrp in efflux of methotrexate at the BBB, i.e., the efflux of [3H]-methotrexate decreased significantly in the Bcrp1 (-/-) mice relative to the wild-type mice (p <0.01) (Figure 4).

Role of Organic Anion Transporters in Elimination of Pemetrexed and Methotrexate From Mouse Brain

Probenecid is a non-specific inhibitor of organic anion transporters, including MRPs, organic anion transporters (Oats) and organic anion transporting polypeptides (Oatps) (Bakos et al., 2000; Sugiyama et al., 2001; Haimeur et al., 2004). In the present study, the involvement of organic anion transporters in brain-to-blood transport of the two antifolates was determined by examining the inhibitory effect of probenecid (Figure 6). In wild-type mice, simultaneous injection of probenecid (100 mM in the injectate) significantly increased the fraction of [3H]-pemetrexed remaining in the brain from 51.1 ± 5.1 % to 72.4 ± 3.3 % and [3H]-methotrexate remaining in the brain from 36.2 ± 5.2 % to 75.8 ± 8.6 %, respectively. The substantially diminished efflux of pemetrexed and methotrexate from the mouse brain in the presence of probenecid indicates the involvement of one or more organic anion transport protein.

Benzylpenicillin is a selective Oat3 substrate/inhibitor (Kikuchi et al., 2003). In the present study, the possible role of Oat3 in the efflux transport of the antifolates was examined via the inhibitory effect of benzylpenicillin. As shown in Figure 6, the elimination of both [3H]-pemetrexed and [3H]-methotrexate from the mouse brain was markedly inhibited in the presence of benzylpenicillin (100 mM in the injectate), with approximately 90% of the dose remaining in
the brain 30 minutes after injection. Effect of various treatments on brain-to-blood transport of pemetrexed and methotrexate are summarized in Table 1 and Table 2, respectively.

**Distribution Volume of Pemetrexed and Methotrexate in Mouse Brain**

The distribution volume of pemetrexed and methotrexate in mouse brain was determined by *in vitro* brain slice uptake technique. Figure 7 shows the time profiles of uptake of [3H]-pemetrexed and [3H]-methotrexate by brain slices. For both antifolates, there was no statistically significant difference in the brain distribution volume between 1 hour and 2 hours incubation, giving a steady-state brain distribution volume of 0.62 ± 0.10 mL/g brain for pemetrexed and 0.85 ± 0.06 mL/g brain for methotrexate. Incorporating the elimination rate constant (k_{eff}) and brain distribution volume (V_{d, brain}) into equation 3, the apparent efflux clearance (Cl_{app, efflux}) is 11.04 μL/min·g brain for PMX and 20.23 μL/min·g brain for MTX (Table 3).

**Discussion**

The potential use of pemetrexed in treatment of primary and secondary brain tumors has gained more interest in recent years. Methotrexate, a classical antifolate widely used for brain metastasis, has restricted brain penetration which necessitates a high systemic dose to achieve a therapeutic effect. In spite of the tremendous need to enhance the brain penetration of these antifolate agents, the mechanisms behind limited brain distribution are still not clear. In the present study, we characterized the brain elimination kinetics of pemetrexed and methotrexate, and explored the involvement of several efflux transporters in the brain distribution of the two agents.
Using the BEI method, we show that both pemetrexed and methotrexate are rapidly eliminated from the brain with their half-lives in the brain being 0.65 hrs and 0.48 hrs, respectively. The short retention time in the brain may be attributed to very limited brain distribution volume. Specifically, the distribution volume ($V_d$) of pemetrexed and methotrexate in mouse brain, as determined by brain slide uptake method, is close to brain interstitial fluid space of 0.8 mL/g brain (Reinoso et al., 1997). On the other hand, efficient efflux transport may also play a role in quickly clearing these antifolate agents from the brain. In the present study, we first confirmed the involvement of active efflux transport in the brain elimination of pemetrexed. The elimination of $[^3H]$-PMX from the brain was almost completely inhibited in presence of 50 mM unlabelled PMX, suggesting that the contribution of passive diffusion to PMX brain efflux transport is very limited and active transport accounts for the majority of the total efflux. This is consistent with physicochemical properties of pemetrexed. As an anionic compound with a $\log P$ of -1.5, it is unlikely that pemetrexed can cross biological membranes readily by passive diffusion. As a matter of fact, intracellular transport of pemetrexed is also facilitated by influx transporters, including reduced folate carrier (RFC), folate receptor (FR) and proton-coupled folate transporter (PCFT). Therefore it is plausible that active drug transporters are required to remove pemetrexed across lipid membranes, and across the BBB out of the brain.

After confirming the involvement of active efflux transport in the brain distribution of pemetrexed, we then explored the potential roles of several efflux transporters at the BBB in eliminating pemetrexed and methotrexate from the brain. The first transporter we investigated is Mrp2. In a recent study, Vlaming et al. have reported that Mrp2 plays an important role in determining the total body clearance of methotrexate (Vlaming et al., 2009). Other in vitro
studies have suggested that both pemetrexed and methotrexate are avid MRP2 substrates with the reported $K_m$ values being 66 µM for pemetrexed and 480 µM for methotrexate (Pratt et al., 2002; El-Sheikh et al., 2007). Given the purported expression of Mrp2 at the BBB, we tested the hypothesis that Mrp2 may transport pemetrexed and methotrexate at the BBB and thus limit the brain penetration of the antifolates. However, contrary to the reported in vitro findings, we did not observe any influence of Mrp2 on the efflux of either pemetrexed or methotrexate from the brain. Although the functional relevance of Mrp2 in the systemic disposition of its substrates has been established via studies using Mrp2 gene knockout mice (Vlaming et al., 2006; Tian et al., 2007; Ieiri et al., 2009; Vlaming et al., 2009), the role of MRP2 in brain distribution of drugs is poorly understood. Our findings further confirm this and advocates for the need of further research to investigate the role of Mrp2 at the BBB that include additional expression studies.

In contrast to MRP2, the role of BCRP as a gate keeper at the BBB has been well established. More importantly, BCRP also mediates the cellular transport of pemetrexed and methotrexate as indicated by in vitro studies (Volk and Schneider, 2003; Li et al., 2011). The current BEI study revealed that Bcrp plays a significant role in the brain elimination of methotrexate, but not pemetrexed. This is not totally unexpected, because even though pemetrexed is a structurally similar antifolate, it may possess different physiochemical properties that affect the binding affinities for the transporters. Based on the available in vitro data using in vitro vesicular uptake method, Bcrp-mediated intrinsic clearance ($V_{max}/K_m$) of methotrexate is about 3-fold higher than that of pemetrexed (manuscript submitted). Therefore, the discrepancy in Bcrp-mediated efflux of the two antifolates may be explained by their different affinities for this transporter.
Considering that pemetrexed and methotrexate are weak acids with an anionic charge at physiological pH, we examined the role of organic anion transporter in brain distribution of the antifolates. We demonstrated the possible involvement of Oat3 in brain elimination of both pemetrexed and methotrexate using the inhibitors probenecid and benzylpenecillin. Oat3 is localized at the abluminal membrane of brain capillary endothelial cells (Kusuhara et al., 1999; Kikuchi et al., 2003; Mori et al., 2003). It mediates the brain-to-blood transport of many hydrophilic organic anions; including indoxyl sulfate (IS), 6-mercaptopurine (6-MP), p-aminohippuric acid (PAH), benzylpenicillin (PCG) and homovanillic acid (HVA) (Ohtsuki et al., 2002; Kikuchi et al., 2003; Mori et al., 2004; Kusuhara and Sugiyama, 2005). Due to the cellular localization of Oat3, it is believed that Oat3 can only transport its substrate from brain extracellular space to brain capillary endothelial cells (Kusuhara and Sugiyama, 2005); and another transporter is needed to remove the molecules from the endothelial cells into the bloodstream. The ABC transporter MRP4 has been shown to transport both pemetrexed and methotrexate. Specifically, MRP4 mediates the transport of methotrexate with $K_m$ of 220 µM and the expression of MRP4 correlates with the in vitro chemosensitivity of tumor cells to pemetrexed (Chen et al., 2002; Hanauske et al., 2007). Given the functional role of Mrp4 at BBB (Leggas et al., 2004; Belinsky et al., 2007), Mrp4 is likely to function at the luminal membrane cooperatively with Oat3 for the brain-to-blood transport of pemetrexed and methotrexate. In summary, the present study examined the role of several active efflux transporters at the BBB in the brain distribution of pemetrexed and methotrexate. The results of this study show that both methotrexate and pemetrexed undergo saturable efflux from brain. Of the two transporters investigated in this study using gene knockout mice, Mrp2 does not play a role in the brain clearance of the two antifolates, whereas Bcrp makes a significant contribution.
to the brain elimination of methotrexate, but not pemetrexed. In addition, the brain-to-blood transport of both agents was sensitive to probenecid and benzylpenicillin, suggesting the involvement of organic anion transporters, with Oat3 being a possibility.

In conclusion, an important mechanism behind the low brain distribution of these antifolates is active efflux transport by multiple efflux systems working in tandem at the BBB. This study isolates several efflux systems that could be involved in the efflux of the two antifolates at the BBB.
Acknowledgements

We want to thank Tom Raub (Eli Lilly and Company) for providing Bcrp- and Mrp2-knockout mice. The authors would like to thank Maureen Riedl for the help with intracerebral microinjection technique.

Authorship Contributions

Participated in research design: Li and Elmquist.

Conducted experiments: Li and Agarwal.

Performed data analysis: Li and Elmquist.

Wrote or contributed to the writing of the manuscript: Li, Agarwal and Elmquist.
References


Ieiri I, Higuchi S, and Sugiyama Y (2009) Genetic polymorphisms of uptake (OATP1B1, 1B3) and efflux (MRP2, BCRP) transporters: implications for inter-individual differences in the pharmacokinetics and pharmacodynamics of statins and other clinically relevant drugs. *Expert Opin Drug Metab Toxicol* **5**:703-729.


Figure Legends.

Figure 1. Brain Efflux Index of Pemetrexed and Methotrexate. The percentage of $[^3]H$-pemetrexed remaining in the ipsilateral cerebrum was studied for up to 90 minutes after intracerebral injection (A). The percentage of $[^3]H$-methotrexate remaining in the ipsilateral cerebrum was studied for up to 60 minutes after intracerebral injection (B). The solid line was obtained by the regression analysis. Data are mean ± S.D. (n=3-4).

Figure 2. Inhibitory effect of unlabeled pemetrexed (1 and 50 mM in the injectate) on the efflux of $[^3]H$-pemetrexed from the mouse brain at 30 minutes postdose. Data are mean ± S.D. (n=3~4). (**, p< 0.01 compared with control; ***, p< 0.001 compared with control)

Figure 3. The percentage of $[^3]H$-pemetrexed and $[^3]H$-methotrexate in the brain of wild-type and Mrp2 deficient ($Mrp2 (-/-)$) mice at 60 or 30 minutes after administration

Figure 4. The remaining percentage of $[^3]H$-pemetrexed and $[^3]H$-methotrexate in the brain of wild-type and Bcrp deficient ($Bcrp1 (-/-)$) mice at 30 minutes postdose (*, p< 0.05 compared with WT control)

Figure 5. Steady-state pemetrexed brain and plasma concentration in wild-type and Bcrp deficient ($Bcrp1 (-/-)$) mice after 2-days or 3-days i.p. administration via osmotic minipump. Inset shows the steady-state brain-to-plasma ratio at 72 hours in wild-type and BCRP knockout mice. Values shown are mean ± S.D. (n=4).

Figure 6. Inhibitory effect of probenecid (100mM in the injectate) and benzylpenicillin (100mM in the injectate) on the remaining percentage of $[^3]H$-methotrexate and $[^3]H$-pemetrexed
in the mouse brain at 30 minutes postdose. Data are mean ± S.D. (n=3~4) (*,  p< 0.05, compared with control)

**Figure 7.** Time course of [³H]-pemetrexed and [3H]-methotrexate uptake by mouse brain slices. Data are mean ± S.D. (n=3~4)
Table 1 Effect of various treatments on brain-to-blood transport of pemetrexed

<table>
<thead>
<tr>
<th>[³H]-pemetrexed</th>
<th>concentration in the injectate (mM)</th>
<th>concentration in the braina (mM)</th>
<th>BEI (%)b</th>
<th>Percent of control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (control)</td>
<td>-</td>
<td>-</td>
<td>48.9 ± 5.1</td>
<td>100.0</td>
</tr>
<tr>
<td>Mrp2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>58.3± 3.3 c</td>
<td>100.4d</td>
</tr>
<tr>
<td>Bcrp&lt;sup&gt;1−/−&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>43.9 ± 2.1</td>
<td>89.7</td>
</tr>
<tr>
<td>WT + pemetrexed</td>
<td>1</td>
<td>0.025</td>
<td>28.3 ± 7.1***</td>
<td>57.8</td>
</tr>
<tr>
<td>WT + pemetrexed</td>
<td>50</td>
<td>1.253</td>
<td>4.3 ± 4.8 ***</td>
<td>8.8</td>
</tr>
<tr>
<td>WT + probenecid</td>
<td>100</td>
<td>2.506</td>
<td>27.6 ± 3.3 ***</td>
<td>56.5</td>
</tr>
<tr>
<td>WT + benzylpenicillin</td>
<td>100</td>
<td>2.506</td>
<td>12.2 ± 4.0 ***</td>
<td>25.0</td>
</tr>
</tbody>
</table>

a) Brain concentrations were estimated by dividing the injectate concentration by the dilution factor of 39.9, as reported previously (Kakee et al. 1996)

b) Percent of pemetrexed eliminated from the ipsilateral cerebrum at 30 minutes

c) Percent of pemetrexed eliminated from the ipsilateral cerebrum at 60 minutes

d) Percent of control normalized by the value of wild-type mice at 60 minutes

***, p < 0.001
Table 2 Effect of various treatments on brain-to-blood transport of methotrexate

<table>
<thead>
<tr>
<th>[3H]-methotrexate</th>
<th>concentration in the injectate (mM)</th>
<th>concentration in the brain (mM)</th>
<th>BEI (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Percent of control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (control)</td>
<td>-</td>
<td>-</td>
<td>63.8 ± 5.2</td>
<td>100.0</td>
</tr>
<tr>
<td>Mrp&lt;sup&gt;2/-/-&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>57.2 ± 6.0</td>
<td>90.0</td>
</tr>
<tr>
<td>Bcrp&lt;sup&gt;1/-/-&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>46.4 ± 7.4 **</td>
<td>72.7</td>
</tr>
<tr>
<td>WT + probenecid</td>
<td>100</td>
<td>2.506</td>
<td>24.2 ± 8.6 ***</td>
<td>37.9</td>
</tr>
<tr>
<td>WT + benzylpenicillin</td>
<td>100</td>
<td>2.506</td>
<td>13.7 ± 8.5 ***</td>
<td>21.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> % of methotrexate eliminated from the ipsilateral cerebrum at 30 minutes, **, p < 0.01; ***, p < 0.001
Table 3 Kinetic parameters of brain distribution of pemetrexed and methotrexate

<table>
<thead>
<tr>
<th></th>
<th>$\text{Cl}<em>{\text{lin}}/\text{Cl}</em>{\text{eff}}$</th>
<th>$\text{k}_{\text{eff}}$ (min$^{-1}$)</th>
<th>$V_{\text{d,brain}}$ (ml/g brain)</th>
<th>$\text{Cl}_{\text{eff}}$ ($\mu$l/min·g brain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pemetrexed</td>
<td>0.106 $^a$</td>
<td>0.0178 ± 0.0031</td>
<td>0.62 ± 0.10</td>
<td>11.04</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.05 $^b$</td>
<td>0.0238 ± 0.0028</td>
<td>0.85 ± 0.06</td>
<td>20.23</td>
</tr>
</tbody>
</table>

a) (Dai et al., 2005)
b) (Devineni et al., 1996)
Figure 1

![Graph A](image1.png)

**Percentage of PMX in the Ipsilateral Cerebrum**

- 100 - BEI (%) [Mean ± SD]
- Time after injection (minutes)

![Graph B](image2.png)

**Percentage of MTX in the Ipsilateral Cerebrum**

- 100 - BEI (%) [Mean ± SD]
- Time after injection (minutes)
Figure 2

![Graph showing the percentage of PMX in the ipsilateral cerebrum 100 - BEI (%) [Mean ± SD] for control, 1 mM PMX, and 50 mM PMX conditions. The graph includes error bars and significance levels (*, **, ***).]
Figure 3

Percentage Remaining in the Ipsilateral Cerebrum

100 - BEI (%) [Mean ± SD]

- Wild-type
- Mrp2 (-/-)

PMX
MTX
Figure 4

Percentage Remaining in the Ipsilateral Cerebrum 100 - BEI (%) [Mean ± SD]

- **Wild-type**
- **Bcrp1 (-/-)**

<table>
<thead>
<tr>
<th></th>
<th>PMX</th>
<th>MTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>45</td>
<td>76</td>
</tr>
<tr>
<td>B</td>
<td>56</td>
<td>38</td>
</tr>
</tbody>
</table>

* indicates a significant difference.
Figure 5

![Bar chart showing steady-state PMX concentration in nM/ml or nM/gm (mean ± SD) for WT and Bcrp1 (-/-) at different time points.](chart.png)
Figure 6

![Bar graph showing percentage remaining in the ipsilateral cerebrum](image)

- **Control**
- **100 mM Probenecid**
- **100 mM Benzylpenicillin**

**PMX** and **MTX**

Percentage Remaining in the Ipsilateral Cerebrum (Mean ± SD)