Distribution of the Novel Antifolate Pemetrexed to the Brain

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Abbreviations used: CNS, central nervous system; AUC, area under concentration-time curve; BBB, blood-brain barrier; MTX, methotrexate; ECF, extracellular fluid; HPLC, high performance liquid chromatography; CL, clearance; DHFR, dihydrofolate reductase; TS, thymidylate synthetase; GARFT, glycinamide-ribonucleotide-formyl transferase; MRP, multidrug-resistance associated protein; OAT, organic anion transporter; hOAT, human organic anion transporter; BCRP, breast cancer resistance protein.

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Abstract

Pemetrexed disodium is a novel antifolate which exhibits potent inhibitory effects on multiple enzymes in folate metabolism. Phase II/III clinical trials have shown that pemetrexed is effective against various solid tumors. Like methotrexate, pemetrexed may be useful in treatment of primary and secondary brain tumors. In this study, we examined the CNS distribution of pemetrexed and the interaction with an organic anion transport inhibitor, indomethacin. Male Wistar rats were administered pemetrexed by either single intravenous bolus or constant intravenous infusion. Unbound pemetrexed in blood and brain were measured by simultaneous arterial blood and frontal cortex microdialysis sampling. In the IV bolus experiments, indomethacin was administered by iv bolus (10mg/kg) followed by iv infusion (0.1mg/kg/hour) in a crossover fashion. In the infusion experiments, the same dose indomethacin was administered after a steady state was reached for pemetrexed. CNS distributional kinetics were analyzed by compartmental and non-compartmental methods. Both bolus and infusion studies showed that pemetrexed has a limited CNS distribution. The mean AUC_{brain} / AUC_{plasma} ratio of unbound pemetrexed is 0.078±0.038 in the iv bolus study. The pemetrexed steady-state brain-to-plasma unbound concentration ratio after iv infusion is 0.106±0.054. The distributional clearance into the brain was approximately 10% of the clearance out of the brain in both the compartmental and non-compartmental analyses. Indomethacin had no effect on either the brain-to-plasma AUC ratio or the steady state brain-to-plasma concentration ratio. The distribution of pemetrexed into the brain is limited and an efflux clearance process, such as an efflux transporter, may be involved.
Introduction

The antifolate agents, such as methotrexate (MTX), have been widely used in the treatment of various tumors (Bertino, 1993; Chamberlain, 1998; DeAngelis, 1999). MTX-based regimens are the most commonly used chemotherapy for primary lymphoma in the central nervous system (CNS) (Park and Abrey, 2002). However, the prognosis for CNS tumors, including primary CNS lymphoma, is still very poor (Bart et al., 2000). The poor response can be attributed to two reasons: low availability of drug at the site of action and intrinsic or acquired resistance the tumor has against the anti-tumor agents.

The natural or acquired resistance is often due to the overexpression or mutation of target enzymes (Melera, 1991; Banerjee et al., 1995; Bart et al., 2000). During the last decade, a novel antifolate compound, pemetrexed (LY-231514, multi-targeted antifolate, Alimta®), has been developed to overcome some of these problems. Pemetrexed targets at least three enzymes in folic acid metabolism (Kaye, 1998). It is conceivable that such a combinatorial effect of inhibiting three enzymes at multiple sites would give this new antifolate an advantage in overcoming acquired or intrinsic resistance if the resistance is due to overexpression or mutation of one enzyme. In fact, it has been shown that pemetrexed has broad antitumor activity in Phase II trials in a wide variety of solid tumors, including mesothelioma, non-small cell lung, breast, cervical, colorectal, head and neck, and bladder cancers (Shih et al., 1998) (Shih et al., 1997) (Adjei, 2000). Given the spectrum of activity of methotrexate, it can be expected that pemetrexed may also have useful applications in the treatment of CNS tumors with a possible benefit of less acquired resistance. This is important not only for the treatment of primary CNS lymphoma, but also for brain metastases, considering lung and breast cancers are the leading source of secondary brain tumors (Greenberg et al., 1999). It is known that more
than 25% of patients with lung cancer and 10-15% of patients with breast cancer develop a brain metastasis during the course of their disease (Greenberg et al., 1999).

Delivery of classic antifolate compounds such as methotrexate to the brain has been a major challenge for the effective use of these compounds in CNS malignancies. Tremendous efforts have been made to increase the penetration of methotrexate into the brain, including the use of Ommaya reservoirs for direct intraventricular infusion (Dakhil et al., 1981; Stone et al., 1999), intrathecal injection and infusion (Wilson and Norrell, 1969; Bleyer et al., 1997), reversible osmotic breaching of the BBB using high dose mannitol (Hasegawa et al., 1979), and very high dose intravenous infusion with leucovorin rescue (Wang et al., 1976; Allen et al., 1980). All of these CNS drug delivery strategies are complicated by serious side effects (Bleyer et al., 1978; Allen et al., 1980; Browne et al., 1987; Bleyer et al., 1997; Stone et al., 1999).

While the low distribution of MTX into brain may be attributed to its hydrophilic nature and its interaction with various transporters expressed in BBB (Hooijberg et al., 1999; Kool et al., 1999; Chen et al., 2002), the factors that influence the distribution of the new antifolate pemetrexed to the CNS have not been mechanistically studied. Compared with MTX, pemetrexed is a structurally similar antifolate, however, it could have different physiochemical properties that may affect its CNS distribution, particularly when considering active transport into or out of the brain. Information regarding CNS penetration and distribution would be important in developing effective therapy for CNS tumors.

In this study, we examined the CNS distribution of this new antifolate. We also investigated the effects of a known inhibitor, indomethacin, of some active organic anion efflux transport systems that exist in the BBB, on the distribution of pemetrexed to the
brain. A more complete and in depth understanding of the CNS distribution of new and possibly more effective antifolate compounds should result in more successful treatments of primary and secondary cancers in the CNS, and in other tissues that have similar issues regarding drug transport and therefore drug delivery.
Materials and Methods

Chemicals:

Pemetrexed disodium (N-[4-[2-(2-amino-4,7-dihydro-4-oxo-1H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]-benzoyl]-L-glutamic acid) and ³H-pemetrexed disodium were kindly provided by Eli Lilly. Raltitrexed (N-[5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl]-L-glutamic acid) was kindly provided by AstraZeneca. Indomethacin was purchased from Sigma Chemicals (St. Louis, Missouri). Solvents were HPLC grade, and all other chemicals were reagent grade or better.

Microdialysis studies

Microdialysis is a powerful tool to measure the unbound drug concentration in the plasma and tissue. The unbound drug in the tissue is the active entity that exerts its pharmacological effect while the unbound drug concentration in the plasma is the driving force for unbound drug to distribute into the tissue. It would be beneficial in the study of CNS drug distribution if the unbound drug in the plasma and brain ECF can be simultaneously measured. This was done in this study using simultaneous blood and brain microdialysis sampling using a novel blood probe (Dai and Elmquist, 2003).

Quantitative microdialysis requires the determination of the recovery of drug that is the fraction of drug that is gained through microdialysis probe from the site of measurement (Wang et al., 1993). One of the approaches to measure the in vivo recovery is through retrodialysis, where the loss of a retrodialysis calibrator is measured and used as the recovery for calculation of the concentration of drug. Therefore, loss of a retrodialysis calibrator has to be shown to be as equal to the recovery of the drug of interest before it
can be rationally used. In this study, raltitrexed was chosen as a retrodialysis calibrator because of its similarity with pemetrexed in chemical structure (figure 1), and was validated in the in vitro and in vivo microdialysis studies described below.

**In vitro microdialysis**

The procedure for in vitro microdialysis was similar to previous published protocols (Dai and Elmquist, 2003). Briefly, the microdialysis probe (CMA-12 or home-made blood probe) was placed in a 2 ml vial containing well-stirred artificial cerebrospinal fluid (119.5 mM NaCl, 4.75 mM KCl, 1.27 mM CaCl$_2$, 1.19 mM KH$_2$PO$_4$, 1.19 mM MgSO$_4$, 1.6 mM Na$_2$HPO$_4$, pH 7.4, for the brain probe) or Ringer’s solution (142 mM NaCl, 4.0 mM KCl, 2.38 mM CaCl$_2$, 2.38 mM NaHCO$_3$, pH 7.2, for the blood probe) for perfusion at 37 °C.

A Harvard 22 syringe pump (Harvard Apparatus, Holliston, Massachusetts) was used to perfuse the probe at different rates. The concentration of the pemetrexed or raltitrexed in the out-flowing dialysate was measured by online HPLC. The mobile phase was 5% acetonitrile, 4.5% tetrahydrofuran in 30 mM sodium phosphate (pH 3) (w/w). The column was a reverse phase C-18 (BDS C18, 150x2mm, pore size of 5μm, Keystone, Bellefonte, Pennsylvania). The UV-absorption of pemetrexed and raltitrexed was measured by UV detection (SPD-10A, Shimadzu) at a wavelength of 245 nm. The loss of each compound through the microdialysis probe was calculated as follows,

\[
\text{Loss} = \frac{(\text{Cin} - \text{Cout})}{\text{Cin}} \quad \text{Eq. (1)},
\]

where Cout and Cin are the concentrations of the compound in the dialysate and in perfusate, respectively. Similarly, the gain of pemetrexed and raltitrexed was measured when a probe was placed in a medium containing pemetrexed (2 μg/ml) or raltitrexed (2
μg/ml), and perfused with drug-free medium. The dialysate was measured for the drug concentrations. The gain was calculated as follows,

\[
\text{Gain} = \frac{C_{\text{out}}}{C_{\text{m}}} \quad \text{Eq. (2)},
\]

where \( C_{\text{out}} \) is the concentration of the compound in the dialysate and \( C_{\text{m}} \) is concentration of the compound in the medium surrounding the probe in the vial, respectively.

**In vivo microdialysis to measure unbound Pemetrexed in blood and brain**

**Microdialysis probe placement.** Male Wistar rats weighing from 275-340 gram were used in this study. The surgical procedures for implantation of the microdialysis probe guide cannula, probe placement, and the cannulation of the femoral vein were similar to Yang et al (Yang et al., 1997) except that an arterial microdialysis probe, instead of a cannula, was placed in the femoral artery (Dai and Elmquist, 2003). All surgical procedures were done using aseptic technique. The stereotaxic coordinates for the frontal cortex were 3mm (anterior) and 1.5 mm lateral (right) to the bregma. The tip of guide cannula was 1 mm ventral from the brain surface. The rat was allowed to recover for 3 to 4 days following the placement of the guide cannula. After this recovery period, the cortical probe was placed in the guide cannula, the femoral vein was cannulated for dosing, and a blood probe was placed in the femoral artery for microdialysis. CMA-12 microdialysis probes (CMA-Microdialysis, Acton, Massachusetts) of 3 mm length were used for cortex sampling. A newly designed blood probe was used for sampling the unbound concentration of pemetrexed in the arterial circulation (Dai and Elmquist, 2003). The blood microdialysis probe was implanted in the femoral artery in a similar procedure.
by which a probe is placed in the femoral vein as previously described (Yang et al., 1997) with slight modification (Dai and Elmquist, 2003).

In the *in vivo* microdialysis, raltitrexed was perfused as the retrodialysis calibrator through both blood and brain probes. The perfusion rate was 0.5 µl/min for the brain probe, and 1 µl/min for the blood probe, so that no net flux of water across the membrane occurred. This was verified gravimetrically. The dialysate was simultaneously collected every 26 minutes and measured for pemetrexed and raltitrexed by two online HPLCs. Retrodialysis recovery of the calibrator (raltitrexed) was calculated using Eq. 1.

**Animal studies**

**Intravenous bolus study:** The rats (*n* = 4) received an intravenous bolus dose of pemetrexed with or without co-treatment of indomethacin in a crossover fashion. In the control phase, pemetrexed disodium (60mg/kg, dissolved in 0.9% sodium chloride at a concentration of 10 mg/ml) was given by intravenous bolus into the femoral vein. After 12 hours of continuous brain and blood sampling using the microdialysis probes, the rats again received pemetrexed as above. However, one-half hour prior to pemetrexed dosing, the rats were pretreated with indomethacin (a bolus dose of indomethacin (10 mg/kg, iv) diluted in 0.9 % saline chloride) followed by a continuous iv infusion of indomethacin (0.1 mg/kg/h) for the duration of the experiment. There was no detectable pemetrexed in either the brain probe dialysate or the blood probe dialysate when the second treatment arm of the crossover began.

**Intravenous infusion:** A separate group of rats (*n* = 4) received pemetrexed by intravenous infusion (20mg/kg/hour, dissolved in 0.9% sodium chloride at a
concentration of 6 mg/ml) for three hours to achieve steady state (control phase I). The elimination half-life of pemetrexed in the rat is approximately 18 minutes, and steady state should be achieved after 90 minutes of constant rate infusion. After the steady state was reached, while the infusion of pemetrexed continued, the rat received an iv bolus dose of indomethacin (10 mg/kg, dissolved in 9% saline), followed by a continuous iv infusion of indomethacin (0.1 mg/kg/h) for another three hours (treatment phase). After a minimum of a 12 hour washout period, another intravenous infusion of pemetrexed (same infusion rate) was given until steady state was achieved (control phase II). This second control phase was to determine if the experimental procedure was stable with time (treatment order effect) and to see if any effect of indomethacin was reversible.

During the whole experiment, the unbound concentrations of pemetrexed in brain extracellular fluid and blood were monitored by simultaneous brain and blood microdialysis. The unbound concentrations of pemetrexed in the brain and blood were calculated after correction for each probe recovery measured with the retrodialysis calibrator, raltitrexed.

**Data analysis:**

**Compartmental analysis.** Compartmental modeling was performed using SAAMII nonlinear regression software (SAAM Institute, Seattle, Washington). First, a two-compartment model with first-order elimination in the central compartment was fit to the unbound pemetrexed plasma concentration-time data (see figure 2). The purpose of this first stage was solely to obtain parameters to be used as a forcing function in the second stage, where a one-compartment model for drug disposition in the brain was fit to the
unbound pemetrexed concentrations in brain ECF (figure 2). For purposes of this modeling, the apparent volume of brain ECF was assumed to be 1.44 ml/kg body weight, which is 20% of brain weight (Wang and Sawchuk, 1995; Malhotra et al., 1997; Bouw et al., 2001), where brain weight in the rat is 1.8 g/250 gram body weight (Sharp and La Regina, 1998). Even though an inaccuracy in this value may affect the estimation of the efflux clearance out of the brain (CL\textsubscript{out}), the comparison between the controls and the treated regarding CL\textsubscript{out} or the ratio of CL\textsubscript{in} to CL\textsubscript{out}, will not be affected by the choice of value for the brain ECF.

**Non-compartmental analysis:** The terminal rate constant is determined by linear regression of the last three or four log-transformed data points. AUC was calculated by using the following equation (26):

\[
\text{AUC} = \sum Ci \cdot \Delta t + C_{\text{last}}/k \quad \text{Eq. (4)},
\]

where \(\Delta t\) is the microdialysis collection interval, \(Ci\) is the concentration at the midpoint of the interval, \(C_{\text{last}}\) is the last concentration time point, and \(k\) is the terminal rate constant. In the infusion study, the ratio of the clearance of entering and efflux out of brain can be calculated from the brain-to-plasma ratio of steady state unbound concentration, i.e.,

\[
\frac{C L_{\text{in}}}{C L_{\text{out}}} = \frac{C_{s s, \text{brain}}}{C_{s s, \text{plasma}}}.
\]

**Statistics**
The nonparametric alternative of the paired t-test, the Wilcoxon signed-rank test, was used to test difference between the control and treated groups, with a chosen level of significance of \(p < 0.05\).
Results

Calibration of the microdialysis probe

First, the suitability of raltitrexed to be used as a retrodialysis calibrator was examined. As seen in figure 3, the loss of both pemetrexed and raltitrexed across the microdialysis probe from the perfusate to the well-stirred medium was similar at different flow rates. These flow rates ranged from 0.5 to 4 µl/minute, and the two compounds tested had similar permeability-area products (dialysis clearance), as indicated by the slopes of the regression lines in figure 3B (0.21 vs 0.22 µl/min). Moreover, the linear correlation between Ln (1-loss) and the reciprocal of flow rates (figure 3) indicates the clearance of both drugs is independent of the flow rate (Sawchuk and Elmquist, 2000). Also, the gain of pemetrexed was shown to be similar to its loss and, importantly, to the loss of raltitrexed as tested in the in vitro microdialysis (figure 4). This suggests that the transport of these two antifolates across the dialysis membrane is a diffusion-controlled process, and indicates the suitability of using raltitrexed as a retrodialysis calibrator for measuring pemetrexed in vivo. This was further confirmed by the in vivo study, where the loss of these two compounds through the probe placed in the frontal cortex was similar (figure 4), though the in vivo loss of pemetrexed and raltitrexed was lower than their in vitro values. Others have also observed that in vivo loss of the calibrator is lower than that of in vitro (Fox et al., 2002), which may be due to the increased resistance to the diffusion of the compound in the cortical tissue (Sun et al., 2001a; Fox et al., 2002).
**Intravenous bolus studies**

Rats were given intravenous bolus administration of pemetrexed (60mg/kg) with or without co-administration of an inhibitor of organic anion transporters, indomethacin, in a crossover fashion. The unbound pemetrexed concentration-time profile in the brain cortex and blood is shown in figure 5. Pemetrexed concentration in the brain ECF rose rapidly to its maximum and declined more slowly than the unbound concentration in the blood. Table 1 summarizes the pharmacokinetic parameters for unbound pemetrexed. The clearances of unbound pemetrexed entering (CL\textsubscript{in}) and exiting the brain ECF (CL\textsubscript{out}) were estimated by compartmental modeling. As shown in table 1, the ratio of CL\textsubscript{in}/CL\textsubscript{out} is 0.094± 0.04 in the control phase and 0.080 ± 0.03 in the indomethacin treated phase.

Table 2 summarizes the non-compartmental determination of the brain penetration of unbound pemetrexed and the terminal rate constant for unbound pemetrexed in brain ECF. Consistent with the ratio of CL\textsubscript{in}/CL\textsubscript{out} determined by compartmental modeling, the equilibrium distribution coefficient of pemetrexed in the brain, expressed by tissue-to-plasma area ratio, is low with the mean±S.D. unbound AUC\textsubscript{brain}/AUC\textsubscript{plasma} ratio being 0.078±0.038. Indomethacin treatment did not affect this ratio or the terminal rate constant of pemetrexed in the brain (Table 2). From these data taken together, it can be concluded that indomethacin treatment did not change the distribution of unbound pemetrexed into brain.

**Intravenous infusion studies**

To further investigate the CNS distribution of pemetrexed, and the possible influence of organic anion transporters on that distribution, an intravenous infusion study
was conducted in combination with indomethacin treatment. As shown in figure 6 and table 3, the mean±S.D. of the $C_{ss\ brain}/C_{ss\ plasma}$ ratio of unbound pemetrexed was 0.106±0.054, which agrees with the $CL_{in}/CL_{out}$ determined from the compartmental modeling from the intravenous bolus experiments (see table 1). Furthermore, the ratio of $C_{ss\ brain}/C_{ss\ plasma}$ was not affected by indomethacin treatment ($P>0.1$) (Table 3). The stability of the experimental system was not affected by time, as seen by a second infusion of pemetrexed administered after a 12 hour washout period, where the brain-to-plasma ratio of steady-state concentrations did not change with time ($P>0.1$, figure 6, table 3). These data confirm that pemetrexed has a limited CNS distribution and that indomethacin does not affect the CNS penetration of pemetrexed.
Discussion

The classic antifolate, MTX, has been used for the treatment of various solid tumors (Bertino, 1993; Chamberlain, 1998; DeAngelis, 1999). However, previous studies have shown that tumor cells can acquire resistance to MTX (Banerjee et al., 1995). It has further been shown that one important resistance mechanism is associated with the overexpression of the target enzyme, dihydrofolate reductase (DHFR), or a variant DHFR with a low affinity for MTX (Melera, 1991; Banerjee et al., 1995). During the last decade, several new antifolates have been developed so that they would overcome the acquired and natural resistance to methotrexate. Pemetrexed is one of these new analogues. It has been demonstrated that pemetrexed inhibits three enzymes: DHFR, thymidylate synthetase (TS), and glycaminde-ribonucleotide-formyl transferase (GARFT). Each is an important enzyme in the folic acid pathway, which is critical in purine and pyrimidine nucleotide synthesis, leading to the replication of DNA, particularly for rapidly dividing cells (Shih et al., 1997; Shih et al., 1998; Adjei, 2000). Inhibition of multiple enzymes by pemetrexed could preclude the development of drug resistance caused by overexpression or mutation of a single enzyme. In this regard, pemetrexed has demonstrated broad spectrum of clinical activities against non-small cell lung, breast, colorectal, neck, bladder and cervical cancer as a first line or second line therapeutic agent (O'Dwyer et al., 1999).

MTX has been used for the treatment of primary CNS lymphomas and other primary or secondary brain tumors (Bertino, 1993; Chamberlain, 1998; DeAngelis, 1999). It is conceivable that pemetrexed might also be used for such tumors with the added benefit of less acquired resistance. However, its therapeutic effect will depend
largely on the targeted bioavailability of the drug to the CNS site of action, and effective
delivery of antitumor compounds across blood-brain barrier to the brain remains a major
challenge in the treatment of brain tumors, including secondary brain tumors (Lesniak et
al., 2001). The purpose of this study was to examine the CNS distribution of pemetrexed
and to identify the factors that may limit the brain penetration of this compound by using
simultaneous arterial blood and brain microdialysis.

Microdialysis is a useful tool to study the tissue distribution of drug, especially
CNS distribution (Sawchuk and Elmquist, 2000). It measures the unbound drug
concentration that is the active moiety in the brain. If only total drug concentration in
plasma or tissue are measured, as previous experiments have done, problems in
interpretation of the distributional processes could arise. For instance, when nonlinear
protein binding or drug-drug interaction in the level of protein binding occurs, the total
drug concentration in the plasma may not reflect the change in unbound concentration in
blood, the driving force for the drug to distribute into the brain. In this regard,
simultaneous blood and brain microdialysis was conducted to measure the unbound drug
concentration in plasma and brain. In doing so, the effect of binding of drug to the
plasma or tissue protein on the distributional kinetics was addressed. With the novel
design of the blood microdialysis probe (Dai and Elmquist, 2003), the simultaneous
sampling by microdialysis of arterial blood and brain extracellular fluid has provided a
powerful tool to study the CNS distributional kinetics in this study.

The microdialysis probe was validated by three criteria: independence of the
recovery on the perfusion flow rate, the equal recovery of the analyte across the dialysis
membrane both regarding loss from, and gain to, the perfusate and equal loss between the
drug and calibrator in either the in vitro or in vivo situation. It is worthy to note that the
in vivo loss from the perfusate to the tissue (brain cortex) is lower than the loss from the perfusate to the aqueous medium in vitro. This is most likely due to the greater resistance of the transport of the drug in the tissue (in vivo) compared with that in the aqueous medium (in vitro). Fox et al and others have reported similar phenomenon (Fox et al., 2002).

This is the first reported study to characterize the brain penetration of pemetrexed in animals. This study demonstrates that pemetrexed has a limited CNS distribution, which is indicated by brain-to-plasma AUC ratio of pemetrexed and the brain-to-plasma ratio of steady state concentrations. The brain penetration of pemetrexed is slightly higher than that of the classic antifolate, MTX. This may be partly due to the fact that pemetrexed is more lipophilic than that of MTX (data not shown). Previous microdialysis studies have shown that the ratio of unbound MTX level in brain versus the total MTX level in plasma ranges from 0.01 to 0.02 in terms of the AUC_{ECF, unbound}/AUC_{plasma, total} when it was administered by intravenous bolus dose (Dukic et al., 2000) (Dukic et al., 1999) (Devineni et al., 1996). In our intravenous bolus study, the AUC_{ECF, unbound}/AUC_{plasma, unbound} is 0.078, which would give a ratio of AUC_{ECF, unbound} /AUC_{plasma, total} of approximately 0.05, given the free fraction of pemetrexed in rat plasma of 0.36 (data not shown). Similarly, in the iv infusion study, the ratio of C_{ss, brain}/C_{ss, plasma} for unbound pemetrexed is about 0.106, further indicating a limited distribution of pemetrexed into brain ECF. Of note, the brain level of pemetrexed rose to maximum rapidly in the iv bolus study, and also achieved a rapid steady state in the infusion study even though the clearance into the brain is low (about 2.9 µl/min/kg, see table 1). This may be due to the small volume of distribution of pemetrexed in the brain, allowing a rapid achievement of distributional equilibrium in this tissue. Moreover, this kinetic
behavior could be a result of an efficient efflux clearance process, such as active efflux transporters at the blood-brain barrier.

Factors limiting the CNS distribution of pemetrexed remain unidentified. Various reasons for the low CNS distribution of pemetrexed could be: protein binding in the blood, low passive permeability (diffusional influx clearance) due to the hydrophilicity of the compound, and high efflux clearance. The results from intravenous bolus study showed that the efflux clearance (Clout) is about 10-fold greater than influx clearance for pemetrexed. The efflux clearance may include components such as bulk flow, possible efflux transport system in BBB, and/or metabolism. The rate of brain ECF bulk flow in the rat is about 0.3 µl/min (Szentistvanyi et al., 1984; Rosenberg, 1990) which is much smaller than efflux clearance (approximately 31 µl/min/kg or 9.2 µl/min for a 300 gram rat, see table 1), and thus is not considered to be a major factor in this process. The active efflux transporters expressed in the BBB and BCSFB could be an important factor. Previously, MTX has been shown to be a substrate of many efflux transporters such as MRP1 (Hooijberg et al., 1999), MRP2 (Hooijberg et al., 1999), MRP3 (Kool et al., 1999), MRP4 (Chen et al., 2002), and OATs (Masuda et al., 1999) (Takeda et al., 2002). As an analogue of MTX, pemetrexed may also be the substrate of these efflux transporters that are expressed in BBB and may limit the distribution of pemetrexed into the CNS.

To examine this hypothesis, indomethacin, an established inhibitor with activity against some organic anion transporters (Hamilton et al., 2001; Sun et al., 2001b; Berger et al., 2003), was co-administered with pemetrexed to see if the CNS distribution of pemetrexed would be affected. Indomethacin has been shown to inhibit effectively the transport of various organic anion compounds in vitro and in vivo, including MRP1 (Draper et al., 1997), MRP2 (Berger et al., 2003), MRP4 (Reid et al., 2003), oapt2
human organic anion transporters (hOAT1, hOAT2, hOAT3, and hOAT4) (Khamdang et al., 2002). It has been shown that indomethacin can sensitize MRP or OAT-overexpressed cell and affect the transport of MTX (Khamdang et al., 2002; Takeda et al., 2002; Sosogi et al., 2003), anionic fluorescent dye carboxy-2',7'-dichlorofluorescein (Payen et al., 2000) and adefovir (Mulato et al., 2000). If pemetrexed is a substrate of indomethacin-sensitive organic anion transporter, its brain penetration would be expected to increase in the presence of indomethacin. Surprisingly, our studies, including intravenous bolus and steady-state infusion, showed that the CNS distribution of unbound pemetrexed was not affected by co-treatment of indomethacin. One concern may be raised about the availability of indomethacin in the brain. Using the published pharmacokinetic parameters of indomethacin in rats (Ogiso et al., 1989), the starting plasma concentration ($C_0$) of indomethacin at the dose given in this study is predicted to be 83.3 µg/ml. Given that indomethacin is about 90% bound in plasma (Mason and McQueen, 1974), the free concentration of indomethacin would be 8.3 µg/ml. It has also been shown that indomethacin can interact with MRP4 at a concentration low as 0.375 µg/ml (1 µM) (Reid et al., 2003). Thus, at this dose regimen, indomethacin seems more than likely to be in sufficient concentration at the site of action to interact with organic transporters. From a pharmacodynamic perspective, it has been shown that at the same dose of indomethacin used in this study, indomethacin levels in the brain are high enough to effectively protect brain from ischemic damage (Chung et al., 2001). Therefore, the lack of effect by indomethacin on the CNS distribution of pemetrexed seems less likely to be due to the inadequate concentration of indomethacin in the brain. However, it is possible that efflux mediated by transporters other than organic transporters or metabolism may be responsible for the limited penetration of pemetrexed in the brain.
Previous studies have indicated that MTX is a substrate of breast cancer resistance protein (BCRP) which is expressed in BBB (Volk et al., 2002; Volk and Schneider, 2003). This warrants further investigation regarding the role of BCRP in the transport of pemetrexed in BBB in vivo.

In summary, our study showed that the novel antifolate, pemetrexed, has a limited CNS distribution, although it is greater than that of MTX. Co-treatment with indomethacin does not significantly affect this distribution, suggesting indomethacin-sensitive organic anion transporters may not play a significant role in limiting the distribution of pemetrexed to the brain.
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Legends for Figures

Figure 1. Chemical structures of pemetrexed (top) and raltitrexed (bottom).

Figure 2. The model used to estimation of kinetic parameters in iv bolus study. A two-compartment model was fit to the plasma data then the parameters obtained were used as a forcing function in the estimation of brain distributional kinetics.

Figure 3. Transport behavior of pemetrexed, and raltitrexed in in vitro microdialysis. The losses of pemetrexed and raltitrexed through a microdialysis probe (CMA-12) at different flow rates were simultaneously measured by online HPLC (Panel A). When –Ln(1-loss) is plotted against the reciprocal of flow rate Q, the slope of the line indicates the permeability-area product for each drug (Panel B).

Figure 4. Comparison of the in vivo and in vitro recovery of antifolates
The gain and loss of pemetrexed through a microdialysis probe CMA-12 were simultaneously measured at a perfusion rate of 0.5 µl/min in the in vitro microdialysis (n=10). In an in vivo experiment, the loss of pemetrexed and raltitrexed through a CMA-12 probe that was implanted in the cortex of a rat were simultaneously measured at a perfusion rate of 0.5 µl/min (n=4). The values are expressed as mean ± SD.

Figure 5. Plasma and brain concentration time profile of unbound pemetrexed in rats. Pemetrexed (60mg/kg) was administered by intravenous bolus without (control, panel A) or with (treated, panel B) the coadministration of indomethacin as described in...
the Materials and Methods. Unbound pemetrexed concentrations in both the plasma and brain ECF were measured by simultaneous microdialysis.

Figure 6. A representative concentration time profile of unbound pemetrexed in plasma and brain ECF in the intravenous infusion study. Pemetrexed (20mg/kg/hr) was given by intravenous infusion. After the steady state was achieved, the rat further received co-administration of indomethacin (a iv bolus dose of indomethacin (10 mg/kg) being followed by a continuous iv infusion of indomethacin (0.1 mg/kg/h)) for another three hours. After 12 hour washout period, another intravenous infusion of pemetrexed (20mg/kg/hr) was given to reach the steady state. During the whole experiment, the unbound concentrations of pemetrexed in brain extracellular fluid (o) and plasma (●) were measured by simultaneous brain and blood microdialysis.
Table 1. Pharmacokinetic parameters of unbound pemetrexed after iv bolus administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>control</th>
<th>treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Cl (ml/min/kg)</td>
<td>9.6 ± 3.5</td>
<td>9.3 ± 3.3</td>
</tr>
<tr>
<td>v1 (ml/kg)</td>
<td>245.7 ± 73.6</td>
<td>308.7 ± 64.6</td>
</tr>
<tr>
<td>k21 (min-1)</td>
<td>0.021 ± 0.006</td>
<td>0.017 ± 0.011</td>
</tr>
<tr>
<td>k12 (min-1)</td>
<td>0.0022 ± 0.0009</td>
<td>0.0012 ± 0.00006</td>
</tr>
<tr>
<td>Koutbrain (min-1)</td>
<td>0.021 ± 0.0047</td>
<td>0.020 ± 0.006</td>
</tr>
<tr>
<td>Kinbrain (min-1)</td>
<td>0.0000011 ± 0.000003</td>
<td>0.000007 ± 0.000002 *</td>
</tr>
<tr>
<td>Clin (µl/min/kg)</td>
<td>2.9 ± 1.6</td>
<td>2.3 ± 1.0</td>
</tr>
<tr>
<td>Clout (µl/min/kg)</td>
<td>30.5 ± 6.8</td>
<td>28.6 ± 9.1</td>
</tr>
<tr>
<td>Clin/Clout</td>
<td>0.094 ± 0.04</td>
<td>0.080 ± 0.003</td>
</tr>
</tbody>
</table>

* P< 0.05 compared with control, the volume of ECF was fixed at 1.33ml/kg
Table 2. Brain penetration of unbound pemetrexed in rats after iv bolus administration

<table>
<thead>
<tr>
<th>Rat #</th>
<th>AUC&lt;sub&gt;brain&lt;/sub&gt;/AUC&lt;sub&gt;plasma&lt;/sub&gt;</th>
<th>Terminal rate constant unbound pemetrexed in ECF (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control treated</td>
<td>control treated</td>
</tr>
<tr>
<td>1</td>
<td>0.073 0.074</td>
<td>0.0244 0.0176</td>
</tr>
<tr>
<td>2</td>
<td>0.083 0.076</td>
<td>0.0179 0.0122</td>
</tr>
<tr>
<td>3</td>
<td>0.032 0.043</td>
<td>0.0152 0.0069</td>
</tr>
<tr>
<td>4</td>
<td>0.125 0.107</td>
<td>0.0075 0.0088</td>
</tr>
<tr>
<td>mean</td>
<td>0.078 0.075</td>
<td>0.0163 0.0114</td>
</tr>
<tr>
<td>SD</td>
<td>0.038 0.026</td>
<td>0.0070 0.0047</td>
</tr>
</tbody>
</table>
Table 3. $C_{ss,\text{brain}} / C_{ss,\text{plasma}}$ of unbound pemetrexed in the intravenous infusion study

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Control I</th>
<th>treated</th>
<th>Control II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.053</td>
<td>0.055</td>
<td>0.061</td>
</tr>
<tr>
<td>2</td>
<td>0.170</td>
<td>0.152</td>
<td>0.178</td>
</tr>
<tr>
<td>3</td>
<td>0.072</td>
<td>0.069</td>
<td>0.064</td>
</tr>
<tr>
<td>4</td>
<td>0.130</td>
<td>0.130</td>
<td>0.150</td>
</tr>
<tr>
<td>mean</td>
<td>0.106</td>
<td>0.101</td>
<td>0.113</td>
</tr>
<tr>
<td>SD</td>
<td>0.054</td>
<td>0.047</td>
<td>0.060</td>
</tr>
</tbody>
</table>
Figure 1.

Pemetrexed

Raltitrexed
Figure 2.
Figure 3.
Figure 4.

![Graph showing recovery of antifolates](image-url)
Figure 5.
Figure 6.