ACTIVE EFFLUX TRANSPORT AND CNS DISTRIBUTION OF THE NOVEL ANTIFOLATE PEMETREXED

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Dedication

This thesis is dedicated to my wonderful parents Yusheng Li and Wenqin Xu and my beloved husband Jinming Liu for their constant support and unconditional love.

Abstract

Pemetrexed (PMX, Alimta®) is a novel multi-targeted antifolate approved for the treatment of malignant pleural mesothelioma and non-small cell lung cancer (NSCLC). Given the high incidence of brain metastasis in NSCLC patients and wide use of the classic antifolate, methotrexate (MTX), in primary CNS lymphoma, the clinical use of PMX may eventually include the treatment of CNS tumors. However, previous studies in our laboratory indicated that, like MTX, PMX has difficulty in penetrating across the blood-brain barrier (BBB). Factors limiting the CNS distribution of PMX remain unidentified. One important determinant of CNS distribution is efflux transport by BBB transporters. The overall objective of this study was to characterize the brain-to-blood efflux transport of PMX and to examine role of BBB efflux transporters such as BCRP, MRP2 and other organic anion transporters in brain distribution of PMX. The interaction of PMX with BCRP was examined *in vitro* and *in vivo. In vitro* results revealed that PMX is a substrate for BCRP-mediated transport. *In vivo* examination indicated that deletion of Bcrpl has little influence on brain penetration of PMX. Using the brain efflux index method, the mechanism responsible for the brain efflux of PMX and MTX was investigated. The results revealed that brain elimination half-life of PMX and MTX were 48 and 32 minutes, respectively and both PMX and MTX undergo saturable efflux transport across the BBB. MRP2 does not play a role in the brain distribution of either antifolate. However, BCRP makes a significant contribution to brain elimination of MTX, but not PMX. In addition, it was observed that brain-to-blood transport of PMX and MTX was markedly inhibited by probenecid and

benzylpenicillin, suggesting the involvement of organic anion transporters, possibly OAT3. Given the knowledge that solid tumors and some physiological barriers have an acidic extracellular environment, the effect of pH on transport activity of BCRP for PMX was examined. In addition, the molecular basis of the observed pH-dependency in BCRP transport activity was explored based on the recent homology model of BCRP. This study has important implications in the handling of PMX and other chemotherapy drug molecules in the acidic environment of tumors and in the distribution and elimination of the drug molecules.

Studies presented in this dissertation provide useful information about the specific mechanisms involved in limiting the brain penetration of PMX and MTX. This new knowledge will help in formulating strategies to improve CNS delivery of these antifolates and maylead to more successful treatment of primary and secondary CNS tumors.

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CHAPTER 1

INTRODUCTION

1.1. The ATP-binding cassette (ABC) Transport System

The family of ATP-binding cassette transporters (ABC-transporters) is a large gene family of structurally related transmembrane proteins, that use the energy of ATP hydrolysis to translocate various substrates across cellular membranes (Jones and George, 2004). To date, 49 members of the ABC transporter family in humans have been discovered (Glavinas, Krajcsi et al. 2004). Based upon phylogenetic analysis, these transporter families have been divided into seven subfamilies which share the root ABC in their names followed by subsequent alphabet from A to G (Dean et al., 2001; Glavinas et al., 2004). For example, breast cancer resistance protein is named as ABCG2, since it belongs to subfamily G and is the second member of that subfamily.

1.1.1 Structural attributes of ABC-transporters

Typical ABC-transporters will contain two transmembrane domains (TMD) and two ATP-binding domains (nucleotide-binding domain (NBD)). The transmembrane domain, which typically contains six transmembrane helices, is thought to contain the main determinants of the substrate specificity (Ujhelly et al., 2003). The ATP-binding domains are located on the cytoplasmic side of the membrane and are composed of three core consensus motifs: the Walker A and Walker B motif that are involved in ATP binding and the C loop (ABC signature motif) (Klein et al., 1999).

1.1.2 Role of ABC-transporters

ABC-transporters were initially described as drug efflux pumps and are well known as crucial factors in the development of multidrug resistance (MDR). Among the various ABC-transporters, P-glycoprotein (P-gp, ABCB1), breast cancer resistance protein (BCRP, ABCG2) and multidrug resistance-associated proteins (MRPs, ABCC family) have been recognized as the most significant transporters for clinical MDR: (Leslie et al., 2005; Robey et al., 2007). In addition to the malignant tissues, ABC-transporters are also expressed in normal tissues. The ubiquitous expression and broad substrate spectrum make the efflux transporters the "front-line" cellular defender against the xenobiotics (Leslie et al., 2005; Evseenko et al., 2006).

1.1.3 Active efflux transporters

P-gp (MDR1, ABCB1) is the first human ABC transporter cloned, and is studied most extensively (Juliano and Ling, 1976; Riordan et al., 1985). It is a full transporter comprised of two TMDs and two ATP-binding domains. It has been demonstrated to be a promiscuous transporter of hydrophobic compounds which are usually uncharged or cationic (Hrycyna et al., 1999; Gottesman et al., 2002). ABCB1 protein is expressed in many tissues such as kidney, liver, intestine, brain capillary where it mediates the absorption, distribution and elimination of the given substrates (Leonard et al., 2003; Callaghan et al., 2008). In cancer cells, P-gp mediates the drug resistance to chemotherapy agents. The prototypical P-gp substrates include etoposide, doxorubicin, and vinblastine and paclitaxel (Taylor, 2002).

The family of Multidrug Resistance-associated Proteins (MRPs, ABCC) consists of 9 protein members, designated MRP1 through MRP9, corresponding to ABCC1 through ABCC6 and ABCC10 through ABCC12, respectively (Kuo, 2009). Most members of this family are able to transport both hydrophobic uncharged molecules and negatively charged organic molecules (Kruh and Belinsky, 2003) . Much like MDR1, beyond conferring drug resistance to cancer cells, MRPs also involve in membrane transport of various substances or ions, thereby exhibiting a wide spectrum of biological functions (Toyoda et al., 2008). Of the MRPs, MRP1 has been extensively studied. It is involved in the transport of natural organic anions such as glutathione and glucuronate conjugates as well as some anticancer drugs. MRP2 has partial substrate overlap with MRP1 and is primarily involved in the transport of conjugated metabolites into the bile canaliculus in the liver. Rats with impaired MRP2 expression show defects in hepatobiliary extrusion of bilirubin glucuronide and develop Dubin–Johnson syndrome (Kartenbeck et al., 1996; Toyoda et al., 2008). Structurally, MRPs can be divided into two major groups: (1) MRP4, MRP5, MRP7 and MRP8 which has typical ABC transporter structure like MDR1, and (2) MRP1, MRP2, MRP, MRP6 and MRP7 which has an additional TMD (Toyoda et al., 2008).

1.2. Breast Cancer Resistance Protein (BCRP)

1.2.1 Nomenclature and Structure of BCRP

Coded by gene ABCG2, BCRP is made of 655 amino acids with a molecular weight of 72kDa. BCRP is claimed to be a half transporter in that it has only one TMD and

one ATP-binding domain (Doyle et al., 1998; Allen and Schinkel, 2002). Recent studies on ABC-transporters have demonstrated that two functional ATP-binding domains are required to power the substrate translocation, thus it is believed that BCRP must at least dimerize to be functionally active (Smith et al., 2002). This theory has been supported by several research groups. It was suggested by Kage et al. that BCRP forms homodimers linked by a disulfide bond (Kage et al., 2002). More recently it was reported that BCRP can form an oligmer and residues 528 or 655 between the 5th and 6th transmembrane segment are responsible for the homooligomerization of the protein (Kage et al., 2005; McDevitt et al., 2006).

1.2.2 Tissue distribution and function

BCRP is present in many human tissues and acts as an important physiological barrier against xenobiotics. By northern blot, the highest levels of BCRP were found in placenta and lower levels in the blood-brain barrier, prostate, small intestine, testis, liver, adrenal gland and stem cells (Doyle et al., 1998; Fetsch et al., 2006). Studies using monoclonal antibodies against BCRP have provided localization information in different tissues. In placenta, BCRP is expressed on the apical surface of the chorionic villus in the placenta syncytiotrophoblast, suggesting a protection role of BCRP in the maternal-fetal barrier (Jonker et al., 2000). In the GI tract organs, it is expressed on apical membrane of the small intestine epithelium and canalicular membranes of liver, suggesting a potential role of BCRP in oral absorption and biliary excretion (Maliepaard et al., 2001) (Jonker et al., 2000). BCRP is abundantly

expressed in various stem cells (hematopoietic, muscle, neural and testicular) (Zhou et al., 2001). As stem cells derived from BCRP knockout mice were less resistant to the chemotherapy agent mitoxantrone, it has been postulated that BCRP may play a protective role for stem cells (Zhou et al., 2002).

1.2.3 Substrates and inhibitors

BCRP exhibits transport activity for both exogenous and endogenous compounds. The endogenous substrates include folic acid, sulfated conjugates of steroids, bile salts, and porphyrins (Jonker et al., 2002; Chen et al., 2003; Suzuki et al., 2003; Yang et al., 2003; Janvilisri et al., 2005).Since BCRP was discovered from anticancer drug-resistant cell line, the first reported exogenous substrates of BCRP were predominantly chemotherapeutic agents such as mitoxantrone, flavopiridol, topotecan, irinotecan and its active metabolite SN-38 (Robey et al., 2001). Later, a large of number of drugs from various therapeutic categories, including antibiotics, anti-hypertensive, and antidiabetics, have been identified as BCRP substrates. The list of representative substrates of BCRP is shown in **Table 1-1**.

Pharmacological inhibitors are useful to modulate transporter-mediated efflux. The first reported BCRP inhibitor was fungal toxin fumitremorgin C (FTC). As a potent BCRP inhibitor, it can completely reverse the mitoxantrone and topotican-resistance in BCRP overexpressing cell line at 1 to 5 μM concentration (Rabindran et al., 2000).

However, the inhibitory effects of FTC have not been assessed clinically due to its neurotoxicity concerns. As a structure analog of FTC, Ko143 was later-developed and has been demonstrated to be a specific and very potent BCRP inhibitor. Importantly, Ko143 is nontoxic at effective *in vitro* and *in vivo* concentrations, which makes it one of the most promising compounds as a clinical modulator of BCRP-mediated efflux (Allen and Schinkel, 2002). Another interesting group of BCRP inhibitors are flavonoids. The potent inhibitors include chrysin and acacetin, which can inhibit BCRP function in micromolar concentrations (Imai et al., 2004). As flavonoids are widely present in food, BCRP inhibitors derived from flavonoids should have the advantage of low toxicity. Some P-gp inhibitors such as elacridar (GF120918) have also been reported to inhibit BCRP activity (de Bruin M, 1999). As BCRP and P-gp often have overlapping substrates, simultaneous inhibition of both efflux transporters make elacridar a very promising modulator in the clinical use in reducing drug resistance.

1.2.4 Structure and function study of BCRP

Structure and function analysis of BCRP can provide better understanding of the molecular mechanism behind clinical drug resistance. However, to date, there is limited information regarding to the structural features of BCRP and how these relate to substrate recognition. Amino acid at position 482 has been reported as a hot spot for substrate specificity. Two BCRP mutants, which have arginine at 482 substituted by threonine or glycine, lose the transport activity for methotrexate, folic acid,

topotecan and SN-38, but acquire the ability to efflux transport of rhodamine, and doxorubicin (Honjo et al., 2001; Allen et al., 2002a). Several studies have been conducted to characterize the mechanism of altered substrate specificity due to the mutation at 482. Using site-directed mutagenesis method, Laczka‘s group generated nine Arg-482 mutants (G, I, M, S, T, D, N, K, Y) of BCRP. All BCRP variants showed cell surface expression, suggesting that Arg-482 is not crucial for protein expression and trafficking of ABCG2 to the plasma membrane (Ozvegy-Laczka et al., 2005; Ejendal et al., 2006). Since Arg-482 is assumed to be located in the beginning of the third transmembrane region, it is feasible that amino acid 482 involved in the substrate-binding pocket interface of BCRP (Ejendal et al., 2006; Li et al., 2007). As arginine is positively charged under physiological condition, and mutation to an uncharged amino acid (glycine or threonine) results in an enhanced transport for mildly positive substrates and a loss of, or reduced, affinity for negatively charged substrate, it is tempting to speculate that Arg-482 is involved in direct substrate binding via electrostatic interaction (Chen et al., 2003). This possibility was argued against by Ejendal and Pozza et al. who reported that the role of Arg-482 mutations on substrate transport may be due to the influence on ATP hydrolysis, but not by changes in direct ligand binding (Ejendal et al., 2006; Pozza et al., 2006). Further studies are needed to understand the mechanisms of this effect.

1.2.5 Single nucleotide polymorphisms (SNPs)

Sequencing of the ABCG2 gene from human samples has revealed more than 80 different, naturally occurring sequence variations (Tamura et al., 2006). Of these, C421A (Q141K), in which the amino acid at 141 changes from lysine to glutamine, was most extensively studied. C421A is predominantly found in Asian population (Honjo et al., 2002; Iida et al., 2002; Kim et al., 2008). *In vitro* studies have indicated that C421A polymorphisms may associate with low BCRP expression, resulting in reduced transport activity for anticancer drugs such as mitoxantrone, topotecan and SN-38 (Imai et al., 2002). The clinical outcome of C421A polymorphism is somewhat contradictory. Altered pharmacokinetics of topotecan, irinotecan, rosuvastatin were observed for C421A carrier (Sparreboom et al., 2005; Zhou et al., 2005; Zhang et al., 2006). However, for some other BCRP substrates such as imatinib, doxorubicin and pitavastatin, no statistically significant difference was observed for patients carrying C421A polymorphism compared with patients carrying the wild-type sequence (Gardner et al., 2006; Ieiri et al., 2007; Lal et al., 2008).

1.2.6 Role of BCRP at the Blood-Brain Barrier (BBB)

Brief description of the BBB and BCSFB

Despite aggressive therapy, the majority of primary and metastatic brain tumor patients have a poor prognosis with brief survival periods. In general, the poor response of CNS tumors to chemotherapy drugs is multifactorial, but the inability to deliver therapeutic agents to the CNS across the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) is certainly a well-known mechanism (Drewes,

2001; Taylor, 2002). BBB is in large part formed by a single layer of brain capillary endothelial cells fused by tight junctions (Pardridge, 2002; Ballabh et al., 2004). The tight junctions, together with the absence of brain endothelial fenestra and limited endothelial vesicular transport, creates a high electrical resistance (2000-8000 $\Omega.\ cm^2$) interface that forces most solutes to transit the brain capillaries either by passive transcellular diffusion or being transported across by various energy dependent or facilitated systems (Butt and Jones, 1992; Smith, 2003). In addition, the vessel wall is surrounded by astrocytic end feet and by pericytes, which further reinforce the barrier function of BBB (Pardridge, 1998).

Over the past decade, traditional thinking on the nature of BBB has changed substantially. It has become increasingly clear that barrier is not just a passive, anatomical lipid phase membrane, but a dynamic interface containing metabolic and functional (transporter) components. Solutes crossing the cell membrane are subsequently exposed to degrading enzymes present in endothelial cells that contain relative high mitochondrial content, metabolically highly active organelles (el-Bacha and Minn, 1999). In addition, the BBB has a high concentration of uptake and efflux drug transporters in the luminal or abluminal membranes of the cerebral capillary endothelium, including organic anion-transporting polypeptide 1A2 (OATP1A2 / SLCO1A2), organic anion transporter 3 (OAT3 / SCL22A8), monocarboxylate transporter 1 (MCT1 / SLC16A1), P-gp (MDR1 /ABCB1), MRP4 (ABCC4) and BCRP (ABCG2) (Urquhart and Kim, 2009a). These transporters can carry drug

molecules in and out of the brain through the capillary endothelial cells, thereby playing an important role in determining the CNS drug exposure and subsequently the efficacy and toxicity of these drugs in the CNS.

Blood-cerebrospinal fluid barrier (BCSFB) consists of choroid plexus and the arachnoid membrane with the former receiving more attention (Johanson et al., 2005). The micro-structure of the BCSFB at the choroid plexus is a monolayer of polarized epithelial cells where high-resistance intercellular junctions form a dynamic interface between two circulating fluids: the choroidal blood and ventricular CSF. However, compared with the BBB, this transepithelial resistance is relatively lower, and consequently hydrophilic drugs may gain access to the CSF but only to a limited extent. Similar to brain endothelial cells, the choroid plexus has a high capacity for drug metabolism and active transporters that substantially affect drug penetration as the drug substrate may be actively pumped into or out of the CSF (Allen and Smith, 2001). A review written by Ito et al. summarized that OATP2 (SLC21A6), OAT3, MRP1 and MRP4 are oriented at the basolateral side of BCSFB, whereas OATP2, organic cation transporter 2 (OCT2, SLC22A2), peptide cotransporter 2 (PEPT2, SLC15A2) and P-gp are at the apical side of the BCSFB (Ito et al., 2005).

BCRP at the BBB

In addition to P-gp and some of the MRPs, BCRP has been found to be expressed at the BBB. High-level expression of BCRP has been detected in brain capillary

endothelial cells of pigs, mice, and humans (Cooray et al., 2002; Eisenblatter et al., 2003; Cisternino et al., 2004). Further confocal microscopic analysis demonstrated BCRP is localized at the luminal surface of the endothelial cells of human brain microvessels (Cooray et al., 2002). This localization closely resembles that of P-gp at the blood-brain barrier, and it is speculated that BCRP may have a similar functional role as P-gp in protecting the brain parenchyma from the exposure to therapeutic agents. The role of BCRP in limiting the brain uptake of its substrates is not completely known and its importance at the BBB needs to be thoroughly evaluated.

1.3 Antifolates

1.3.1 General introduction of antifolates

Folic acid is an essential vitamin to maintain body function. Acting as one carbon carrier, folate is required for methylation reactions, nucleic acid synthesis, and the cell proliferation (Appling, 1991). Thus, by inhibiting the folate dependent enzyme and subsequently hindering DNA, RNA synthesis and cell division, the antifolate drug have found widespread clinical use as anti-proliferative and anti-microbial agents. Given this working mechanisms of antifolates, they have a greater toxic effect on rapidly dividing cells (such as malignant and myeloid cells, and GI & oral mucosa) and have proved successful in various oncologic disorders(Gangjee et al., 2007).

The first antifolate of significance was aminopterin, a 4-amino analog of folic acid. It acts by inhibition of folate dependent enzyme dihydrofolate reductase (DHFR) and

has been used for the treatment of childhood leukemia. However, for toxcitiy reasons, aminopterin was soon replaced by its 10-methyl congener, methotrexate. Methotrexate (MTX, amethopterin) is a 4-amino-10-methyl substituted analog of folic acid. MTX has the identical mechanism of action to aminopterin, i.e., exerting pharmacological activity by competitively inhibiting the target enzyme DHFR (Kamen, 1997). Over the last 50 years, MTX has been widely used in the treatment of multiple malignancies, including lymphoma, choriocarcinoma, childhood acute lymphoblastic leukemia (ALL), gastric, bladder, head and neck cancers. In addition, MTX is one of the most effective and commonly used medicines to treat various forms of arthritis and other rheumatic conditions.

Despite the widespread application of MTX, the intrinsic or acquired resistance to MTX has been often observed from clinical practice. The mechanisms of resistance include: 1) decreased potency due to mutation or up-regulation of target enzyme DHFR (Goker et al., 1995), 2) decreased cellular concentration due to the change in the expression of influx or efflux transporters (Assaraf, 1993; Assaraf, 2007), and 3) Decreased accumulation of active cellular metabolites and shorter cell retention time due to the mutation of polyglumation enzyme folypolyglutamate synthase (FPGS) (Waltham et al., 1997). Due to the limitation of these early antifolates, especially the development of drug resistance, a significant effort has been made to develop more effective antifolates. To date, more than 10 antifolates have been investigated extensively and entered clinical trials, with the pharmacological features summarized

in **Table 1-2**. The novel antifolates were designed to overcome the drug resistance via different mechanisms. For example, unlike the classic antifolates which are hydrophilic and rely on the uptake transporters to get into cells, some novel antifolate such as trimetrexate and nolatrexed are lipophilic with the activity independent of the polyglutamation within cells. As such, they can overcome the drug resistance associated with the alteration in uptake transporters and polyglutamation enzymes.

1.3.2 Mechanism of action for pemetrexed

Pemetrexed (PMX, Alimta®) is a structurally novel analog of the classic antifolate, originally described by Taylor and developed by Eli Lilly as multi-targeted antifolate (Taylor et al., 1992). It has a pyrrole ring that replaces the pyrazine ring in the pterine portion of folic acid and a methylene group that replaces the benzylic nitrogen in the bridge portion of folic acid (Paz-Ares et al., 2003). PMX has demonstrated antitumor activity in various tumor types including breast, cervical, colorectal, head and neck, and bladder cancers as a single agent and in combination with other chemotherapeutic agents (Adjei, 2000; Pivot et al., 2001; Martin et al., 2003; Rollins and Lindley, 2005). In 2004, pemetrexed was approved by FDA for the treatment in malignant pleural mesothelioma, a type of tumor of the lining of the lung, in combination with cisplatin. In 2008, the FDA granted approval as a first-line treatment, in combination with cisplatin, against of locally-advanced and metastatic non-small cell lung cancer (NSCLC), in patients with non-squamous histology.

Unlike the classic antifolate drug methotrexate, which selectively targets a single enzyme, pemetrexed exerts its effect by inhibiting multiple enzymes in the folate cascade. PMX primarily inhibits thymidylate synthase (TS), and to a lesser extent, glycinamide ribonucleotide formyltransferase (GARFT), dihydrofolate reductase (DHFR) and aminoimidazole carboxamide ribonucleotide formyl transferase (AICARFT) (Goldman and Matherly, 1985; Taylor et al., 1992; Rustum et al., 1997; Shih et al., 1997). It is conceivable that such a combinatorial effect of inhibiting three enzymes at multiple sites would provide an advantage in overcoming the drug resistance due to overexpression or mutation of one single enzyme (Schultz et al., 1999).

1.3.3 Cellular transport of pemetrexed

PMX is a hydrophilic molecule, carrying two negative charges from the glutamate moiety of the molecule at physiological pH. Therefore, the magnitude of passive diffusion is very limited. As the structural analogue of folic acid, PMX utilizes the folate influx transporters to gain entry into the cell. The affinities of PMX for these transporters are summarized in **Table 1-3**, and are compared with that of folic acid and MTX.

Reduced folate carrier (RFC)

RFC was the first folate uptake transporter defined and characterized in mammalian cells in late 1960s. Broadly expressed in both normal and malignant tissues, RFC has

been long known to play a major role in the uptake of reduced folate cofactors and many antifolates (Goldman et al., 1968; Westerhof et al., 1995; Whetstine et al., 2002). RFC mediates the uphill transport of folate and hydrophilic antifolates by binding and exchanging organic phosphates such as adenine nucleotides (Yang et al., 1984), therefore RFC is considered to be a low capacity transporter compared to ABC transporters which translocate the substrate via ATP hydrolysis. RFC exhibits a high affinity *(Km,*1-10 μM) for natural reduced folate cofactors and antifolates such as MTX (Sirotnak, 1985). PMX is also a good substrate of RFC with an affinity about 1 μM (Wang et al., 2004b). A characteristic feature of the RFC is its poor affinity for folic acid, 1-2 orders of magnitude less than the affinity for reduced folate.

Folate receptor (FR)

A second route of folate influx transport is via folate receptor (FR, membrane folate binding protein). FR is a membrane protein which mediates transport by a receptor-mediated endocytosis. FR is coded by three distinct genes known as FRα, FR_{β} and FRγ. Among them, FRγ is a secreted protein, thus only FRα and FRβ are involved in folate transport. Unlike the family of RFC, which are ubiquitously expressed in tissues and tumors, FRs exhibits a relatively narrow and selective tissue expression profile. FRs display a high affinity for PMX (~ 1nM), with more than 3 orders of magnitude greater than RFC. However, due to the complex process for endocytosis, the maximum rate of transport into cells mediated by FR is one hundredth the rate mediated by RFC (Mineo and Anderson, 2001). Therefore, in presence of RFC, the

contribution from FR is considered to be minor. However, FR may play an important role in delivery of PMX when the function of RFC is impaired due to the acquired drug resistance or in certain types of tumors where the expression of FR is highly elevated (Spinella et al., 1995; Chattopadhyay et al., 2007).

Proton-coupled folate transporter (PCFT, low pH folate transporter)

The gene of the PCFT transporter was cloned and termed as proton-coupled folate transporter (PCFT, SLC46A1) in 2006 (Qiu et al., 2006). It has high affinity for both folate and antifolate, and functions optimally at acidic pH (5.5). PCFT has been shown to be involved in the intestinal absorption of folate and is mutated in individuals with hereditary folate malabsorption (Qiu et al., 2006). The affinity of PMX for this transporter is much higher at acidic pH relative to physiological pH (Zhao et al., 2008). As there is a low pH in the core of human solid tumors, this transport activity of PCFT is of considerable interest with respect to the therapeutic effect of PMX, in that it provides routes for the preferential delivery of PMX to tumor cells (Zhao et al., 2004a). In addition, studies suggested that the PCFT-mediated transport of PMX represent an important route of PMX tumor delivery when RFC activity is impaired or lost (Chattopadhyay et al., 2007).

Other possible influx transporters

Some organic anion transporters, such as the organic anion-transporting polypeptide (OATP, SLC210) and organic anion transporter (OAT, SLC22), have been reported

to transport folate and antifolate (VanWert and Sweet, 2008). OAT3 and OAT4 have been reported to transport folate and MTX with affinities comparable to that of RFC (Takeda et al., 2002; Matherly and Goldman, 2003). Therefore, these organic anion transporters likely play an important role in the transport of folate and antifolates.

Multidrug resistance-associated proteins (MRPs)

MRPs are a family of ATP-dependent transporters that export a broad range of neutral as well as anionic compounds out of the cell. Among the nine members of this family (MRP1~MRP9) which have been identified to date, at least 5 MRP proteins have been reported to transport folate and antifolate, including MTX (Bakos et al., 2000; Zeng et al., 2001; Chen et al., 2002; Wielinga et al., 2005). Using MRP overexpressing membrane vesicles, the transport kinetic properties of various MRPs for folate and MTX have been determined (Table 5). Despite the fact that the affinities of the MRPs for folate and antifolates are orders of magnitude lower than that of RFC, MRPs produce a substantial depression of concentrative transport of folate and antifolates due to the high transport capacity relative to that of RFC (Jedlitschky et al., 2006; Assaraf, 2007). The cellular transport of PMX is also mediated by MRP. In particular, PMX is a substrate of MRP2 and MRP5 with comparable efficacy to MTX (Pratt et al., 2002). One interesting phenomenon about MRP-mediated drug resistance is that a significant level of drug resistance to hydrophilic antifolates was observed only when the duration of exposure was brief, and the resistance did not persistent after long drug exposure (>24hours) (Hooijberg

et al., 1999; Wielinga et al., 2005). Later studies have illustrated that this is a polyglutamation-based phenomenon and the possible mechanisms will be discussed in section 1.3.4.

Possible efflux transporter: Breast cancer resistance protein (BCRP)

BCRP has been shown to confer resistance to a variety of chemotherapeutic agents. MTX is a prototypical substrate of BCRP (Volk and Schneider, 2003; Ifergan et al., 2004; Ifergan et al., 2005; Rhee and Schneider, 2005). More recently, it was found that folate and other antifolates including tomudex and GW 1843 are also substrates of BCRP (Volk and Schneider, 2003; Shafran et al., 2005; Bram et al., 2006). Given the structural similarity, it is possible that BCRP will also mediate the efflux transport of PMX. The interaction of PMX with BCRP will be examined in this project by using *in vitro* and *in vivo* methods.

1.3.4 Intracellular metabolism of pemetrexed

Polyglutamation is a unique property of the classic antifolates. Via this reaction, the antifolate is converted to its polyglutamated form by addition of glutamic acid residues catalyzed by folylpolyglutamate synthetase (FPGS). PMX is an excellent substrate for FPGS with two orders of magnitude greater affinity than MTX (Habeck et al., 1995). The polyglutamation has two important consequences. First, these polyglutamate derivatives have shown a much higher affinity for the target enzyme, for example, the pentaglutamate form of PMX (predominant intracellular form) is 60-

fold more potent in its inhibition of the primary target TS than is the monoglutamate form (Shih et al., 1997). Secondly, it results in a prolonged drug action in that polyglutamates are better retained in the cell (Jolivet et al., 1982). One reason for prolonged cellular retention may be the loss of affinity of these polyglutamtate derivatives for the efflux transporters. For example, for MTX, the addition of two glutamate residues from intracellular polyglutamation results in the loss of affinity with MRP2 and MRP5 (Chen et al., 2002) and the addition of a third glutamate residue leads to loss of affinity with BCRP (Volk and Schneider, 2003).

1.4 CNS delivery of antifolates

1.4.1 CNS delivery of methotrexate

The antifolate agents, in particular MTX, have been widely used for the treatment of primary and secondary CNS tumors. However, delivery of MTX to the brain has been a major challenge for its effective use in CNS malignancies. To date, tremendous efforts have been made to increase the brain penetration of MTX, including the use of intrathecal injection (Bleyer et al., 1997), direct intraventricular infusion via Ommaya reservoirs (Stone et al., 1999) and intra-arterial administration combined with osmotic disruption of BBB (McAllister et al., 2000; Kraemer et al., 2001). Intrathecal delivery usually leads to high lumber drug concentration, low ventricular (CSF) concentration and sometimes causes series of adverse effects such as progressive paraplegia, anemia, Balint's syndrome and long-term cerebral metabolite changes (Kerr et al., 2001). Direct intraventricular administration of MTX provides higher and more consistent

ventricular CSF MTX concentrations than does intrathecal dosing. However, unpredictable spatial distribution and variation in drug concentration and resulting neurotoxicity has limited the broad application of this method (Kerr et al., 2001). As the capacity of MTX to enter the brain extracellular space from the CSF is limited, both routes of administration mentioned above are ideal for situations in which the target is within the subarachnoid space such as carcinomatous meningitis, and are inefficient for intraparenchyma brain tumors (Groothuis, 2000). For osmotic disruption of BBB, increased brain penetration of MTX has been observed, but efficacy and improved survival still need to be confirmed (Siegal et al., 1997; Zylber-Katz et al., 2000).

Besides the clinical studies mentioned above, there are also several preclinical studies which shed light on potential therapeutic strategies to improve delivery and efficacy of MTX against CNS. Wang et al. reported that intranasal delivery of MTX may be an appealing administration option to achieve adequate concentration in the CSF. The *in vivo* studies using Sprague Dawley rats showed that the ratio of the AUC_{CSF} value between the intranasal route and the intravenous injection was 13.76, and the drug targeting index (DTI) of nasal route was 21.7 (Wang et al., 2003a). Dukic et al. examined the influence of dosing regimen on MTX brain penetration using Wistar rats implanted with C6 Glioma cell lines. The results suggested that AUC values for brain tumor ECF was approximately 5-fold higher and the mean MTX penetration in tumor ECF (AUC_{ECF} /AUC_{Plasma}) was approximately 3-fold

higher after i.v. bolus (over 2 minutes) than after i.v. infusion (over 4 hours) administration. The investigators concluded that i.v. bolus administration schedules promote MTX delivery in brain tumor tissue (Dukic et al., 2000).

The drug distribution into the brain is dependent upon a variety of factors: (i) physicochemical properties of the drug, such as molecular weight and lipophilicity; (ii) the transport by influx and efflux transporters at the BBB and BCSFB; (iii) protein binding in the blood; and (iv) physiological variables such as blood flow and membrane integrity (Motl et al., 2006).It is believed that only small molecules (400~600 Da) with high lipophilicity can cross the membrane barrier to reach a pharmacological active concentration in the brain (Pardridge, 1997). In addition, the polar surface area and the number of hydrogen bonds on the drug molecule also influence the CNS permeability (Lipinski, 2000). The marketed CNS drugs on average have logP around 2.5, polar surface area at 60–70 $Å^2$ through 90 $Å^2$ and on average 2.12 H-bond acceptors and 1.5 H-bond donors (Pajouhesh and Lenz, 2005). MTX has a logP around -1.85, a polar surface area of 211 $Å^2$, 12 H-bond acceptors and 5 H-bond donors (information obtained from Drugbank, Pubchem). Clearly, the low membrane permeability due to hydrophilic nature of MTX can be a reason for the limited brain distribution. Besides tight junctions which restrict the entry of polar molecules from entering the brain, reduced drug delivery to the CNS could also be due to the expression of efflux transporters at the BBB and BCSFB. Studies using recently developed gene knockout animal models have provided compelling

evidence supporting the important role of active efflux transporters in limiting the CNS penetration of the substrate molecules (Loscher and Potschka, 2005c; de Vries et al., 2007; Kim et al., 2008; Chen et al., 2009a). Of the known efflux transporters at the BBB and BCSFB, MRPs (MRP1-5), BCRP and OAT3 have been demonstrated to transport MTX (Volk et al., 2002; Assaraf, 2007; VanWert and Sweet, 2008). It has been suggested that active transport systems at BCSFB are involved in governing MTX efflux from the CSF and that they can be inhibited by probenecid and acetazolamide (Bode et al., 1980). More studies are needed to characterize the role of these efflux transporters on brain distribution of MTX and other antifolate compounds.

1.4.2 CNS delivery of pemetrexed

Given the wide use of MTX in the treatment of primary CNS lymphoma and secondary CNS tumor, the novel antifolate PMX is currently being evaluated for anti-tumor activity for a variety of CNS malignancies. It can be expected that PMX may be eventually applied for the treatment of CNS tumors with added benefits of increased potency and less resistance (Kuo and Recht, 2006). However, studies have shown that similar to MTX, PMX also exhibited limited brain penetration. Stapleton reported that CSF penetration of PMX in non-human primate was less than 2% (range 0.33-1.58%) (Stapleton et al., 2006). In our laboratory, Dr. Dai examined the CNS distribution of PMX in the rat model. In the intracerebral microdialysis studies, the mean area under concentration-time curve $(AUC)_{brain}$

$/AUC_{plasma}$ ratio of unbound PMX was 0.078 ± 0.038 in the i.v. bolus study and steady-state brain-to-plasma unbound concentration ratio after i.v. infusion was 0.106 ± 0.054 (Dai et al., 2005). In this report, it was also found that the CNS distribution of PMX was not influenced by concomitant high does indomethacin treatment, indicating that various transporters inhibited by indomethacin (some of the MRP transporters) may not be critical in limiting the CNS distribution of PMX (Dai et al., 2005). This further stimulated our study of the influence of BCRP on PMX CNS delivery.

1.5 Methods to study drug transport mechanisms in CNS

1.5.1 Membrane vesicles

By using vesicles derived from transfected cells, the membrane vesicle technique has been used to characterize the efflux transport mediated by various transporters such as MRP, P-gp and BCRP (Ding et al., 1999; Wheeler et al., 2000; Kohler and Stein, 2003; Volk and Schneider, 2003). In this method, inside-out membrane vesicles generated from wild type or transfected cells are incubated with a compound of interest, and at predetermined time points, the amount of a substance accumulated in the vesicles is measured. This assay has a major limitation for hydrophobic substrates due to significant nonspecific binding and rapid leakage of the compounds from the vesicles (Peterson and Hawkins, 2003). Another disadvantage is that membrane transporter activities are usually measured in the

absence of regulatory factors and may not be reflective of the actual *in vivo* fluxes (Glazier and Sibley, 2006).

However, compared with using intact cells, membrane vesicles offer two significant advantages for the study of PMX transport. First, it can circumvent the interference from metabolic processes which is particularly suitable for PMX due to extensive intracellular metabolism. Second, due to the hydrophilic nature of the compound, although there are active influx transporters involved, the intracellular accumulation of PMX is limited, and when there is active efflux transport involved, the accumulation is even smaller which requires either increased concentration of stock solution or a more sensitive analysis method. However, when using membrane vesicles, the hydrophilic nature of PMX becomes a positive characteristic due to the inside-out orientation of the efflux transporter in the vesicles. The vesicular uptake will be predominantly from active transport with little contribution from passive diffusion, and once inside the vesicles, there will be no rapid leakage of PMX from the vesicles by passive diffusion. This method has been proven invaluable in our studies of the interaction of PMX with BCRP.

1.5.2 Brain efflux index (BEI)

BEI, also referred to as intracerebral microinjection technique, is a novel technique to study mechanisms of brain-to-blood efflux transport at the BBB (Kakee et al., 1996). In the experiment, the radiolabeled test and reference compounds are introduced

directly into the brain by microinjection. At various time points following injection, animals are sacrificed and the amount of test/reference compound remaining in the brain is determined (Kakee et al., 1996). The BEI value is defined as the percentage of test drug effluxed at the BBB to test drug injected into the brain. To minimize inter-individual differences in the amount of test substrate injected, a BBB-impermeable reference compound is injected simultaneously into the brain cortex. Therefore the value 100-BEI (%), which represents the percentage of the test drug remaining in the ipsilateral cerebrum, was calculated as amount of test drug remaining in the brain divided by the amount of test drug injected into brain and normalized by the correspondent ratio of reference compound (Equation 1-1). The BBB efflux rate constant, $k_{eff,}$ can be obtained from the slope of the semilogarithmic plot of the value of (100-BEI) versus time.

$$100 - \text{BEI}(\%) = \left(\frac{\text{amount of drug in the brain} / \text{amount of reference in the brain}}{\text{amount of drug injected} / \text{amount of reference injected}} \right) * 100$$

(Equation 1-1)

Among all *in-vivo* methods, BEI is the most useful method for determining efflux clearance and clarifying the transport function in that it can isolate the efflux process directly, rather than considering efflux as modulator of brain uptake (Kitazawa et al., 1998) (Kusuhara et al., 1997). Therefore, this method enables one to directly characterize the kinetics of brain efflux transport of the test compound and reveal the possible efflux transport mechanisms at the BBB. This technique also allows for the inclusion of a specific transport inhibitor with the test substrate to access the drug–

drug interactions at the level of efflux transport (Kitazawa et al., 1998; Golden and Pollack, 2003). However, in order to validate the BEI method, several concerns need to be taken into consideration (Kakee et al., 1996). First, after intracerebral microinjection of substrates, there are several possible elimination pathways: 1) the brain-to-blood transport across the BBB, 2) leakage from the injection site to circulating blood via disturbed capillaries caused by microinjection, and 3) diffusion from the injection site to CSF bulk flow and subsequently undergoes the elimination by CSF turnover and /or efflux via choroid plexus. In order to make sure that the efflux transport is mainly from pathway 1 (BBB transport), the following criteria have been assessed: the reference compound is not eliminated from the brain for the period of time examined and apparent efflux clearance of test compound selectively reflects the efflux transport process across BBB, not via BCSFB. Given the information above, a specific region of the brain (Par2 in rat, S2 in mouse) is chosen as the site of injection where the recovery of reference marker was maximum in the cerebrum and miminum in the CSF (Kakee et al., 1996). Second, the BEI method cannot be used for substrates that are significantly metabolized in the cerebrum. Third, due to the small injectate volumes introduced, substrate solubility can be a limiting factor.

1.6 Research rational and objective

Given the wide use of MTX in the treatment of primary and secondary CNS tumors, the clinical use of PMX may eventually include the CNS malignancies. Previous studies in our laboratory using the microdialysis method have shown that PMX has a limited brain distribution, and the brain-to-plasma unbound concentration ratio at steady state is 0.106 ± 0.054. Many factors may affect the drug availability to the brain. Efflux transport systems at the BBB are an important factor that restricts the CNS exposure to the xenobiotics and toxic metabolites.

The overall objective of this study was to characterize the brain-to-blood efflux transport of PMX and to examine the role of efflux transporters such as BCRP, MRP2 and organic anion transporters, in brain distribution of PMX. Our hypothesis is that various drug efflux transporters at the BBB may limit CNS distribution of PMX and therefore, effective inhibition of these transporters may improve PMX targeted delivery to the CNS.

1.7 Specific aims

- Specific aim 1: characterize the interaction of PMX with BCRP by using an *in vitro* membrane vesicle model, and evaluate the contribution of BCRP to brain distribution of PMX, using an ABCG2-deficient mouse model. This specific aim was presented and fulfilled in **Chapter 2**.

- Specific aim 2: characterize the kinetics of brain efflux transport of antifolates (PMX and MTX), and examine the role of efflux transporters such as BCRP, MRP2 and organic anion transporters, in brain elimination of PMX and MTX, using *in vivo* brain efflux index method. This specific aim was presented and fulfilled in **Chapter 3**.

- Specific aim 3: examine the influence of pH on transport activity of BCRP for PMX and explore mechanisms of the apparent pH-dependency. This specific aim was presented and fulfilled in **Chapter 4**.

The completion of this study will provide useful information about specific mechanisms involved in limiting the brain penetration of PMX. This would help in formulating strategies to improve the delivery of PMX to the brain and lead to more successful treatment of primary and secondary CNS tumors.

Table 1-1. Representative substrates of ABCG2

	Reference
Anti-HIV	
Zidovudine (AZT)	(Wang et al., 2003b)
Lamivudine	(Wang et al., 2004a)
Abacavir	(Pan et al., 2007)
Anti-cancer	
Mitoxantrone	(Doyle and Ross, 2003)
Topotecan	(Ma et al., 1998)
Methotrexate	(Volk et al., 2002)
Doxorubicin	(Doyle et al., 1998)
Imatinib	(Burger et al., 2004)
Dasatinib	(Chen et al., 2009a)
Antibiotics	
Ciprofloxacin	(Merino et al., 2006)
Ofloxacin	(Merino et al., 2006)
Grepafloxacin	(Ando et al., 2007)
Statins	
Pitavastatin	(Hirano et al., 2005)
Rosuvastatin	(Huang et al., 2006)
Anti-diabetics	
Glyburide	(Zhou et al., 2008)
Anti-hypertensive	
Prazosin	(Litman et al., 2000)
Other molecules	
Estrone-3-sulfate	(Suzuki et al., 2003)
Hoechst 33342	(Scharenberg et al., 2002)
Pantoprazole	(Breedveld et al., 2004)

Table 1-2. Representative antifolates used in preclinical and clinical studies

Molecular class	Drug	Transport mechanism	Substrate for FPGS?
DHFR inhibitor	Methotrexate	RFC, folate receptor	Yes, K_m 166μM
	Trimetrexate	Lipophilic	No
	Piritrexim		No
	Edatrexate	RFC	Yes, K_m 30 μM
	PDX	RFC	Yes, K_m 6 μM
TS inhibitor	CB3717		Yes, K_m 40 μM
	Raltitrexed	RFC	Yes, K_m 1.3 μM
	Plevitrexed		No
	Nolatrexed	Lipophilic	No
Purine biosynthesis inhibitors	Lometrexol	RFC, folate receptor	Yes, K_m 16 μM
	LY309887	RFC, folate receptor	Yes, K_m 6.5 μM
	AG2034		Yes, K_m 6.4 μM
Multiple targets (DHFR, TS, GARFT, AICARFT)	Pemetrexed	RFC, folate receptor PCFT	Yes, K_m 0.8 μM

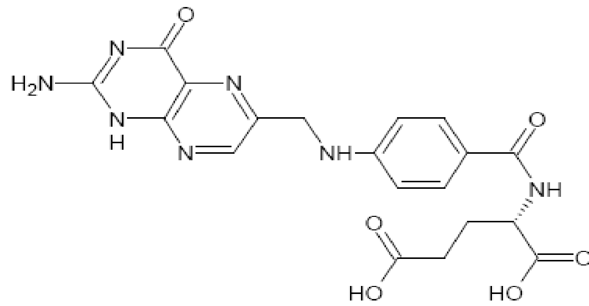
Table 1-3. Affinities of folic acid, MTX and PMX for folate influx transporters

	MTX (μM)		Folic acid (μM)		PMX (μM)	
RFC	0.0017	(Wang et al., 2004b)	0.142	(Wang et al., 2004b)	0.001	(Wang et al., 2004b)
FR	0.3	(Wang et al., 1992)	0.001	(Wang et al., 1992)	0.001	(Goldman and Zhao, 2002)
PCFT	131	(Qiu et al., 2006) (Zhao et al., 2004b)	56.2	(Qiu et al., 2006)	12.7	(Zhao et al., 2008)
PCFT (pH= 5.5)	3.4	(Qiu et al., 2006)	1.3	(Qiu et al., 2006)	0.8	(Zhao et al., 2008)

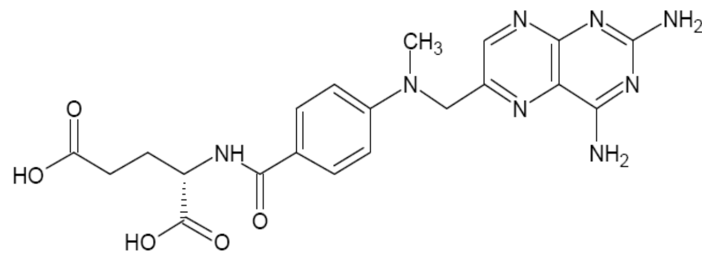
NOTE: Data represent either direct measurements with radiolabeled substrates (Kt) or the inhibitory effects ofsubstrates on influx of methotrexate (Ki).

Table 1-4. Summary of transport kinetic properties of MRPs for folic acid, MTX and PMX

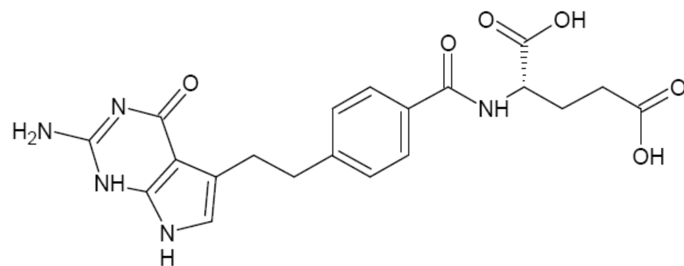
MRPs	MTX		Reference	Folic acid		Reference	PMX		Reference
	Km (μM)	Vmax (pmol/mg/min)		Km (μM)	Vmax (pmol/mg/min)		Km (μM)	Vmax (pmol/mg/min)	
MRP1	2150 ± 790	2050 ± 910	(Zeng et al., 2001)	N.D.[a]	N.D.	-	N.D.	N.D.	-
MRP2	2500-3000	~1000	(Bakos et al., 2000)	N.D.	N.D.	-	66	18	(Pratt S, 2002)
MRP3	620 ± 230	2930 ± 320	(Zeng et al., 2001)	1960 ± 50	1710± 50	(Zeng et al., 2001)	N.D.	N.D.	-
MRP4	220 ± 10	240 ± 50	(Chen et al., 2002)	170 ± 20	680 ±140	(Chen et al., 2002)	N.D.	N.D.	-
MRP5	1300 ± 300	780 ± 70	(Wielinga et al., 2005)	1000 ± 100	875 ± 75	(Wielinga et al., 2005)	387	50	(Pratt S, 2002)



Folic acid



methotrexate (MTX)



pemetrexed (PMX)

Figure 1-1 Chemical structures of folic acid, MTX and PMX

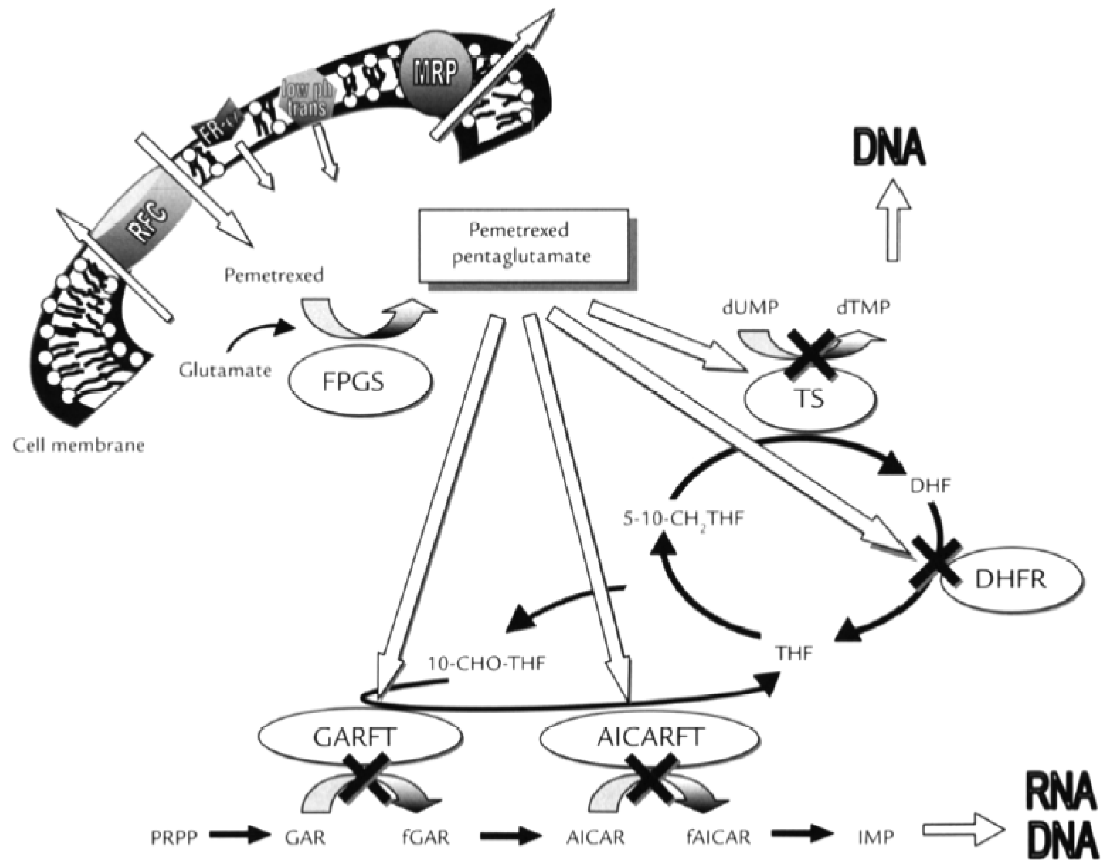


Figure 1-2 Mechanism of action for PMX (Adapted from (Rollins and Lindley, 2005)).

PMX gets in the cell by use of the folate transporters, including the reduced folate carrier (RFC), folate receptor-α (FR-α) and a low pH transporter (recently cloned and termed as proton-coupled folate transporter, PCFT). Once in the cell, PMX is rapidly metabolized to its polyglutamated form catalyzed by enzyme FPGS. The predominant form of intracellular metabolites of PMX is PMX-glu(5). Parent and cellular metabolites of PMX can simultaneously inhibit several folate-dependent enzymes including TS, DHFR, GARFT and AICARFT, preventing the formation of RNA and DNA synthesis. The cellular transport of PMX is also mediated by efflux transporter

MRPs, but it has been suggested that PMX is better retained in the cells once coverted to its polyglutamated forms in that these metabolites are no long the substrates for MRP mediated transport.

FPGS= folylpolyglutamate synthetase; TS = thymidylate synthase (TS); dTMP= deoxythymidine monophosphate; dUMP = deoxyuridine monophosphate; GARFT= glycinamide ribonucleotide formyltransferase; AICARFT= 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; THF = tetrahydrofolate; DHF = dihydrofolate; DHFR = DHF reducatase; PRPP = phosphoribosyl pyrophosphate; GAR = glycinamide ribonucleotide; fGAR = *N-formylglycinamide* ribonucleotide; AICAR = 5-aminoimidazole-4-carboxamine ribonucleotide; fAICAR = 5-formylaminoimidazole-4-carboxamide ribonucleotide; IMP = inosine monophosphate

CHAPTER 2

BCRP-MEDIATED ACTIVE EFFLUX TRANSPORT AND BRAIN DISTRIBUTION OF PEMETREXED

Pemetrexed (PMX, Alimta®) is a novel multi-targeted antifolate approved for the treatment of malignant pleural mesothelioma and non-small cell lung cancer (NSCLC). Given the high incidence of brain metastasis in NSCLC patients and wide use of another antifolate, methotrexate, in the primary CNS lymphoma, the clinical use of PMX may eventually include the treatment of CNS tumors. To understand if BCRP may mediate the distribution of PMX to the brain, we have examined the interaction of PMX with BCRP *in vitro* and *in vivo*. Accumulation in a membrane vesicle system showed BCRP can actively transport parent PMX, but intracellular metabolites conjugated with more than triglutamate residues were not transported. Induced mutations at arginine-482, an amino acid residue critical for activity in BCRP, resulted in loss of transport activity. *In vivo* brain distribution studies in wild-type mice showed that CNS distribution of PMX is limited, with a steady-state brain-to-plasma concentration ratio of 0.086 ± 0.032. No significant difference was found in brain-to-plasma ratios between wild-type mice and BCRP knockout mice, implying that the presence of BCRP alone may not limit PMX brain distribution. In conclusion, BCRP can actively in transport PMX and BCRP alone may play at most a minor role in effluxing PMX from brain. Other organic anion transporters may assume a more prominant role in CNS distribution of PMX. Our study underscores the fact that the relevant physiological function of one active drug transporter needs to be evaluated relative all other transporters present in the same tissue.

2.1 Introduction

Pemetrexed (PMX, Alimta®) is a novel multi-targeted antifolate used for the treatment of malignant pleural mesothelioma as well as non-small cell lung cancer (NSCLC). CNS metastases are a common complication of NSCLC, occurring in approximately 30% to 50% of patients (Taimur and Edelman, 2003). The clinical use of PMX may eventually be included for the treatment of metastatic CNS tumors. Several clinical trials have been launched to test the activity of PMX on brain metastases from NSCLC. Among them, Bearz et al have collected 39 patients with evidence of CNS localization from NSCLC and investigated the role of PMX on brain metastases. They reported an overall clinical benefit in 69% of patients, which is highly suggestive of activity of PMX on brain metastases (Bearz et al., 2009). In addition, methotrexate, another related antifolate compound, has been widely used in the treatment of primary CNS lymphoma, indicating that PMX may have potential for primary CNS tumor treatment as well (Hoang-Xuan et al., 2004; Apostolidou et al., 2007). Given the possible application in CNS tumors, it is of interest to determine the CNS distribution of PMX and characterize the possible mechanisms that may limit the penetration of PMX across the blood-brain barrier.

Previous studies in our laboratory using the microdialysis method have shown that the PMX steady-state brain extracellular fluid to unbound plasma concentration ratio after intravenous infusion is 0.106 ± 0.054, indicating that the brain distribution of PMX is limited. Kinetic analysis indicates that this limitation could be due to the presence of

an active efflux clearance process (Dai et al., 2005). There are several efflux transporters that are expressed at the brain capillary endothelial cells (blood-brain barrier), such as P-glycoprotein (P-gp / MDR1/ *ABCB1*), multidrug resistance-associated proteins (MRPs), and breast cancer resistance protein (BCRP/*ABCG2*) (Sun et al., 2003; Zhang et al., 2004; Loscher and Potschka, 2005a; Loscher and Potschka, 2005b). These efflux transporters can actively extrude xenobiotic compounds from brain to the circulating blood, thereby limiting the central nervous system (CNS) exposure to these compounds (Scherrmann, 2005).

BCRP is a member of the ATP-binding cassette family of transport proteins (Doyle et al., 1998). It mediates the transport of a wide variety of therapeutic agents such as mitoxantrone, topotecan, prazosin, pantoprazole, imatinib and dasatinib (Doyle et al., 1998; Litman et al., 2000; Burger et al., 2004; Chen et al., 2009b; Vlaming et al., 2009a). BCRP is also able to transport folate and various antifolates including methotrexate, raltitrexed (Tomudex), plevitrexed (GW 1843), and trimetrexate (Volk and Schneider, 2003; Shafran et al., 2005; Bram et al., 2006). It is therefore quite likely that BCRP will also transport PMX, thereby acting as a functional barrier for delivery of PMX to the brain, and possibly elucidate the mechanism that led to the microdialysis findings. In order to address the possible role of BCRP in brain distribution of PMX, we first studied the interaction of PMX with BCRP using an *in vitro* vesicular transport assay. Next we used a growth inhibition assay to examine how the presence and absence of BCRP on the membrane affects PMX sensitivity in

intact cells. Finally we studied the influence of active efflux by BCRP at the blood-brain barrier by comparing the brain distribution of PMX in wild-type *Bcrp1* (+/+) versus knockout *Bcrp1* (-/-) mice.

2.2 Material and methods

2.2.1 Chemicals

[^{3}H]-PMX, unlabeled PMX, tri-glutamated PMX and penta-glutamated PMX were provided by Eli Lilly and Company. [^{3}H]-Methotrexate was obtained from Moravek Biochemicals (Brea, CA). Unlabeled MTX was purchased from Sigma-Aldrich (St. Louis, MO). GF120918 (N-[4-[2-(6, 7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-l)ethyl]phenyl]-5-methoxy-9-oxo-10H-acridine-4-carboxamide) was a gift from GlaxoSmithKline (Research Triangle, NC). Ko143 (a fumitremorgin C analog) was kindly provided by Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) (Allen et al., 2002b). GF120918 and Ko143 were dissolved in DMSO and diluted to the desired concentration with assay buffer (25 mM $NaHCO_3$, 122 mM NaCl, 10 mM Glucose, 10 mM HEPES, 1.2 mM $MgSO_4$, 3 mM KCl, 1.4 mM $CaCl_2$, and 0.4 mM K_2HPO_4, pH 7.4). The final concentration of DMSO in all reaction solutions was less than 0.1%. All other chemicals used were HPLC or reagent grade.

2.2.2 Cell lines

HEK293 cells transfected with ABCG2-R482, ABCG2-R482G, ABCG2-R482T and plasmid vector (pc-DNA) were obtained from Dr. Susan E. Bates (National Cancer Institute, Bethesda, MD). Stable transfectants were maintained in Dulbecco's modified Eagle's medium (Mediatech, Inc., Herndon, VA) fortified with 10% heat-deactivated fetal bovine serum (SeraCare Life Sciences, Inc., Oceanside, CA), 100

U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO) at 37°C with 5% CO_2 under humidifying conditions. The medium for ABCG2 transfected cells was supplemented with geneticin (G418) at a concentration of 2 mg/ml to maintain positive selection pressure for BCRP expression.

Epithelial Madin-Darby Canine Kidney (MDCKII) cells were used in growth inhibition studies. Wild-type and Bcrp1-transfected cells were kindly provided by Dr. Alfred H. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Cells were cultured in DMEM media supplemented with 10% fetal bovine serum (SeraCare Life Sciences, Inc., Oceanside, CA), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Sigma-Aldrich, St. Louis, MO) and maintained at 37°C with 5% CO_2 under humidifying conditions.

2.2.3 Animals

Male *Bcrp1*(-/-) (Bcrp1 knockout) and wild-type (C57BL/6) mice were provided by Taconic Farms. Inc. (Germantown, NY) and were a gift from Eli Lilly and Company. Animals were maintained under temperature-controlled conditions with a 12-h light/dark cycle and were allowed food and water ad libitum. All 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 of the University of Minnesota (IACUC).

2.2.4 Membrane vesicle preparation

Vector controls or BCRP-transfected HEK293 cells were cultured in 500 cm^2 plates (Corning, Lowell, MA). Cells were rinsed twice with PBS and then scraped into PBS. After centrifugation at 1700 rpm at 4°C for 5 min, the cell pellet was diluted with hypotonic buffer containing 1 mM sodium bicarbonate at pH 8 and 1% EDTA free protease inhibitors. The cells were allowed to swell on ice for 5 minutes followed by vigorous shaking for 20 times. The resultant cell lysate was centrifuged at 1700 rpm at 4°C for 5 min to remove nuclei and unlysed cells. The supernatant (crude membrane fraction) was layered over 40% (w/v) sucrose solution and centrifuged at 25000 rpm at 4°C for 30 min. The turbid layer at the interface was collected and suspended in Tris/sucrose buffer (250 mM sucrose containing 50 mM Tris/HCl, pH 7.4), and centrifuged at 25000 rpm for 40 min. The membrane fraction was collected and resuspended in a small volume (150–250 µl) of Tris-sucrose buffer. The membrane vesicles were made by slowly passing the suspension through a 27 gauge needle 20 times. The vesicle solution was aliquoted into 100 µL portions and was frozen at -80 ºC. The amount of membrane vesicles was quantified by measuring membrane protein using the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL).

2.2.5 Vesicular uptake study

The vesicular uptake study was performed using the rapid filtration method. In brief, the vesicular uptake was started by addition of 20 µg inside-out membrane

vesicles (BCRP- expressing vesicles or pc-DNA vector control vesicles) to 20 µL Tris-HCl sucrose buffer containing [^{3}H]-PMX, 20 mmol/L $MgCl_2$, 4 mmol/L ATP, 10 mM phosphocreatine, 100 µg /mL creatine kinase. The pH of vesicular transport buffer was adjusted to 7.4 by adding aliquots of HCl (37% w/w) or 2 M NaOH. Reactions were carried out at 37 °C for 60 minutes and stopped by addition of 200 µL ice-cold Tris-HCl sucrose buffer (pH 7.4). Vesicles were separated from free drug by passing through 0.22 µm Durapore membrane filters using a 96 channel cell harvester (PerkinElmer, Boston, MA). The filters were washed three times with ice-cold Tris-HCl sucrose buffer and dried at room temperature overnight. Radioactivity associated with the membrane vesicles was measured by a liquid scintillation counter (TOPCOUNT, PerkinElmer, Boston, MA). ATP-dependent uptake was calculated by the difference in transport with or without the presence of ATP.

Inhibitor assays. [^{3}H]-PMX uptake into BCRP-expressing or control vesicles was measured in the presence or absence of BCRP inhibitors (200 nM Ko143 or 5 µM GF120918) using the vesicular uptake method described above.

Kinetic studies. [^{3}H]-PMX uptake into BCRP or control vesicles was measured after 15 minutes exposure to 0, 100, 1000, 2000, 4000 and 6000 µM unlabeled PMX. [^{3}H]-MTX uptake into BCRP or control vesicles was measured after 5 minutes exposure to 0, 50, 250, 500, 1500, 2250 and 3000 µM unlabeled MTX.

Kinetic parameters (K_m and V_{max} together with the corresponding values of standard deviation) were determined by fitting the Michaelis-Menten model to the data with nonlinear regression analysis using Sigma Plot (version 9.0.1, SYSTAT software).

Metabolite competition studies. Tri-glutamated or penta-glutamated PMX (500 μM) were added to stock solutions and the vesicular uptake was compared with control samples using methods described above.

2.2.6 Growth inhibition study

A growth inhibition assay (cytotoxicity study) was assessed by the MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation assay, Promega Corporation, Madison, WI, USA). Briefly, MDCKII wild-type and Bcrp1-transfected cells were seeded in 96-well plates at a density of 2,000 cells/well. After 24 hours, PMX was added to the medium with a broad range of concentrations (e.g., 0.001~10 μM) and the cells were further incubated with the drug in a humidified tissue culture chamber for 72 hours. For the 4-hour drug exposure experiment, the drug-containing medium was aspirated after 4 hours drug exposure, following which three careful washes at 10-minute intervals with drug free medium were done. Drug-free medium was added and cultures were incubated for 68 hours at 37 °C. At the end of incubation, 20 μl of CellTiter 96® Aqueous One Solution Reagent containing a tetrazolium compound (MTS) were added to each well and the cells were incubated for 2 hours at 37 C. The absorbance at 490 nm was then recorded in an ELISA microplate reader. Cell

viability was expressed as a percent of the viability of untreated cells. The inhibition curves were analyzed with sigmoidal dose-response model using Prism 4 (GraphPad Software Inc., San Diego, CA) giving EC_{50} values (the drug concentrations giving 50% maximal inhibitory effects) together with the corresponding values of standard deviation.

2.2.7 Determination of PMX concentration in plasma and brain homogenate

2.2.7.1 Long term intraperitoneal administration via osmotic minipump

To achieve a steady-state condition, an Alzet® 1003D osmotic minipump (Durect, Palo Alto, CA) was selected to release the drug continuously over 72 hours at a flow rate of 1 μL/h (25 mg/kg/day). Wild-type, and *Bcrp1*(–/–) mice (n = 5) were anesthetized with ketamine/xylazine (100/10 mg/kg i.p.). The pump was implanted into the peritoneal cavity of wild-type and *Bcrp1*(–/–) mice. The animals were euthanized after 72 hours by using a CO_2 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 plasma and whole brain samples were stored at -80°C until analysis by LC-MS/MS. In the infusion study, the ratio of the clearance into and efflux out of the brain can be defined from the brain-to-plasma ratio of steady state unbound concentration, i.e., $CL_{in} / CL_{out} = C_{ss, brain} / C_{ss,plasma}$.

2.2.7.2 LC-MS/MS Assay

Mouse plasma and brain samples were analyzed by LC-MS/MS. Whole brain samples were first homogenized in solution composed of water and ethanol (20:80) with a volume ratio of 1:3. Acetonitrile containing 20 ng/mL internal standard was mixed with 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) and solvent B containing acetonitrile, trifluoroacetic acid and 1 mM ammonium bicarbonate (2000:8:2, v:v). The injection volume was 10 μL. PMX 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.

2.2.8 Statistical Analysis

Statistical analysis was conducted using SigmaStat, version 3.1(Systat Software, Inc., Point Richmond, CA). Statistical comparisons between two groups were made by using two-sample t test at $p < 0.05$ significance level. If groups failed the normality test, then the nonparametric alternative of two-sample *t* test, the Mann-Whitney rank sum test was used.

2.3 Results

2.3.1 BCRP-mediated transport of pemetrexed

The ability of BCRP (ABCG2-R) to transport PMX was determined using the vesicular transport assay. BCRP functions in intact cells to efflux drugs and metabolites from the cytosol to the external environment. However, for many hydrophilic compounds, a membrane vesicle assay is typically used. We employed a vesicular system containing inside-out membrane vesicles as described under Methods. The inverted nature of these vesicle results in the membrane orientation of the BCRP transporter with its drug ligand site accessible on the external medium resulting in drug transport into the vesicle. Thus, uptake transport activity (net accumulation) detected in this assay indicates an efflux activity in the intact cell. The vesicular transport model was first validated with a known BCRP substrate, i.e., methotrexate (MTX) (Volk and Schneider, 2003). **Figure 2-1** shows the ATP-dependent transport of PMX by BCRP-expressing vesicles and wild-type vesicles (control vesicles). Compared with the uptake into control vesicles, a significantly higher amount of PMX was found in the BCRP-expressing vesicles, indicating BCRP is able to transport PMX. Osmotic sensitivity study is a classical approach used to examine whether the vesicular uptake is caused by drug import into the lumen of vesicle or due to nonspecific binding to the membrane surface (Horio et al., 1988; Lelong et al., 1992).The rational of this method is that high osmotic pressure shrinks membrane vesicles, thereby reducing the intravesicular space available for the substrate accumulation(Lelong et al., 1992). Under the same conditions, the non-

specific binding of the drug to the membrane is barely affected. Therefore, the osmotic sensitivity of the [^{3}H]-PMX accumulation in the membrane vesicles was examined to determine that the uptake of PMX was due to transport or binding. As shown in **Figure 2-2**, ATP-dependent uptake of PMX was measured at various osmolarity conditions. In the presence of ATP, the uptake decreased as osmolarity increased, suggesting that the uptake of PMX observed was, indeed, attributed to the transport into the lumen of the membrane vesicles, rather than to non-specific binding to the membranes. To further confirm that the active uptake into BCRP vesicles is a BCRP-mediated process, a selective BCRP inhibitor (Ko143) was used (**Figure 2-3A**). Ko143 (200 nM) significantly reduced the uptake of PMX in BCRP-expressing membrane vesicles, confirming BCRP involvement in PMX transport. An additional BCRP inhibitor, GF120918, was also examined. The uptake of PMX in BCRP membrane vesicles was significantly decreased in the presence 5 μM GF120918, again confirming that PMX is a substrate for BCRP-mediated transport (**Figure 2-3B**). To characterize BCRP transport kinetics of PMX, a concentration dependent study was performed. As shown in **Figure 2-4A**, the BCRP-mediated transport of PMX in the concentration range from 0 to 6 mM follows the Michaelis-Menten model and the kinetic parameters Km and Vmax were determined to be 1.52 ± 0.27 mM and 678 ± 40.68 pmol/ mg protein/ min, respectively. Compared with the classic antifolate methotrexate (MTX), which is also a substrate for BCRP (MTX: Km; 1.76 ± 0.48 mM, Vmax; 1840 ± 221 pmol/ mg protein/min), BCRP has similar affinity for PMX

as MTX (**Figure 2-4B**), but the capacity for PMX is less than that of methotrexate (in this cell model).

2.3.2 Effect of BCRP active site mutation on transport of pemetrexed

It is well established that amino acid position 482 is a polymorphic hot spot for BCRP activity that can be associated with altered substrate specificities and transport activities (Robey et al., 2003; Ozvegy-Laczka et al., 2005). Vesicular uptake studies showed that ATP-dependent uptake of PMX by mutant forms of BCRP (ABCG-G and ABCG-T) was indistinguishable from that by the vector-control vesicles (pc-DNA), indicating that the mutant forms of BCRP (ABCG-G and ABCG-T) did not transport pemetrexed to any significant extent **(Figure 2-5)**.

2.3.3 Effect of cellular metabolism on BCRP-mediated transport of pemetrexed

One important characteristic of the hydrophilic antifolates is that polyglutamation reactions within the cell leads to active metabolites. Through this intracellular metabolism, the antifolate is metabolized from the monoglutamated (parent) form into its polyglutamated forms that generally exhibit higher affinity for the target enzymes. As polyglutamation of antifolates has a great impact on cellular retention and drug potency (Schirch and Strong, 1989), it would be of great interest to know if BCRP is able to efflux these active metabolites. Towards this end, the ability of BCRP to transport polyglutamated derivatives of PMX was examined in the vesicular uptake assay via a competition method. We found that the presence of 500 μM

PMX-glu$_3$ or PMX-glu$_5$ did not produce any significant change in PMX uptake as compared to the control in each case, indicating that the polyglutamated metabolites were unable to competitively inhibit the BCRP-mediated uptake of PMX, and therefore BCRP may not be able to transport these cellular metabolites of PMX (**Figure 2-6**). Furthermore, from the growth inhibition study, after a short term (4 hours) exposure to PMX, the EC_{50} of BCRP-transfected cells (1006 ± 533 μM) was significantly higher than that in wild-type cells (16.76 ± 6.93 μM), suggesting that the apparent lack of efficacy of PMX in BCRP-transfected cells is due to PMX efflux by BCRP (**Figure 2-7A**). In contrast, after longer (72 hours) exposure times, a similar EC_{50} value was observed for BCRP transfected cells (0.23 ± 0.06 μM) as compared to wild-type cells (0.16 ± 0.02 μM) (**Figure 2-7B**), suggesting that the active cellular metabolites of PMX may not be effluxed by BCRP.

2.3.4 PMX brain distribution in mice

The brain distribution of PMX was determined in wild-type and *Bcrp1* (-/-) mice after 3-days of continuous intrapertioneal administration via an osmotic minipump. For wild-type mice, both brain and plasma concentrations at day 2 were not significantly different from the values at day 3, indicating that the steady-state condition in both brain and plasma was achieved by day 2. As shown in **Figure 2-8**, the steady-state PMX plasma concentrations at 3 days were 653 ± 356 and 1313 ± 406 ng/mL in wild-type and *Bcrp1*(-/-) mice, respectively. The steady-state PMX brain concentration was 46 ± 10 ng/mL in wild-type mice and 49 ± 13 ng/mL in *Bcrp1*(-/-) mice. The

steady-state PMX brain-to-plasma concentration ratio, a measure of the brain distribution, is 0.086 ± 0.032 in wild-type mice and was not significantly different from 0.0401 ± 0.017, the value found for BCRP-deficient mice.

2.4 Discussion

The interaction of PMX with BCRP was investigated using both *in vitro* and *in vivo* methods. The results from *in vitro* inside-out membrane vesicles showed that BCRP can actively transport PMX and the transport activity can be modulated by the BCRP inhibitors Ko143 and GF120918. The vesicular uptake of PMX by BCRP was osmotically sensitive and followed Michaelis-Menten kinetics. The K_m and V_{max} values of 1.52 ± 0.27 mM and 678 ± 40.68 pmol/mg protein/min, respectively, suggest that BCRP is a low-affinity, high-capacity transporter for PMX in this membrane vesicle system.

Numerous studies have shown that the mutation at amino acid 482 significantly changes substrate specificity and leads to altered drug resistance and transport capacity (Honjo et al., 2001; Allen et al., 2002a; Ozvegy et al., 2002; Chen et al., 2003; Miwa et al., 2003; Ozvegy-Laczka et al., 2005). In particular, BCRP variants R482G and R482T confer additional transport activity for rhodamine and anthracyclines, but lose affinity for methotrexate and folic acid (Doyle et al., 1998; Chen et al., 2003; Ozvegy-Laczka et al., 2005). In the present study, we have shown that the substitution of arginine by mutant forms (R482G, R482T) led to diminished transport for the antifolate PMX. Although BCRP mutations at amino acid 482 have not been found in clinical specimens (Lemos et al., 2008), the impact of a single amino acid mutation on the substrate profile implies that polymorphisms of the ABCG2 gene could be a factor in transport activity.

The propensity of antifolates to undergo intracellular polyglutamation is critical to their therapeutic activity. This is because the polyglutamate derivatives normally have a much higher affinity for the target enzyme. For example, the pentaglutamate form of pemetrexed (predominant intracellular form) is 60-fold more potent in its inhibition of the primary target enzyme thymidylate synthase (TS) than is the monoglutamate form (Shih et al., 1997). The second therapeutic benefit from polyglutmation is prolonged drug action because polyglutamates are retained in the cell. The reasons behind this are multifactoral. First, as polyanions, the polyglutamate derivatives can no longer traverse cell membranes by passive diffusion. Second, long-chain ($n>3$) polyglutamate derivatives of methotrexate are no longer substrates of efflux transport systems, such as the MRPs (Zeng et al., 2001; Wielinga et al., 2005) or BCRP (Chen et al., 2003; Volk and Schneider, 2003). For example, in the case of methotrexate, the addition of two glutamate residues from intracellular polyglutamation results in the loss of affinity with MRP2 and MRP5 (Chen et al., 2002) and the addition of the third glutamate residue leads to loss of affinity with BCRP (Volk and Schneider, 2003). In the present study, from the competitive inhibition vesicular transport assay, it was shown that BCRP is not able to transport the tri- and penta- glutamated PMX. This was further confirmed by the growth inhibition study, where the BCRP-transfected cells displayed low sensitivity (as indicated by high EC_{50} values) to PMX upon a 4 hour exposure due to the active efflux transport by BCRP, when, in contrast, higher sensitivity (as indicated by low EC_{50} values) to PMX was observed upon 72 hours drug exposure. We hypothesize

that this is because after the long term incubation (72 hours), the majority of intracellular PMX has been converted to active polyglutamated metabolites that can no longer be effluxed by BCRP.

After the identification of the *in vitro* BCRP substrate status of PMX, it is valuable to ascertain the role of BCRP on the *in vivo* disposition of PMX. We used the Bcrp1 knockout mice model (Bcrp1(-/-)) to examine the influence of BCRP-mediated efflux transport on the brain distribution of PMX. The steady-state plasma concentration in BCRP knockout mice is about 2-fold higher than that in wild-type mice. Given the knowledge that BCRP is also expressed at the kidney, the higher plasma concentration in BCRP knockout mice may be attributed to the decreased renal excretion due to BCRP deletion. The brain distribution of PMX, depicted by the steady-state brain-to-plasma concentration ratio (brain partition coefficient, Kp), is 8% in the wild type mice, indicating a limited brain distribution. This is consistent with the previous studies in our laboratory using the microdialysis method, which have shown that the PMX steady-state brain-to-plasma unbound concentration ratio after iv infusion is 0.106 ± 0.054 (Dai et al., 2005). Another relevant study was conducted by Stapleton, et al and they reported the cerebrospinal fluid (CSF) penetration of PMX was less than 2% (range 0.33–1.58%) in a non-human primate model; again, indicative of poor CNS penetration of PMX (Stapleton et al., 2007).

The 8% steady-state brain-to-plasma concentration ratio indicates that the efflux clearance (CL_{out}) of PMX out of the brain is about 10-fold greater than the influx clearance for PMX, as defined by whole tissue concentrations. Possible mechanisms for efflux clearance are active transport, bulk flow or metabolism in the brain. Regarding the contribution of bulk flow to the larger Cl_{out} than $Cl_{in,}$ it was determined in our previous study that the magnitude of bulk flow is much smaller than total brain efflux clearance and thus is not considered to be a major factor in this process (Dai et al., 2005). Moreover, the brain partition coefficient (Kp) of PMX determined after 4 hours intravenous infusion using the microdialysis method in the rat is quite similar to the value obtained from the 72-hour infusion study in the mouse. This indicates that time-dependent cellular metabolism is not major determinant for low brain concentration of PMX. Therefore, active transport processes for the transport of PMX from brain-to-plasma across the BBB or blood-CSF barrier (BCSFB) may be involved. In the current study, we used BCRP-deficient mice to determine if BCRP contributes to the efflux clearance of PMX from the brain. Contrary to the expected result suggested by *in vitro* study, the *in vivo* study showed that the brain distribution of BCRP knockout mice is not significantly different from that of wild-type mice. This result suggests that BCRP by itself does not play an important role in PMX efflux transport across the BBB.

Recently, the significance of BCRP for the brain penetration of its substrates has been questioned. For a number of compounds such as imatinib, abacavir, topotecan and

alfuzosin, which have been shown to be a substrate for BCRP *in vitro*, no significant difference was observed in brain penetration (brain-to-plasma ratio) between wild-type mice and BCRP-deficient mice (Bihorel et al., 2007; de Vries et al., 2007; Giri et al., 2008; Nicolazzo and Katneni, 2009; Zhao et al., 2009). One possibility is that, for chemicals which are dual substrates for both BCRP and P-gp, the residual activity of P-gp at the BBB in BCRP-deficient mice may override any impact of BCRP deletion, masking the functional role for BCRP at the BBB (Nicolazzo and Katneni, 2009; Vlaming et al., 2009a). This speculation was supported by several research groups. Our laboratory also found a compensatory effect in the brain distribution of a dual P-gp/BCRP substrate, dasatinib. Compared to wild-type mice, the brain distribution of dasatinib was 3-fold higher in P-gp knockout (Mdr1a/b(–/–)) mice, with no significant change in Bcrp1-knockout mice, but about 10-fold increase in Mdr1a/b(–/–)/Bcrp1(–/–) knockout mice was observed (Chen et al., 2009a). This suggests that for some dual substrates, P-gp is a dominant transporter in comparison with BCRP, and P-gp and BCRP may work together to limit xenobiotic exposure to the brain. Therefore, it is feasible to propose that BCRP may play a role at BBB, but the importance of BCRP for brain penetration of any given substrate will depend on the relevance of other transporters in the BBB. Based on current knowledge, other transport systems such as MRPs, organic anion transporter (OAT) and organic anion-transporting polypeptide (OATPs) are also expressed at BBB and may play a role in the transport PMX across the BBB. In a recent paper published by the Schinkel group, they showed the significant role of BCRP and MRP2 in the elimination of

methotrexate and a compensatory effect between the two transporters (Vlaming et al., 2009b). As PMX is a substrate for both MRP2 and MRP5 (Prat et al., 2002), it is possible that BCRP and the MRPs (e.g., MRP4 or MRP5) work in concert to limit the CNS exposure of PMX.

2.5 Conclusion

In summary, we have shown that the novel antifolate, PMX, has a limited CNS distribution in the mouse model. *In vitro* studies clearly demonstrate that PMX is a substrate for BCRP-mediated transport. However, from *in vivo* studies using BCRP knockout mice, the deletion of BCRP alone had little influence on the brain penetration of PMX, suggesting that BCRP acting alone may not play a significant role in limiting the distribution of PMX to the brain. Other transporters such as other organic anion transporters may assume a more prominant role in CNS distribution of PMX. This will need to be confirmed in a future study. The present work also underscores the fact that the relevance of a proposed physiological function, suggested from *in vitro* data, of one active drug transporter known to be present in a tissue, needs to be evaluated relative to all other active transporters present in the same tissue.

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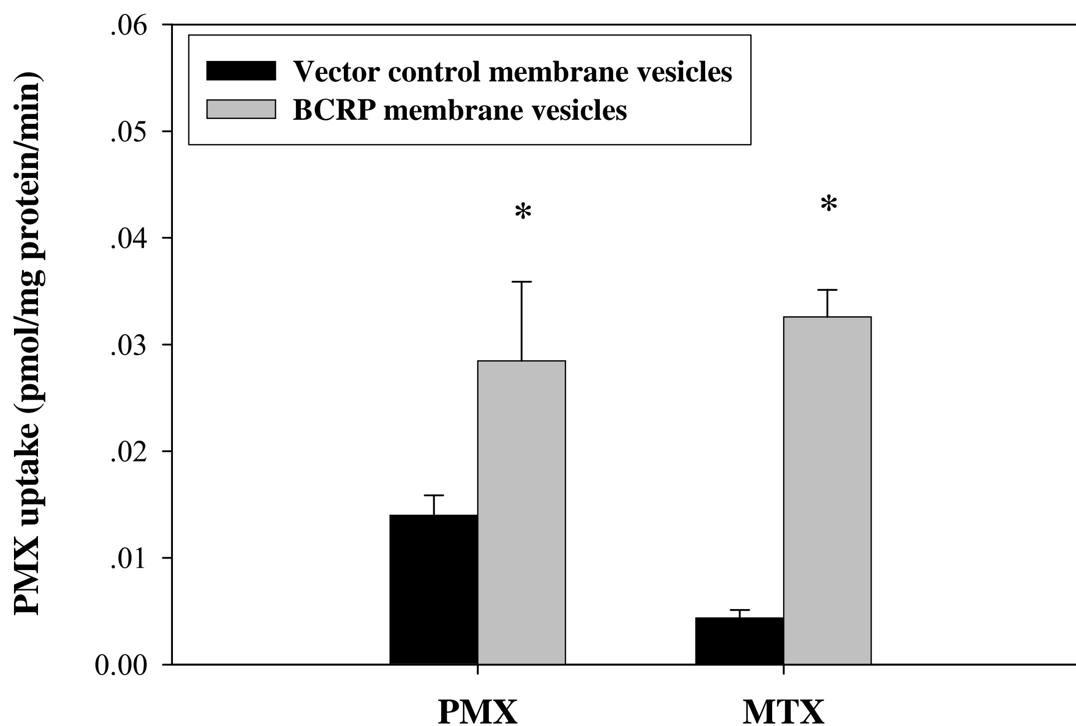


Figure 2-1 ATP-dependent uptake of PMX and MTX (positive control) in wild-type and BCRP-expressing membrane vesicles at physiological pH.

HEK293-BCRP (ABCG2-R482) membrane vesicles and vector control (pc-DNA) membrane vesicles were incubated with [^{3}H]-PMX or [^{3}H]-MTX at pH 7.4 at 37°C for 60 minutes in the presence or absence of 4 mM ATP. Values shown are mean ± S.D. of each experiment (n=4) (*, $p<0.05$ compared to vector control).

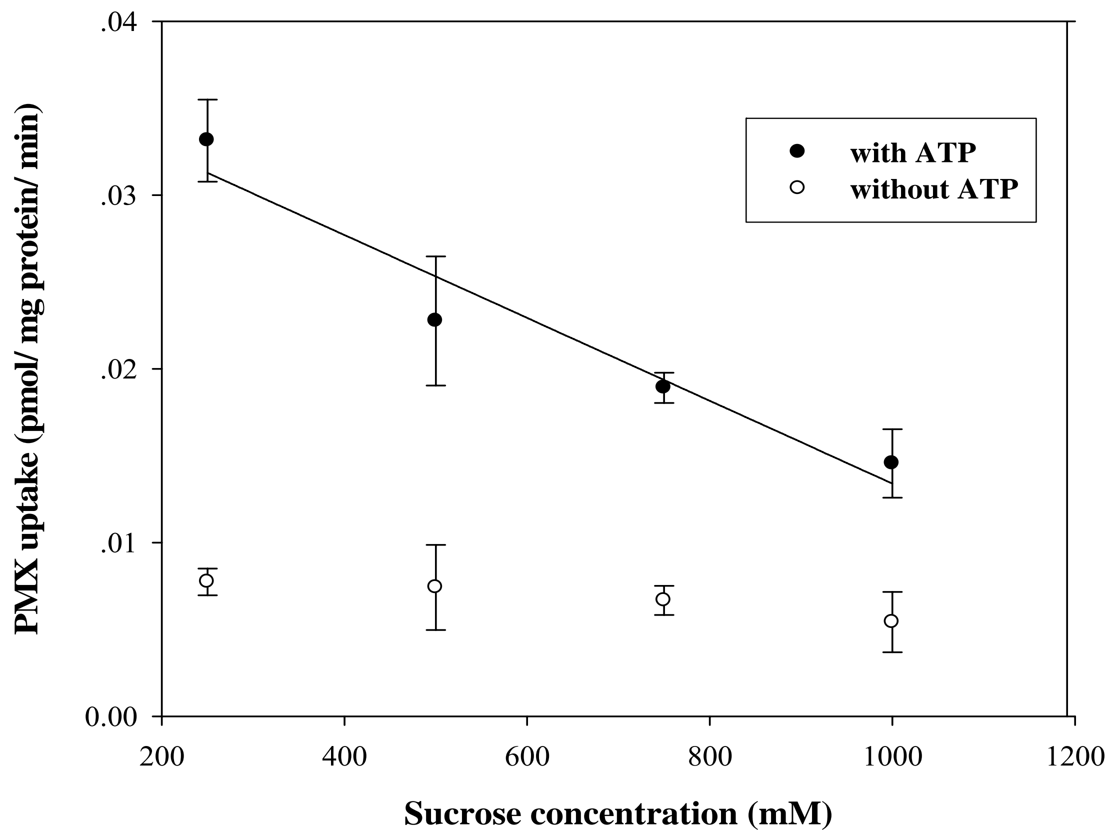


Figure 2-2 Effect of osmolarity on ATP-dependent transport of PMX in BCRP-expressing membrane vesicles.

HEK293-BCRP (ABCG2-R482) membrane vesicles were incubated with [^{3}H]-PMX at pH 7.4 at 37°C for 60 min in the presence or absence of 4 mM ATP. Values shown are mean ± S.D. of each experiment (n=3)

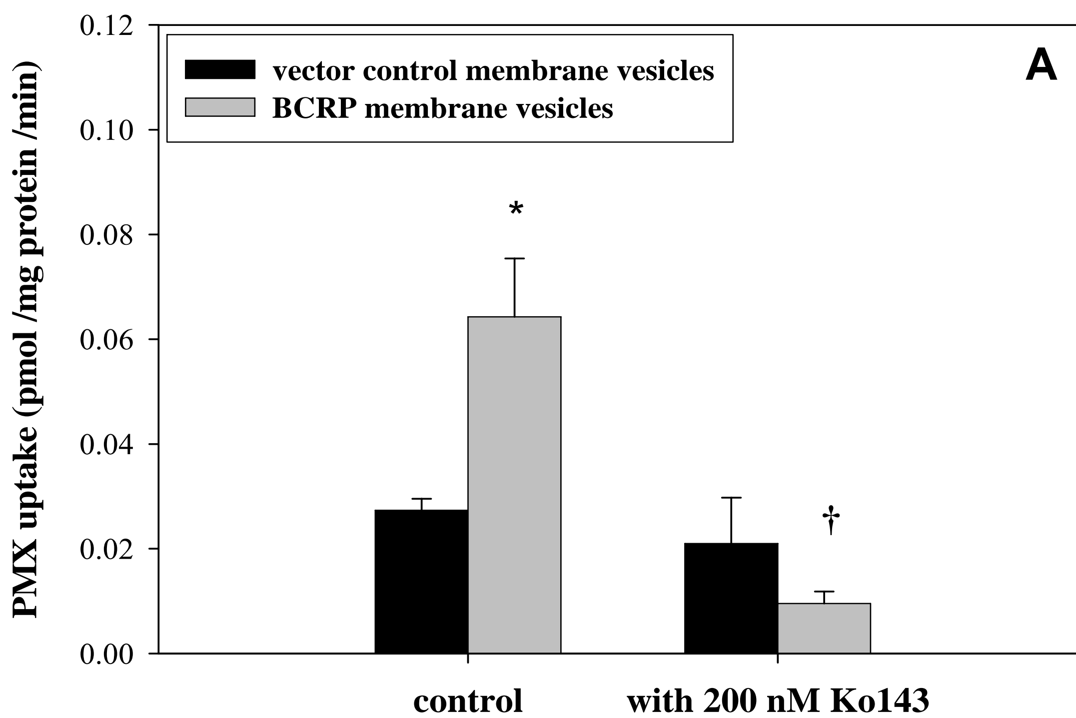


Figure 2-3 Effect of BCRP inhibitors on ATP-dependent PMX uptake in wild-type and BCRP-expressing membrane vesicles.

(A) Effect of 200 nM Ko143 on ATP-dependent PMX uptake in wild-type and BCRP-expressing membrane vesicles. HEK293-BCRP (ABCG2-R) membrane vesicles and pc-DNA (wild-type) membrane vesicles were incubated with [^{3}H]-PMX at pH 7.4 at 37°C with or without 200 nM Ko143 for 60 min in the presence or absence of 4 mM ATP. Values shown are mean ±S.D. of each experiment (n=4). (*, $p < 0.05$ compared to wild-type control. †, $p < 0.05$ compared to treatment control).

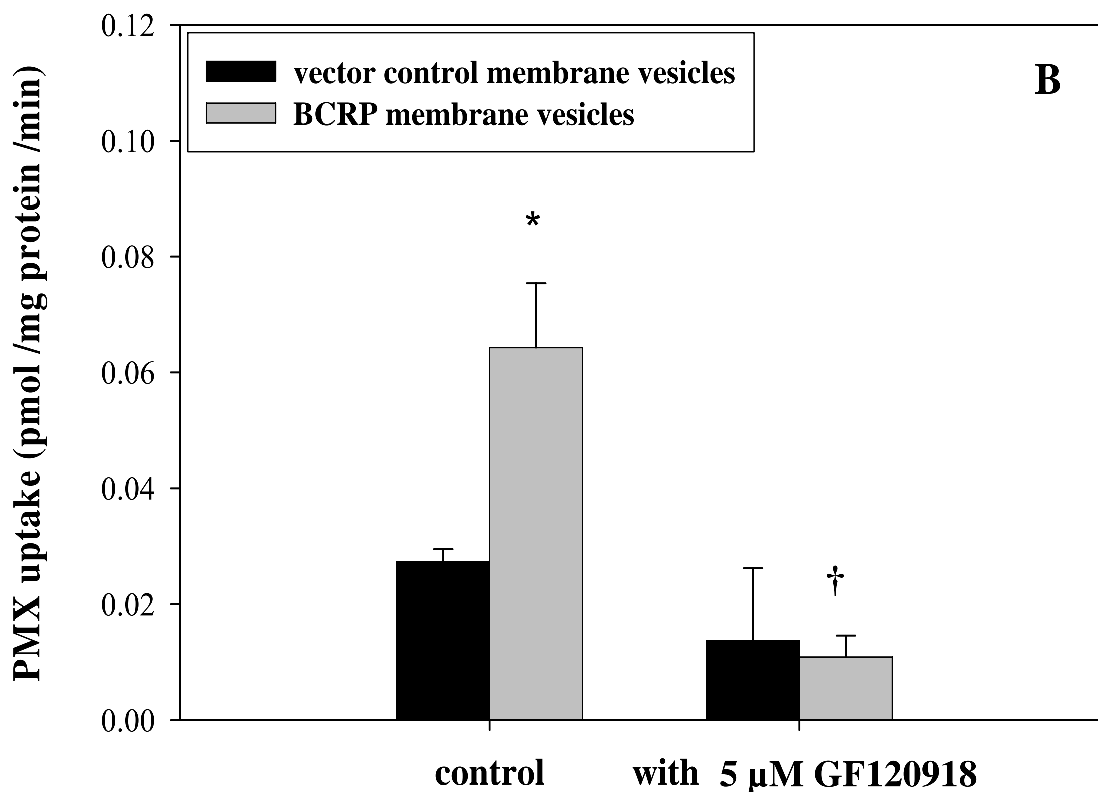


Figure 2-3 Effect of BCRP inhibitors on ATP-dependent PMX uptake in wild-type and BCRP-expressing membrane vesicles.

(B) Effect of 5 µM GF120918 on ATP-dependent PMX uptake in wild-type and BCRP-expressing membrane vesicles. HEK293-BCRP (ABCG2-R) membrane vesicles and pc-DNA (wild-type) membrane vesicles were incubated with [^{3}H]-PMX at pH 7.4 at 37°C with or without 5 µM GF120918 for 60 min in the presence or absence of 4 mM ATP. Values shown are mean ± S.D. of each experiment (n=4). (*, $p < 0.05$ compared to wild-type control. †, $p < 0.05$ compared to treatment control).

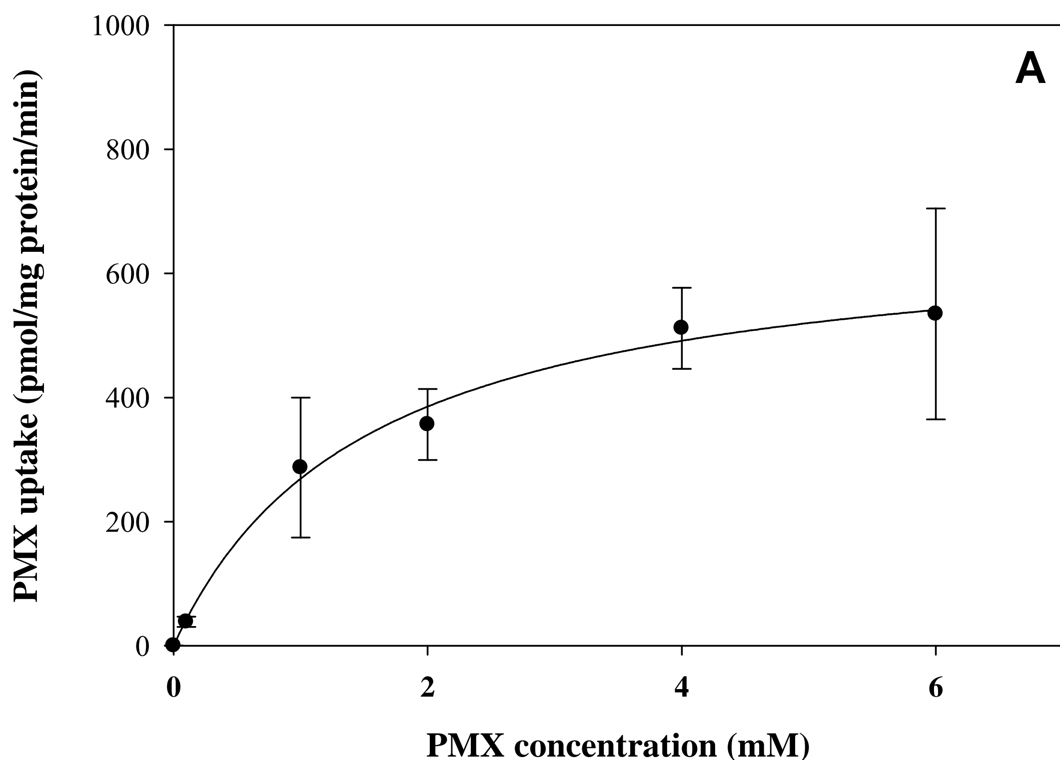


Figure 2-4A Concentration dependence of BCRP-mediated transport of PMX

HEK293-BCRP (ABCG2-R) membrane vesicles and control vesicles were exposed for 15 min to various concentrations of [^{3}H]-PMX, ranging from 0 to 6 mM, at pH 7.4 at 37°C in the presence or absence of 4 mM ATP. Values shown are mean ± S.D. of each experiment (n=4). The solid line represents a best fit Michaelis-Menten plot of the net initial velocity relative to increasing substrate concentrations.

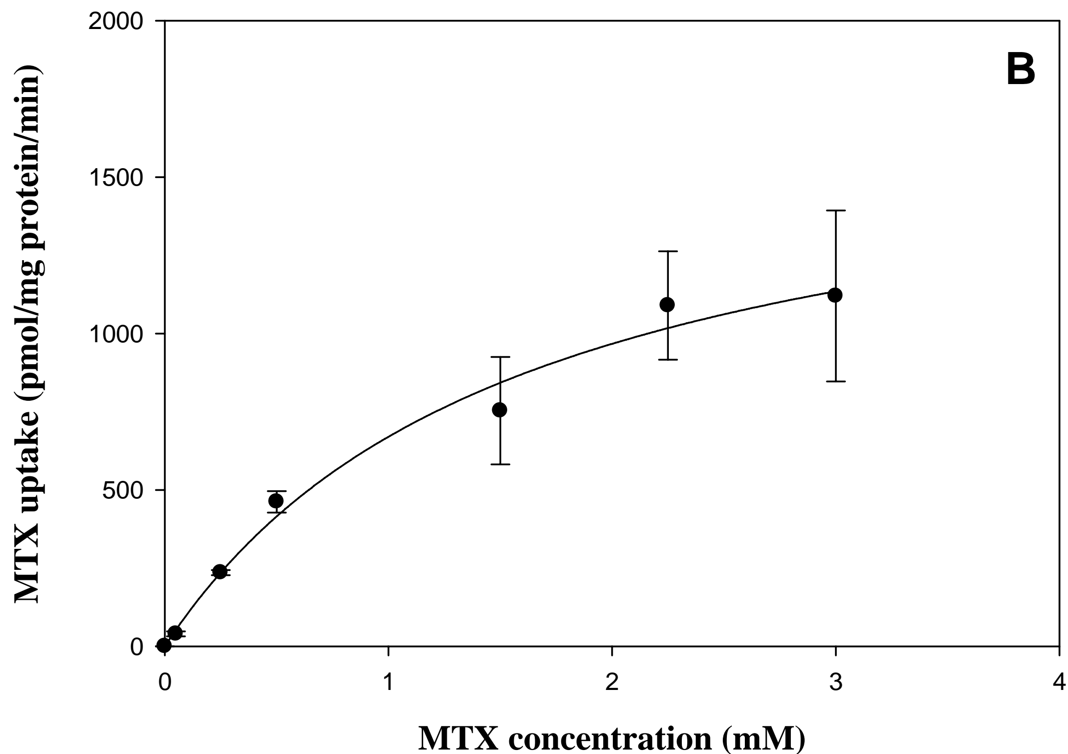


Figure 2-4B Concentration dependence of BCRP-mediated transport of MTX.

HEK293-BCRP (ABCG2-R) membrane vesicles and control vesicles were exposed for 5 min to various concentrations of [^{3}H]-MTX, ranging from 0 to 3 mM, at pH 7.4 at 37°C in the presence or absence of 4 mM ATP. Values shown are mean ± S.D. of each experiment (n=4). The solid line represents a best fit Michaelis-Menten plot of the net initial velocity relative to increasing substrate concentrations.

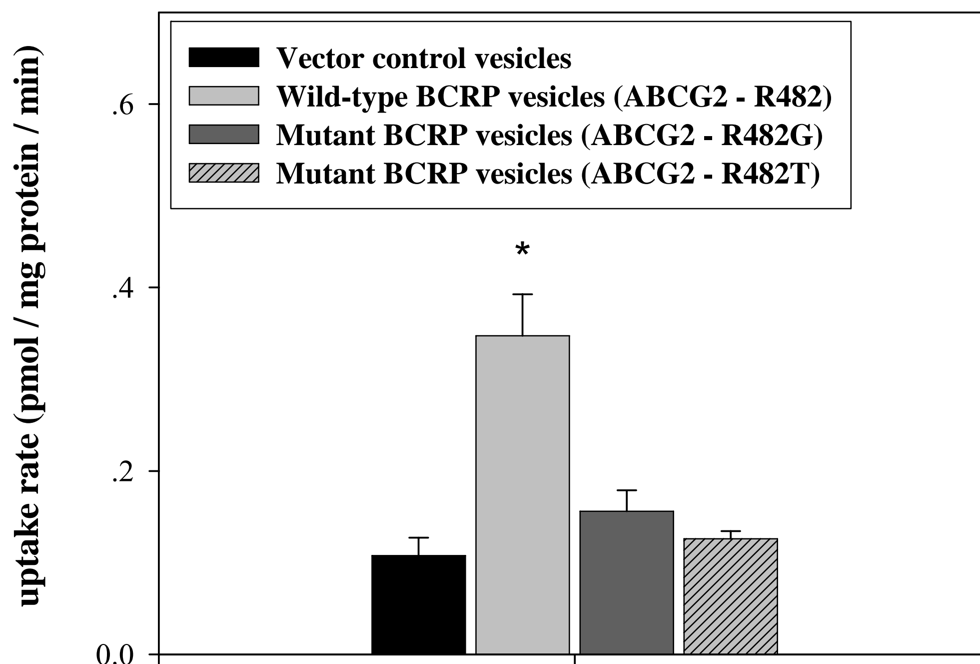


Figure 2-5 Effect of mutation at amino acid 482 on ATP-dependent uptake of PMX in wild-type and BCRP-expressing membrane vesicles.

HEK293-BCRP (ABCG2-R482, ABCG2-R482G and ABCG2-R482T) membrane vesicles and vector control (pc-DNA) membrane vesicles were incubated with [^{3}H]-PMX at pH 7.4 at 37°C for 60 minutes in the presence or absence of 4 mM ATP. Values shown are mean ± S.D. of each experiment (n=4). (*, $p<0.05$ compared to vector control).

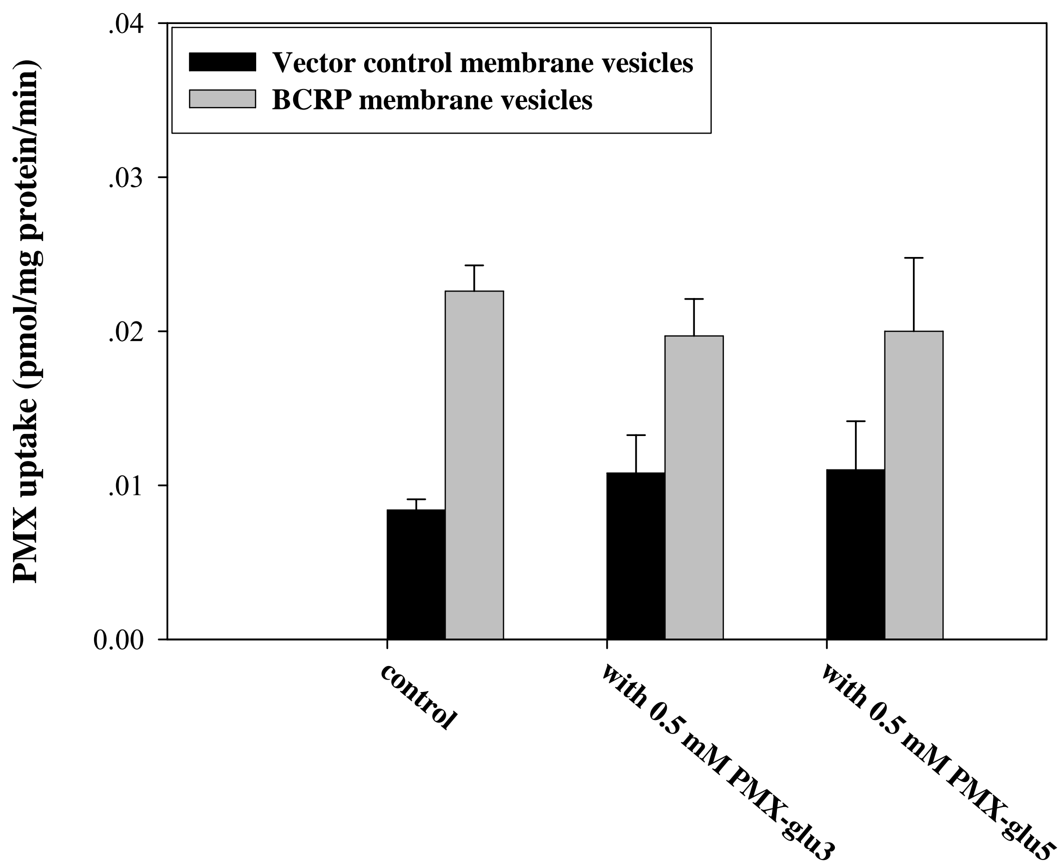


Figure 2-6 Effect of polyglutamated metabolites on ATP-dependent transport of PMX in wild-type and BCRP-expressing membrane vesicles.

HEK293-BCRP (ABCG2-R) membrane vesicles and pc-DNA (vector-control) membrane vesicles were incubated with [^{3}H]-PMX at pH 7.4 at 37°C with or without 500 μM PMX-glu3 or PMX-glu5 for 60 min in the presence or absence of 4 mM ATP. Values shown are mean ± S.D. of each experiment (n=4).

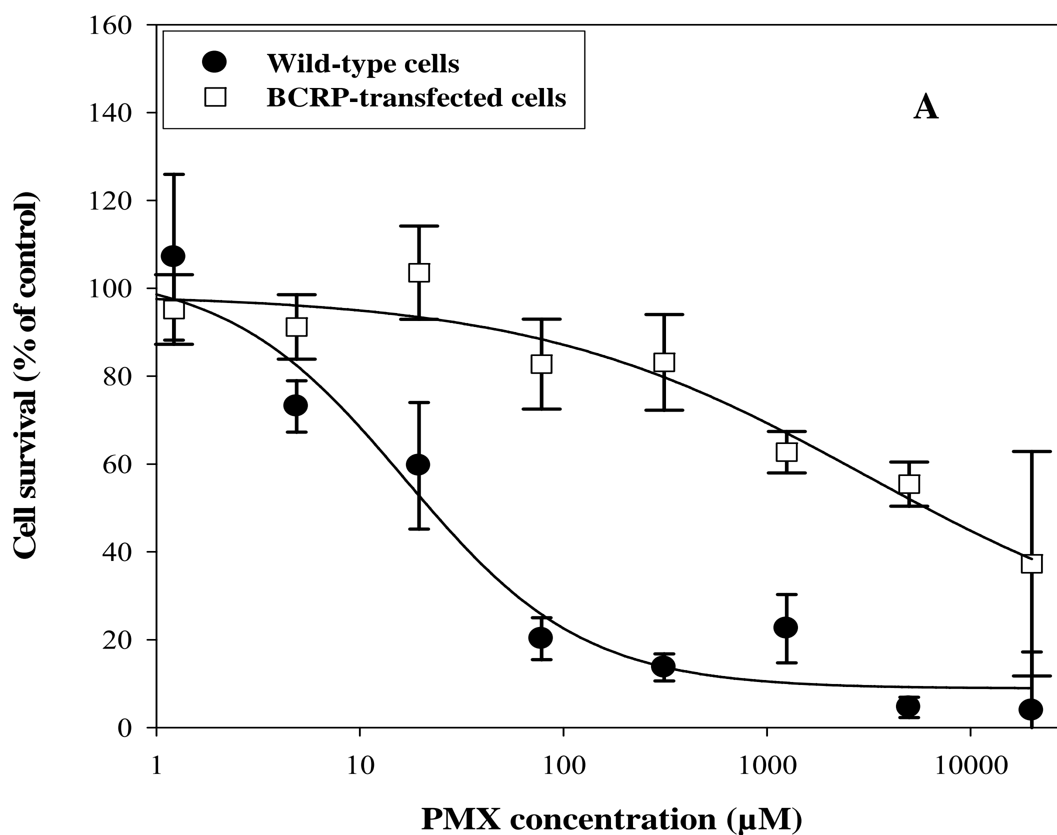


Figure 2-7A Cellular growth inhibition by PMX in BCRP transfected cells and wild-type cells after 4 hr drug exposure.

Wild-type and BCRP transfected cells were exposed for 4 hours to various concentration of PMX. Cells were then washed and drug-free medium was added and cultures were incubated for 68 hours at 37°C. The variable cell numbers were determined by MTS assay. Values shown are mean ± S.D. of each experiment (n=3).

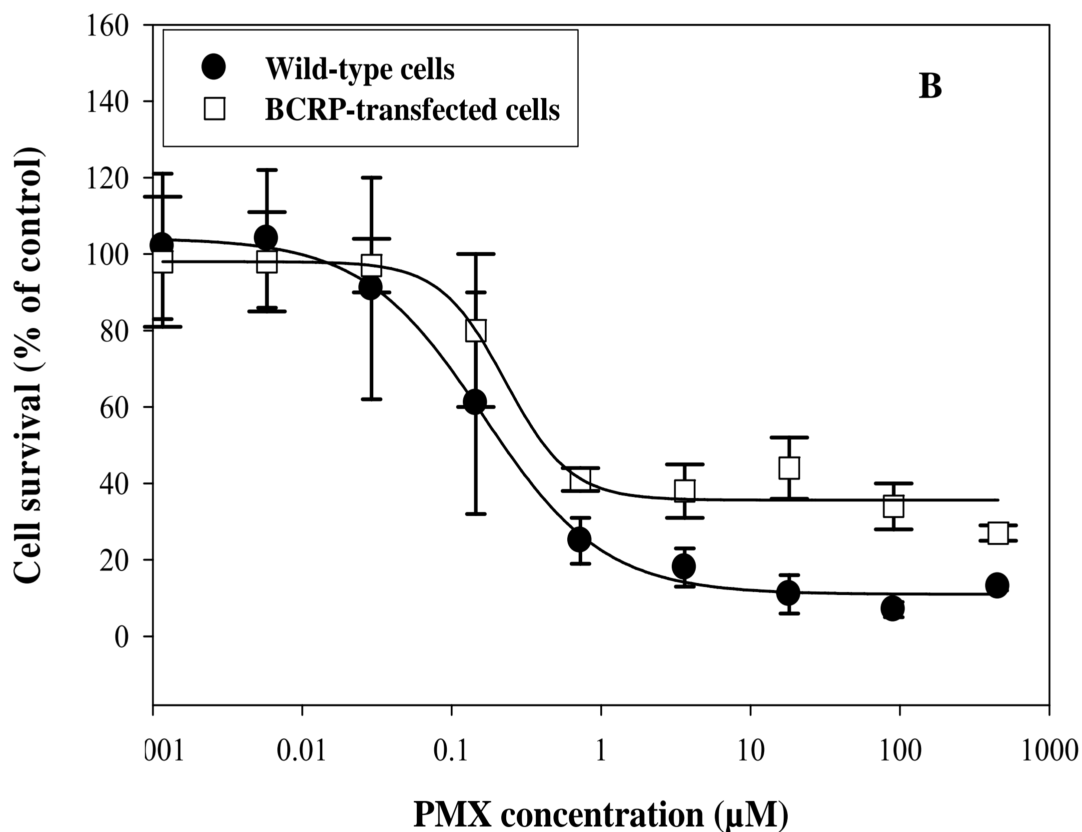


Figure 2-7B. Cellular growth inhibition by PMX in BCRP transfected cells and wild-type cells after 72 hr drug exposure.

Wild-type and BCRP transfected cells was exposed for 72 hours to various concentration of PMX. The variable cell numbers were determined by MTS assay. Values shown are mean ± S.D. of each experiment (n=3).

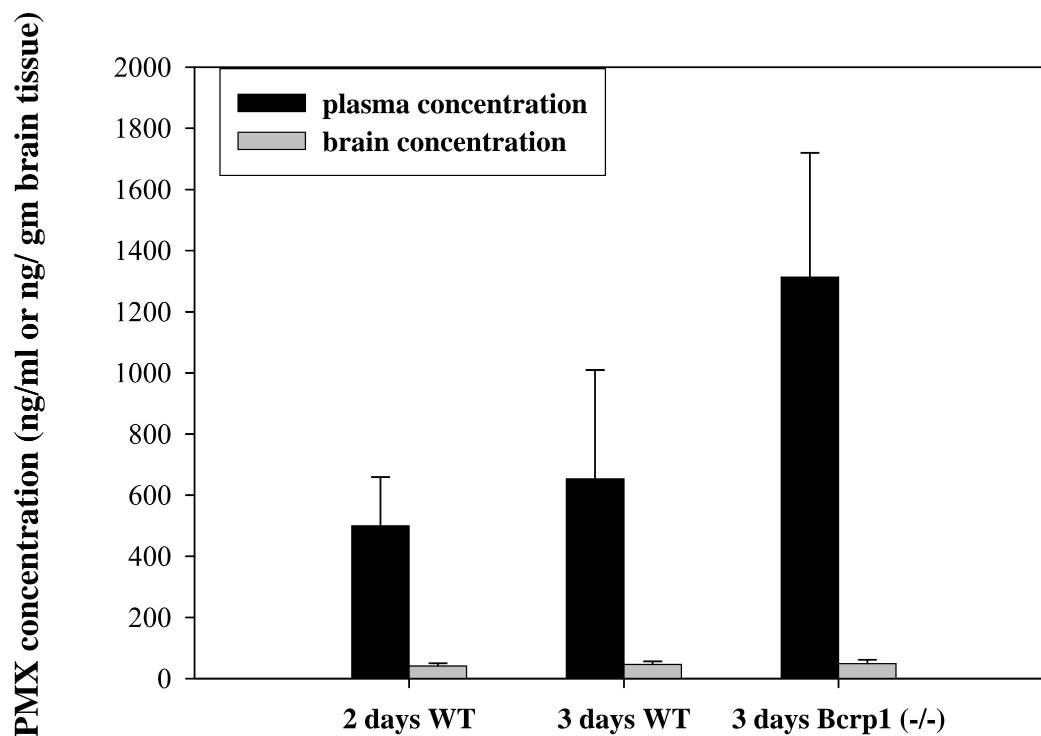


Figure 2-8 Steady-state PMX brain and plasma concentration in wild-type and BCRP knockout mice after 2-days or 3-days i.p. administration via osmotic minipump. Values shown are mean ± S.D. (n=4).

CHAPTER 3

ROLE OF ACTIVE EFFLUX TRANSPORT IN BRAIN DISTRIBUTION OF PEMETREXED AND METHOTREXATE

Antifolate agents, in particular methotrexate (MTX), have been widely used for the treatment in the primary and secondary tumors in the central nervous system (CNS). The clinical use of the novel antifolate pemetrexed (PMX) may eventually include a variety of CNS malignancies. However, studies have shown that both of these antifolates have a very limited brain distribution and active efflux may be involved. The objective of this study was to characterize the brain-to-blood efflux transport of PMX and MTX and to examine the role of the efflux transporters in the elimination of the antifolates across the blood-brain barrier (BBB) using the intracerebral microinjection technique (brain efflux index method).

The results of this study revealed that the elimination half-life of PMX from the brain is 48 minutes. MTX is eliminated from brain more rapidly than PMX with half-life of 32 minutes. Both PMX and MTX undergo saturable efflux transport across the BBB. Using gene knockout mice, it was shown that MRP2 does not play a role in the brain distribution of both antifolates. However, BCRP makes a significant contribution to the brain elimination of MTX, but not PMX. In addition, it was observed that the brain-to-blood transport of both antifolates was dramatically inhibited by probenecid and benzylpenicillin, suggesting the involvement of organic anion transporters, possibly OAT3.

It is likely that one of the underlying mechanisms of the low brain distribution of these antifolates is due to the active efflux transport by BCRP and/or a

benzylpenicillin sensitive transport system. Therefore, reducing the efflux transport from the brain by potent inhibitors may be a useful strategy to enhance the brain delivery of these antifolates.

3.1 Introduction

Despite aggressive therapy, the majority of primary and metastatic brain tumor patients have a poor prognosis with short survival periods. In general, the poor response of CNS tumors to chemotherapy drugs is multifactorial, but the inability to 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 formed in large part by brain capillary endothelial cells, and characterized by three major components, i.e., highly developed tight junctions, astrocytic end feet, and pericytes surrounding the blood vessels. Active efflux transporters that mediate active drug efflux into the systemic circulation across the BBB are also important in limiting drug distribution (Pardridge, 1999; Allen and Smith, 2001; Golden and Pollack, 2003). These transporters include P-glycoprotein (P-gp; MDR1/ABCB1), breast cancer resistance protein (BCRP/ABCG2), multidrug resistance-associated proteins (MRP2/ABCC2, MRP4/ABCC4) and organic anion transporters, such as organic anion-transporting polypeptide 2 (OATP2, SLC21A6) and organic anion transporter 3 (OAT3, SLC22A8) (Miller et al., 2000; Sugiyama et al., 2001; Urquhart and Kim, 2009b).

Antifolate drugs have long been used for anticancer treatment. The classic antifolate, methotrexate (MTX), is widely used for the treatment of primary CNS lymphoma and secondary CNS tumor. A very recent study reported that high-dose MTX alone or in combination with other therapies is the most effective treatment available for primary

CNS lymphoma (PCNSL) (Gerstner et al., 2008). However, studies have shown that MTX has a very limited brain penetration; in particular, only 5% of free MTX in the plasma can reach the brain at steady state (Devineni et al., 1996; Dai et al., 2005). Recently, another antifolate, pemetrexed (PMX, Alimta®), has been evaluated for anti-tumor activity for a variety of CNS malignancies (Kuo and Recht, 2006). Although slightly higher than MTX in terms of brain penetration, PMX brain distribution is still very limited with steady-state brain-to-plasma unbound concentration ratio of 0.106 ± 0.054 (Dai et al., 2005). The physiochemical properties of these antifolates such as their hydrophilic nature and anionic charge at physiological pH may contribute to the low BBB permeability. In the current study, we hypothesize that active efflux transport at BBB is involved in the elimination of antifolates from the brain and thereby, in part, account for the low brain distribution of PMX and MTX.

The objective of this study was to characterize the brain-to-blood efflux transport of antifolates (PMX and MTX) and to examine the role of several efflux transporters on the elimination of antifolates across the BBB using the intracerebral microinjection technique (brain efflux index method). Accordingly, the brain concentration of PMX and MTX after microinjection was compared between wild-type, *Mrp2(-/-)* and *Bcrp1(-/-)* mice. In addition, the involvement of organic anion transporters in the efflux processes was suggested by examining the inhibitory effect of probenecid and benzylpenicillin.

3.2 Theory about brain efflux index

3.2.1 Definition of brain efflux index (BEI)

BEI is an *in vivo* procedure designed to study mechanisms of brain-to-blood efflux transport at the BBB. The BEI value is defined as the percentage of test drug effluxed from the ipsilateral cerebrum to the circulating blood across the BBB, compared to the amount of test drug injected into the cerebrum (Equation 3-1) (Kakee et al., 1996).

$$BEI(\%) = \frac{\text{amount of drug effluxed at the BBB}}{\text{amount of drug injected in the brain}} * 100 \qquad \text{(Equation 3-1)}$$

Because the amount of drug effluxed from the brain is equal to the amount of drug injected minus the amount of drug retained in the brain, Equation 3-1 can be rearranged to Equation 3-2.

$$BEI(\%) = (1 - \frac{\text{amount of drug retained in the brain}}{\text{amount of drug injected in the brain}}) * 100 \qquad \text{(Equation 3-2)}$$

A BBB-impermeable reference compound is injected simultaneously into the brain cortex in order to minimize the inter-individual differences in the amount of drug injected. Therefore, BEI (%) value obtained from Equation 3-2 is normalized by the corresponding ratio of the reference compound (Equation 3-3).

$$BEI(\%) = (1 - \frac{\text{amount of drug in the brain} / \text{amount of reference in the brain}}{\text{amount of drug injected} / \text{amount of reference injected}}) * 100$$

(Equation 3-3)

3.2.2 Determination of BBB efflux clearance

100-BEI (%) is defined as the percentage of the test drug remaining in the ipsilateral cerebrum compared to the amount of drug injected (Equation 3-4). The apparent BBB efflux rate constant, $k_{eff,}$ can be obtained from the slope of the semilogarithmic plot of the value of (100-BEI) versus time.

$$100 - BEI(\%) = \left(\frac{\text{amount of drug in the brain} / \text{amount of reference in the brain}}{\text{amount of drug injected} / \text{amount of reference injected}} \right) * 100$$

(Equation 3-4)

3.2.3 Dilution factor

In concentration dependent studies or inhibition studies, the unlabeled drug or inhibitor will be given concurrently with the radiolabeled drug into the brain cortex. As compounds diffuse in the cerebrum after injection, the effective concentrations in the cerebrum become lower than that in the injection solution (Kusuhara et al., 2003). Therefore, the effective drug/inhibitor concentration in the brain is estimated by dividing the injectate concentration by a dilution factor. Kakee et al. estimated the dilution factor by examining the spreading of trypan blue in the cerebrum after microinjection. The dilution factors corresponding to different injection volumes were listed in the following table (Kakee et al., 1996).

Estimation of the dilution effect after a microinjection into Par2 of rat hemisphere

Injection volume (μL)	Time after administration (min)	Diffusion volume[a] (μL)	Dilution factor[b]
0.2	2	8.5 ± 1.1	42.4 ± 5.7
	20	8.0 ± 0.4	39.9 ±2.2
0.5	2	15.1 ± 2.2	30.3 ± 4.4
	20	23.1 ± 0.6	46.2 ± 1.1
1.0	2	28.6 ± 3.2	28.6 ± 3.2
	20	34.9 ± 5.7	34.9 ± 5.7

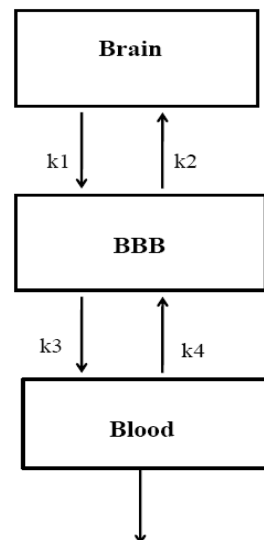
Trypan blue (4mg/ml) in 0.85% saline was injected intracerebrally for 1 sec to normal male rats. Rats were decapitated 2 and 20 min after administration. Each value represents the mean ± S.E. of four experiments.

a. Diffusion volume was determined as the wet weight of the trypan blue-stained region of the brain, assuming the specific gravity equals 1.0.

b. Dilution factor is defined as the ratio of diffusion volume to the injection volume.

3.2.4 Rate-limiting step

The elimination from cerebrum after microinjection consists of two steps: the uptake from cerebrum interstitial space to brain capillary endothelial cells through abluminal membrane (abluminal clearance), and the subsequent excretion into the blood through luminal membrane (luminal clearance). Therefore, assuming the reuptake from blood to brain is negligible, the apparent brain efflux rate constant k_{eff} can be described by Equation 3-6.



$$k_{eff} = \frac{k_1 * k_3}{k_1 + k_2 + k_3} \qquad \text{(Equation 3-6)}$$

When k_3 (excretion process, luminal elimination rate constant) is much larger than $k_1 + k_2$, k_{eff} is approximately equal to k_1. Under this condition, the uptake from cerebrum into the brain capillaries (abluminal clearance) will be the rate-limiting step to control the overall elimination from the brain. If this is the case, then the results obtained from the BEI study reflect mainly the transport across the abluminal membrane. The inhibition of excretion process through the luminal membrane of the barrier will hardly affect the brain elimination time profile (Kakee et al., 1996; Kusuhara et al., 2003). This is different from the systemic administration method, where there is singificant drug transport from blood to the brain. As such, both luminal and abluminal transport contributes to the overall brain elimination. Caution is needed when inconsistent results are observed between these two *in vivo* methods(Kusuhara et al., 2003).

One such example is the brain elimination of quinidine. When quinidine was given intravenously, a potent P-gp inhibitor PSC-833 increased the brain distribution of quinidine (K_p) by approximately 15-fold. In contrast, when quinidine was directly introduced into the brain extracellular fluid, the treatment of PSC-833 had no significant effect on the brain efflux of quinidine as measured by the BEI method (Kusuhara et al., 1997). This apparent contradiction can be explained by the theoretical model of rate-limiting step across the BBB, i.e., when the abluminal membrane clearance is the rate-limiting step for brain elimination of quinidine, the inhibition of P-gp at luminal membrane by PSC833 may not significantly affected the apparent brain efflux rate constant evaluated by BEI.

3.3 Materials and method

3.3.1 Chemicals

[^{3}H]-PMX was provided by Eli Lilly and Company. [^{3}H]-MTX, [^{3}H]-valproic acid and [^{14}C]-carboxyl-inulin were obtained from Moravek Biochemicals (Brea, CA). Probenecid and benzylpenicillin were purchased from Sigma-Aldrich (St.Louis, MO).

3.3.2 Animals

Male *Bcrp1*(-/-), *Mrp2(-/-)* and wild-type (C57BL/6) mice were provided by Taconic Farms. Inc. (Germantown, NY) and were a gift from Eli Lilly and Company. Animals were maintained under temperature-controlled conditions with a 12-h light/dark cycle and were allowed food and water ad libitum. All 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 of the University of Minnesota (IACUC).

3.3.3 Brain Efflux Index (BEI) Method

The brain efflux study was performed using the intracerebral microinjection technique, as reported previously (Kakee et al., 1996). Wild-type, *Mrp2(-/-)* and *Bcrp1*(-/-) mice were anesthetized with ketamine/xylazine (100/10 mg/kg i.p.), 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 2 (S2) region. Then, 0.2 µL of a mixture of [^{3}H]-PMX (2 nCi/mouse) and [^{14}C]-carboxyl-inulin (2 nCi/mouse)

dissolved in extracellular fluid (ECF) buffer (122 mM NaCl, 25 mM $NaHCO_3$, 3mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 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 done. After microinjection, the injection needle was left in place for additional 4 minutes to minimize the backflow of injected solution along the injection track. At designated time points, the mice were decapitated and the right (ipsilateral), left (contralateral) cerebrum and cerebellum were collected. The brain samples were 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 [^{3}H]-PMX across BBB was examined using 1 mM and 50 mM unlabelled PMX in the injectate. To examine the inhibitory effect of probenecid and benzylpenicillin on the elimination of [^{3}H]-PMX from the brain, 100 mM probenecid or benzylpenicillin was dissolved in injection solution. The pH of the solution was adjusted to 7.4. The inhibitory effect was evaluated by comparing the residual percentage of [^{3}H]-PMX with respect to the control value.

100-BEI (%) was calculated as the amount of test drug ([^{3}H]-PMX or [^{3}H]-MTX) retained in the ipsilateral cerebrum divided by the amount of test drug injected, normalized by the corresponding ratio of the BBB impermeable marker (^{14}C]-carboxyl-inulin). 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 non-linear regression function in SigmaPlot (version 9.0.1, SYSTAT software). The value of dilution factor used in the present study is 39.9 (see section 3.3.3) (Kakee et al., 1996).

3.3.4 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 $p < 0.05$.

3.4 Results

3.4.1 Time course of PMX and MTX brain elimination

The kinetic analysis of the brain-to-blood efflux transport of [^{3}H]-PMX and [^{3}H]-MTX was investigated by the brain efflux index method. **Figure 3-1A** shows the time course of the remaining percentage of [^{3}H]-PMX in the ipsilateral cerebrum after microinjection into the S2 region of the mouse brain. Approximately 60% of the administered dose of [^{3}H]-PMX was eliminated from the ipsilateral cerebrum within 90 minutes. The apparent elimination rate constant (k_{eff}) of [^{3}H]-PMX was found to be 0.0143 ± 0.0043 min^{-1} (mean ± S.D.) from the slope of the semilogarithmic plot of (100-BEI%) values versus time. The brain elimination half-life of [^{3}H]-PMX was 48 minutes, calculated from k_{eff} value. MTX was eliminated from the brain more rapidly than PMX after intracerebral administration. The k_{eff} value for MTX was 0.0271± 0.0184 min^{-1} with half-life of 32 minutes (**Figure 3-1B**). Valproic acid was used here as a positive control for method validation (**Figure 3-2**). The brain elimination half-life of valproic acid in the present study was determined to be 2 minutes, which is consistent with the reported value (3.7 minutes) from previous studies using the same technique (Kakee et al., 2002).

3.4.2 Concentration-dependent efflux of PMX from the brain

To further characterize the efflux transport of PMX from the brain, the concentration dependent study was performed. At 30 minutes postdose, 51.1 ± 5.1% of [^{3}H]-PMX was retained in the ipsilateral cerebrum (control case). However, in the presence of 1

mM or 50 mM unlabeled PMX (injectate concentration), the percentages of [^{3}H]-PMX in the brain increased to 71.7 ± 7.1% and 95.7 ± 4.8%, respectively, which were both significantly greater than that in the control case ($p < 0.001$) (**Figure 3-3**, **Table 3-1**). These results suggest that the brain elimination of PMX was saturable and carrier-mediated transport was involved.

3.4.3 Effect of MRP2 on PMX and MTX efflux from the brain

The contribution of MRP2 to the elimination of PMX and MTX from the brain across the BBB was investigated using *Mrp2(-/-)* mice. At 60 minute postdose, the percentages of [^{3}H]-PMX remaining in the ipsilateral cerebrum were 44.1 ± 2.0% in wild-type mice and 41.7 ± 3.3% in *Mrp2(-/-)* mice, and these two values were not statistically different (**Figure 3-4A**). This indicates that a deficiency of MRP2 alone does not affect the brain efflux transport of PMX. A similar result was found for MTX; at 30 minute postdose, the remaining percentage of [^{3}H]-MTX in the brain of *Mrp2(-/-)* mice (42.8 ± 6.0%) was not significantly different from that of wild-type mice (36.2 ± 5.2 %) (**Figure 3-4B**).

3.4.4 Effect of BCRP on PMX and MTX efflux from the brain

The effect of BCRP on the brain-to-blood efflux transport of PMX was examined using *Bcrp1(-/-)* mice. At 30 minutes postdose, the percentage of [^{3}H]-PMX remaining in the ipsilateral cerebrum of *Bcrp1(-/-)* mice was not significantly different from that of wild-type mice **(Figure 3-5A).** In contrast, a significantly

higher amount of [^{3}H]-MTX was found in the brain of *Bcrp1(-/-)* mice than that in wild-type mice ($p < 0.01$) (**Figure 3-5B**), suggesting a role of BCRP in brain elimination of MTX.

3.4.5 Effect of organic anion transporters on PMX and MTX efflux from the brain

The effect of probenecid, a general inhibitor of organic anion transporters, on brain-to-blood transport of PMX and MTX is shown in **Figure 3-5**. With the presence of probenecid (100 mM in the injectate), the percentage of [^{3}H]-PMX and [^{3}H]-MTX remaining in the brain after 30 minutes administration increased from 51.1 ± 5.1% to 72.4 ± 3.3% and 36.2 ± 5.2% to 75.8 ± 8.6%, respectively. These results indicate that probenecid significantly inhibited the efflux of PMX and MTX from the brain and some organic anion transporters may be involved. A similar trend was observed for *Bcrp1 (-/-)* mice.

Another organic anion, benzylpenicillin, was also used to further characterize the possible transporters involved in the efflux transport of the antifolates. As shown in **Figure 3-6**, the elimination of both [^{3}H]-PMX and [^{3}H]-MTX from the mouse brain was dramatically inhibited by benzylpenicillin (100 mM in the injectate), with about 90% of the dose remaining in the brain at 30 minutes administration.

Taken together, the effect of various treatments on brain-to-blood efflux of PMX and MTX (BEI %) was summarized in **Table 3-2** and **Table 3-3**. Of the treatments, deletion of MRP2 did not significantly affect the brain elimination of PMX and MTX. However, deletion of BCRP significantly attenuated the brain efflux of MTX, but not PMX. Brain-to-blood transport of PMX and MTX was sensitive to organic anions probenecid and benzylpenicillin. In particular, brain elimination was reduced by 43.5% for PMX and 62.1% for MTX with treatment of 100 mM probenecid in the injectate (2.5 mM in the brain). 100 mM benzylpenicillin (2.5 mM in the brain) resulted in more significant inhibitory effect and reduced the brain efflux of PMX and MTX by 75.0% and 78.6%, respectively. The most significant inhibition of the brain elimination of PMX was observed with the treatment of unlabeled PMX, where 50 mM unlabeled PMX (1.25 mM in the brain) decreased the brain efflux of PMX by 91.2%.

3.5 Discussion

Validity of the BEI method in the application of the present study

In this study, the efflux transport of PMX and MTX from brain to the circulating blood was characterized with the brain efflux index method (BEI). BEI, also referred to as the intracerebral microinjection technique, is a novel technique to study mechanisms of brain-to-blood efflux transport at BBB (Kakee et al., 1996; Kusuhara et al., 2003). Because it can isolate efflux processes directly, rather than considering efflux as modulator of brain uptake, BEI is considered to be an excellent method for determining efflux clearance and clarifying the transport function. 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).

During the course of the efflux studies, less than 0.5% of injected [^{3}H]-PMX or [^{3}H]-MTX was found in the contralateral cerebrum and cerebellum, suggesting that diffusion into the rest of CNS from the injection site was very limited. Up to 90 minutes after intracerebral injection, the remaining percentage of [^{14}C]-carboxyl-inulin (BBB impermeable marker) in the brain did not change significantly, indicating little damage to the BBB. In addition, the validity of BEI technique was also examined by the application of valproic acid. The brain elimination half-life of

valproic acid determined in the current study is consistent with the previous report by Kakee et al. (Kakee et al., 2002). These results validate that the use of the brain microinjection technique for the examination of mechanism responsible for brain efflux of PMX and MTX.

Time course of PMX and MTX efflux from the brain

The apparent brain elimination rate constant (k_{eff}) of PMX was determined to be 0.0143 ± 0.0043 min^{-1} from the kinetic analysis study. This observed value is in agreement with reported value obtained from brain microdialysis study in rats (0.021 ± 0.0047 min^{-1}) (Dai et al., 2005). MTX and PMX are structural analogs with similar physicochemical properties. However, MTX was eliminated from the brain more rapidly than PMX, with k_{eff} as of 0.0271 ± 0.0184 min^{-1}. Given the hydrophilic nature of PMX and MTX, it is likely that the volumes of distribution of these two antifolates in the brain (V_{brain}) are similar and both are close to the brain extracellular space. Therefore the intrinsic brain efflux clearance of MTX, calculated as the product of k_{eff} and V_{brain}, may be greater than that of PMX. Using intracerebral microdialysis method in rat model, the brain distribution index depicted by $AUC_{ECF, unbound}/AUC_{plasma, total}$ was 0.01- 0.02 for MTX (Devineni et al., 1996; Dukic et al., 1999). With the same *in vivo* method, Dai et al. have shown that $AUC_{ECF,unbound}/AUC_{plasma,unbound}$ was 0.078 (Dai et al., 2005). Given the free faction of PMX in plasma is 0.36, the $AUC_{ECF,unbound}/AUC_{plasma,total}$ for PMX is about 0.05 (Dai et al., 2005), indicating that brian penetration of PMX is slightly higher than that of MTX.

Based on the present study, the lower brain distribution of MTX compared to PMX may be attributed to the greater efflux clearance of MTX from the brain, although possible differences in uptake clearance into the brain must also be considered.

Concentration-dependent efflux of PMX from the brain

The brain-to-blood efflux of [^{3}H]-PMX was inhibited by unlabelled PMX in a concentration-dependent manner, indicating that PMX undergoes carrier-mediated efflux across the BBB. In addition, with 50 mM unlabelled PMX in the injectate, the elimination of [^{3}H]-PMX from the brain was almost completely inhibited, 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 in line with the physicochemical properties of PMX. Given the fact that PMX is an anionic compound with ***log P*** of -1.5, it is likely that magnitude of passive membrane permeability is very limited, and active transporters are involved to allow an efficient membrane transport to remove PMX from the brain.

Role of MRP2 in brain-to-blood transport of the antifolates

In a recent paper, Vlaming et al. have demonstrated that BCRP and MRP2 play an important role in determining the total body clearance of MTX (Vlaming et al., 2009b). Results from *in vitro* studies showed that both PMX and MTX have relative strong affinity with MRP2, i.e., the K_m values are 66 μM for PMX and 480 μM for MTX (Pratt and Chen, 2002; El-Sheikh et al., 2007). Given the knowledge that MRP2

may be expressed at the BBB, we first examined the role of MRP2 in brain elimination of PMX and MTX (Pratt and Chen, 2002; El-Sheikh et al., 2007). Surprisingly, in the present study, no significant change in brain elimination of PMX and MTX was observed in *Mrp2(-/-)* mice as compared to wild-type mice, indicating that MRP2 did not play a significant role in brain-to-blood efflux of these antifolates. Although the functional relevance of MRP2 in substrate body disposition has been established via several studies using MRP2 gene knockout mice (Vlaming et al., 2006; Tian et al., 2007; Ieiri et al., 2009; Vlaming et al., 2009b), the role of MRP2 in drug disposition into the brain is defined poorly. One of the reasons is the controversial findings in the BBB expression of this transporter. MRP2 has been detected on the luminal surface of fish, rat and pig brain capillary endothelium (Miller et al., 2000). However, it was not detected in bovine and human brain capillaries (Zhang et al., 2000; Sugiyama et al., 2003; Aronica et al., 2004; Nies et al., 2004). In a recent study, it was observed that MRP2 expression in the murine brain is strain-specific, i.e., C57BL/6, Swiss and SvJ mice revealed a prominent MRP2 staining in the BBB, whereas FVB mice is lack of expression of this transporter in the brain capillary (Soontornmalai et al., 2006). As the C57BL/6 mouse is the animal model used in the present study, the lack of effect of MRP2 on brain elimination of the antifolates should not be due to the absence of expression at the BBB. It is likely that another efflux transporter at BBB may play a more dominant role in efflux transport of the antifolates and thereby masking the functional role of MRP2.

Role of BCRP in brain-to-blood transport of the antifolates

The second efflux transporter we investigated was BCRP. From the results of a BEI study, deletion of BCRP led to a 30% decrease in brain efflux transport of MTX ($p < 0.01$), indicating the significant role of BCRP in MTX brain distribution. For PMX, a reduced efflux transport from brain was also observed in BCRP deficient mice, but the change was not statistically significant. This is consistent with the results from the systemic administration study (chapter 2), where no significant effect on PMX brain distribution was observed in *Bcrp1 (-/-)* compared to wild-type mice. From *in vitro studies*, the intrinsic clearance of BCRP for MTX, represented by the ratio of V_{max} over K_m, is about 3-fold higher than that of PMX (chapter 2). Therefore, the greater transport activity of BCRP for MTX may account for the significant contribution of BCRP in brain elimination of MTX.

Effect of organic anion transporters on PMX and MTX efflux from the brain

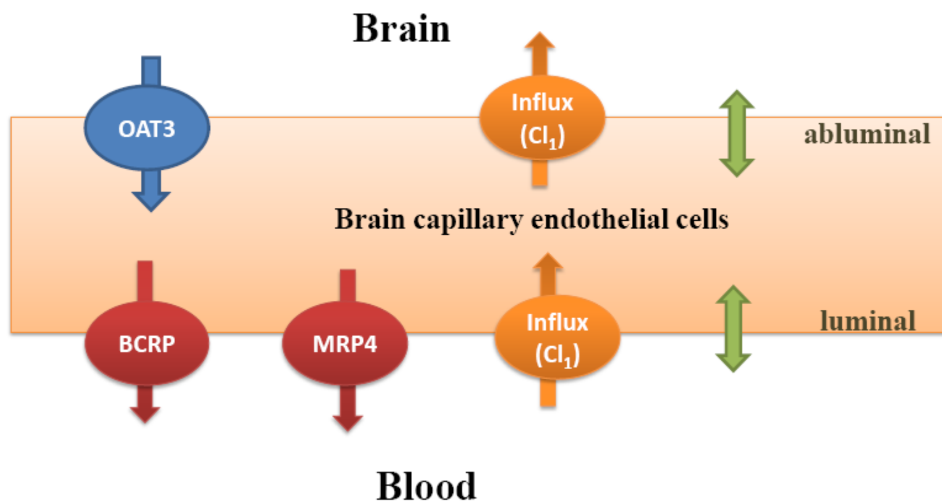
Given the fact that PMX and MTX are weak acids with anionic charge at physiological pH, in the present study, we also examined the role of organic anion transporters in brain distribution of the antifolates. Probenecid is a nonspecific inhibitor of organic anion transporters, such as MRPs, organic anion transporters (OATs) and organic anion transporting polypeptides (OATPs) (Bakos et al., 2000; Sugiyama et al., 2001; Haimeur et al., 2004). The results from current BEI studies showed that brain elimination of PMX and MTX were markedly inhibited by probenecid, indicating the involvement of organic anion transporters.

Benzylpenicillin is an OAT3 substrate. Using the BEI technique in rat, it has been demonstrated that the brain elimination of benzylpenicillin is mediated by OAT3 (Kikuchi et al., 2003). In the present study, 100 mM benzylpenicillin in the injectate solution dramatically reduced the brain efflux transport of PMX and MTX, suggesting that OAT3 on the BBB may play an important role in the efflux transport of the antifolates.

OAT3 is localized at the abluminal membrane of brain capillary endothelial cells (Kusuhara et al., 1999; Kikuchi et al., 2003; Mori et al., 2003) and has been shown to mediate the brain-to-blood transport of many hydrophilic organic anions such as indoxyl sulfate (IS), 6-mercaptopurine (6-MP), p-aminohippuric acid (PAH), benzylpenicillin (PCG) and homovanillic acid (HVA), using BEI method (Ohtsuki et al., 2002; Kikuchi et al., 2003; Mori et al., 2004; Kusuhara and Sugiyama, 2005). OAT3 has relatively strong affinities for MTX with K_m of 60 μM (VanWert and Sweet, 2008) and has been suggested to play an important role in MTX renal clearance (Nozaki et al., 2004). It is therefore conceivable that OAT3 is involved in the brain efflux transport of MTX and PMX. However, we cannot exclude the possibility that other transport systems at the BBB are also sensitive to benzylpenicillin, therefore, the exact role of OAT3 in the brain efflux of the antifolates need to be further confirmed in OAT3 gene knockout mice.

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. Other than BCRP, it has been suggested that the ABC transporter MRP4 is likely to function at the luminal membrane cooperatively with OAT3 (Ohtsuki and Terasaki, 2007). The functional role of MRP4 at BBB has been demonstrated by several studies using *Mrp4 (-/-)* mice (Leggas et al., 2004; Belinsky et al., 2007). Moreover, MRP4 interacts with both PMX and MTX. In particular, MRP4 mediates the transport of MTX (K_m ~220 μM^{-1}) and the expression of MRP4 correlates with the *in vitro* chemosensitivity of tumor cells to PMX (Chen et al., 2002; Hanauske et al., 2007). Taken together, it is possible that MPR4 may play a role in the brain-to-blood transport of PMX and MTX.

Coordination between efflux transporters at the BBB to mediate the brain elimination of the antifolates -- a simulation study



As shown in the diagram above, PMX and MTX may enter the brain via passive diffusion (PS) and active influx transport (Cl_1). Brain elimination of PMX and MTX could result from uptake from brain interstitial fluid by OAT3 at abluminal membrane and the subsequent excretion by BCRP and MRP4 at the luminal membrane. The brain distribution of antifolates (K_p), depicted by the brain-to-plasma AUC ratio, is determined by the clearance into and out of the brain. As shown in Equation 3-7, the contribution of each individual transporter, including OAT3, BCRP and MRP4, to the overall brain elimination of antifolates will depend on its magnitude of transport activity relative to the magnitude of overall efflux on the same side of the membrane. The effect of inhibition of individual transporter on brain distribution of antifolates was described by the drug targeting index value (DTI), which is the ratio of K_p with specific transporter fully inhibited to that in the control case (Equation 3-8).

$$K_p(WT) = \frac{Cl_{influx}}{Cl_{efflux}} = \frac{Cl_{influx,\ abluminal} * Cl_{influx,luminal}}{Cl_{efflux,abluminal} * Cl_{efflux,luminal}} = \frac{(PS + Cl_{in})(PS + Cl_{in})}{(Cl_{OAT3} + PS) * (Cl_{BCRP} + Cl_{MRP4} + PS)}$$

(Equation 3-7)

$$DTI = \frac{K_p(\text{with efflux transport inhibited})}{K_p(\text{control})} = \frac{(AUC_{brain}/AUC_{plasma})_{\text{with inhibited efflux transport}}}{(AUC_{brain}/AUC_{plasma})_{control}}$$

(Equation 3-8)

If we assume that OAT3 and MRP4 have the greatest transport activity for the antifolates ($Cl_{MRP4} = Cl_{OAT3} = 20$), and BCRP and the influx transporter have moderate transport activity ($Cl_{BCRP} = Cl_{in} = 4$), both of which are markedly higher than that of passive diffusion ($Cl_{PS} = 1$), then the enhancement in brain distribution of antifolates due to the inhibition of each efflux transporter can be calculated by the following equations (Equation 3-9 ~Equation 3-12).

$$DTI(\text{OAT3 inhibition}) = \frac{K_{p\ (\text{OAT3 inhibition})}}{K_{p\ (\text{control})}} = 1 + \frac{Cl_{OAT3}}{PS} = 1 + \frac{20}{1} = 21$$

(Equation 3-9)

$$DTI(\text{BCRP inhibition}) = \frac{K_{p\ (\text{BCRP inhibition})}}{K_{p\ (\text{control})}} = 1 + \frac{Cl_{BCRP}}{Cl_{MRP4} + PS} = 1 + \frac{4}{20 + 1} = 1.2$$

(Equation 3-10)

$$DTI(\text{MRP4 inhibition}) = \frac{K_{p\ (\text{MRP4 inhibition})}}{K_{p\ (\text{control})}} = 1 + \frac{Cl_{MRP4}}{Cl_{BCRP} + PS} = 1 + \frac{20}{4 + 1} = 5$$

(Equation 3-11)

$$\text{DTI (BCRP and MRP4 inhibition)} = \frac{K_{p\ (BCRP,\ MRP4\ inhibition\ )}}{K_{p\ (control)}} = 1 + \frac{Cl_{BCRP} + Cl_{MRP4}}{PS} = 1 + \frac{4+20}{1} = 25$$

(Equation 3-12)

As shown from this simulation study, the inhibition of OAT3 resulted in a 21-fold increase in brain penetration of antifolates compared to the control. This is because the magnitude of OAT3-mediated transport is much greater than passive diffusion; therefore, the defect of OAT3 leads to a dramatic reduction in the overall abluminal efflux clearance. On the luminal membrane, inhibition of BCRP only improved the drug accumulation to the brain by 1.2-fold. It is reasonable that the impact of BCRP is not significant, considering that MRP4 makes a greater contribution to the net efflux of antifolates than BCRP at the BBB, as we assumed in this simulation study (Cl_{MRP4}= 20 > Cl_{BCRP}= 4). However, given that BCRP-mediated efflux is greater than the passive diffusion (Cl_{BCRP}= 4 > Cl_{PS}= 1) at the BBB, BCRP can play a more significant role when the function of MRP4 is restrained. This is why the brain distribution of the antifolates increased only by 5-fold when MRP4-mediated transport is fully blocked, despite that intrinsic transport activity of MRP4 for antifolates (Cl_{MRP4}= 20) is assumed to be the same as that of OAT3 (Cl_{OAT3}= 20). Inhibition of both BCRP and MRP4 led to a 25-fold increase in the brain distribution of antifolates. This apparent synergistic effect is determined by the magnitude of MRP4- and BCRP-mediated efflux compared to passive diffusion. Therefore, as illustrated in this simulation study, the contribution of individual transporter to brain penetration of its given substrate is determined by its impact on the net efflux on the

same side of the membrane. If the transporter is not a dominant driving force compared with others, such as BCRP in this example, the importance this transporter can be underestimated by using single knockout mice. Under such circumstance, the combination knockout mice are invaluable tools for these types of studies (Lagas et al., 2009).

3.6 Conclusion

In summary, the present study examined the role of active efflux transport at the BBB in the brain distribution of PMX and MTX. The results of this study revealed that both MTX and PMX undergo saturable efflux from brain, and MTX is eliminated from brain more rapidly than PMX. Of the two transporters investigated in this study using gene knockout mice, MRP2 does not play a role in the brain clearance of both antifolates and BCRP makes a significant contribution to the brain elimination of MTX, but not PMX. In addition, it was observed that the brain-to-blood transport of both antifolates was sensitive to probenecid and benzylpenicillin, suggesting the involvement of organic anion transporter, possibly OAT3. It is likely that one of the underlying mechanisms of the low brain distribution of these antifolates is due to the active efflux transport by BCRP and/or a benzylpenicillin sensitive transport system. Therefore, reducing the efflux transport from the brain by potent inhibitors may be a possible strategy to enhance brain delivery of these antifolates.

3.7 Acknowledgements

We want to thank Tom Raub (Eli Lilly and Company) for providing BCRP and MRP2 knockout mice.

I would like to thank Maureen Riedl for kindly teaching me the intracerebral microinjection technique and Sagar Agarwal for helping me with the mice surgery.

Table 3-1 Effect of unlabeled PMX on the efflux of [^{3}H]-PMX from mouse brain at 30 minutes postdose

PMX concentration in the injectate (mM)	PMX concentration in the brain (mM)[a]	100-BEI (%)
Control (trace only)	-	51.1 ± 5.1
1	0.025	71.7 ± 7.1 ** [b]
50	1.253	95.7 ± 4.8 *** [b]

a. The brain concentration was estimated from the injectate concentration divided by the dilution factor, i.e., 39.9, which was reported previously (Kakee et al., 1996)

b. **, $p < 0.01$ compared with control; ***, $p < 0.001$ compared with control

Table 3-2 Effect of various treatments on brain-to-blood transport of PMX

PMX	concentration in the injectate (mM)	concentration in the brain[a] (mM)	BEI (%)[b]	Percent of control (%)
WT (control)	-	-	48.9 ± 5.1	100.0
MRP2 $^{(-/-)}$	-	-	58.3± 3.3 [c]	100.4[d]
BCRP $^{(-/-)}$	-	-	43.9 ± 2.1	89.7
WT + PMX	1	0.025	28.3 ± 7.1***	57.8
WT + PMX	50	1.253	4.3 ± 4.8 ***	8.8
WT + probenecid	100	2.506	27.6 ± 3.3 ***	56.5
BCRP $^{(-/-)}$ + probenecid	100	2.506	19.6 ± 6.9 ***	40.1
WT + benzylpenicillin	100	2.506	12.2 ± 4.0 ***	25.0

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. % of PMX eliminated from the ipsilateral cerebrum at 30 minutes

c. % of PMX 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 3-3 Effect of various treatments on brain-to-blood transport of MTX

MTX	concentration in the injectate (mM)	concentration in the brain (mM)	BEI (%)[a]	Percent of control (%)
WT (control)	-	-	63.8 ± 5.2	100.0
MRP2 $^{(-/-)}$	-	-	57.2 ± 6.0	90.0
BCRP $^{(-/-)}$	-	-	46.4 ± 7.4 **	72.7
WT + probenecid	100	2.506	24.2 ± 8.6 ***	37.9
BCRP $^{(-/-)}$ + probenecid	100	2.506	24.2 ± 1.8 ***	37.9
WT + benzylpenicillin	100	2.506	13.7 ± 8.5 ***	21.4

a. % of MTX eliminated from the ipsilateral cerebrum at 30 minutes

**, $p < 0.01$; ***, $p < 0.001$

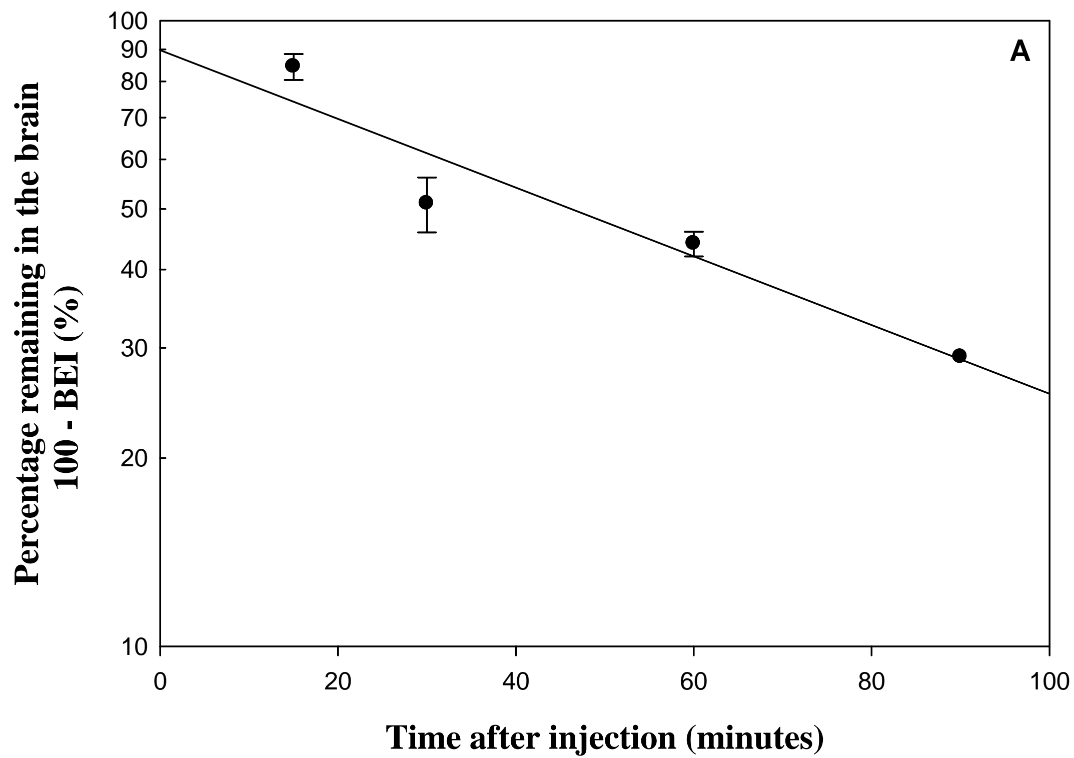


Figure 3-1A The percentage of [^{3}H]-PMX remaining in the ipsilateral cerebrum for up to 90 minutes after intracerebral injection. The solid line was obtained by the regression analysis. Data are mean ± S.D. (n=3~4)

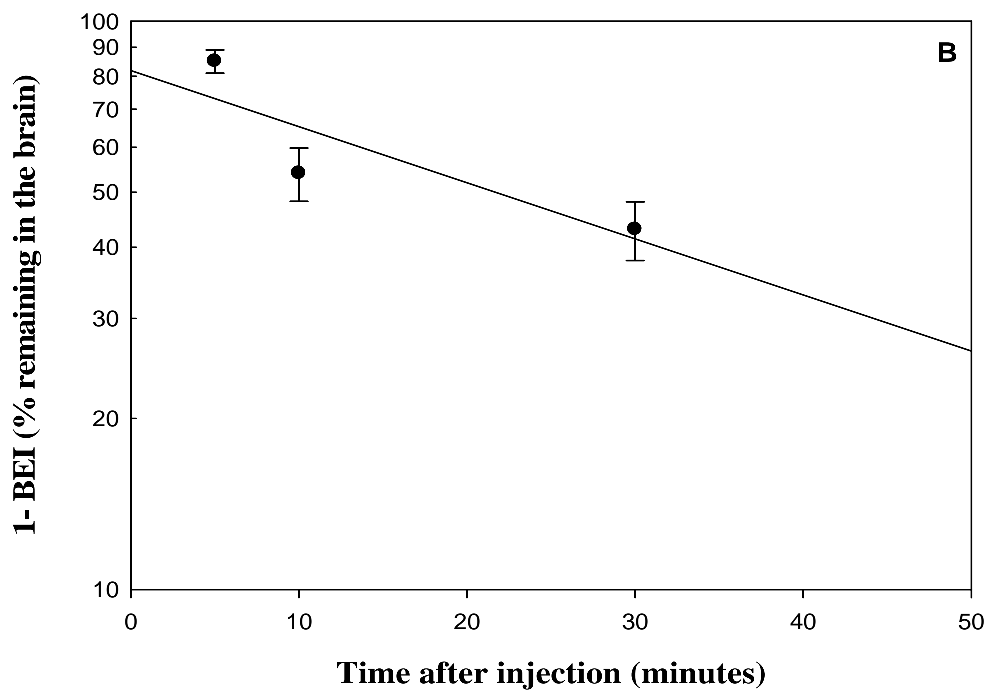


Figure 3-1B The percentage of [^{3}H]-MTX remaining in the ipsilateral cerebrum for up to 30 minutes after intracerebral injection. The solid line was obtained by the regression analysis. Data are mean ± S.D. (n=3~4)

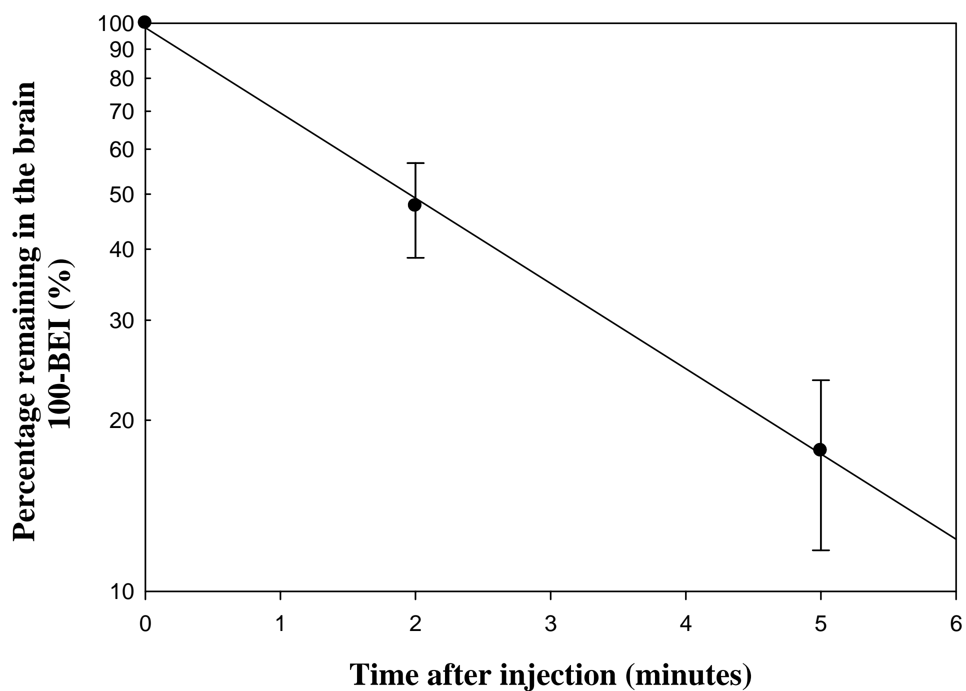


Figure 3-2 The percentage of [^{3}H]-valproic acid remaining in the ipsilateral cerebrum for up to 5 minutes after intracerebral injection. The solid line was obtained by the regression analysis. Data are mean ± S.D. (n=3~4)

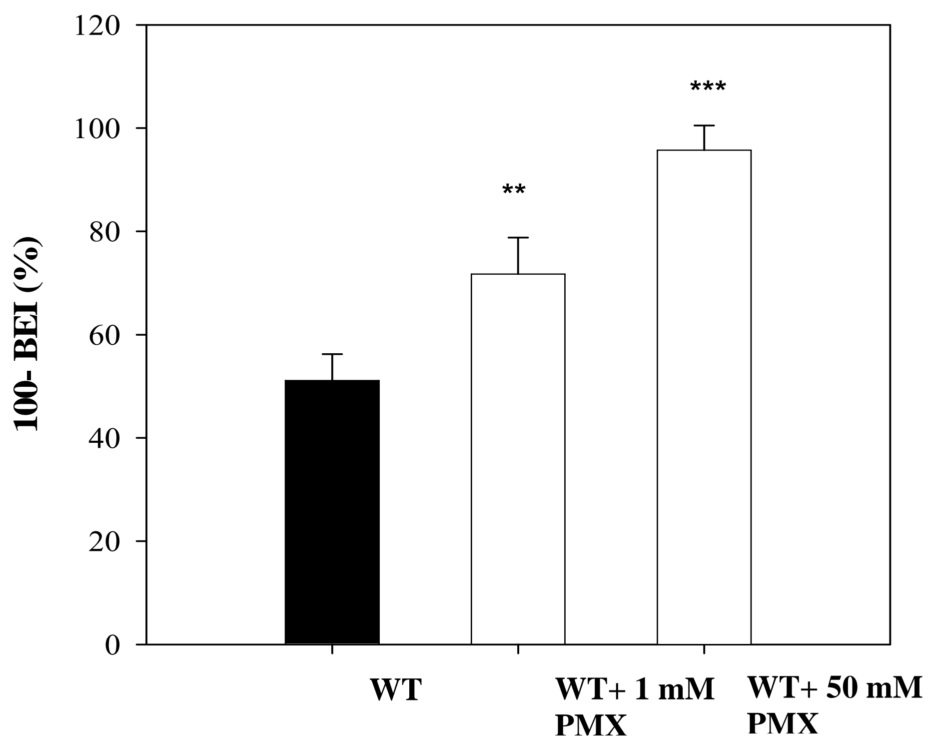
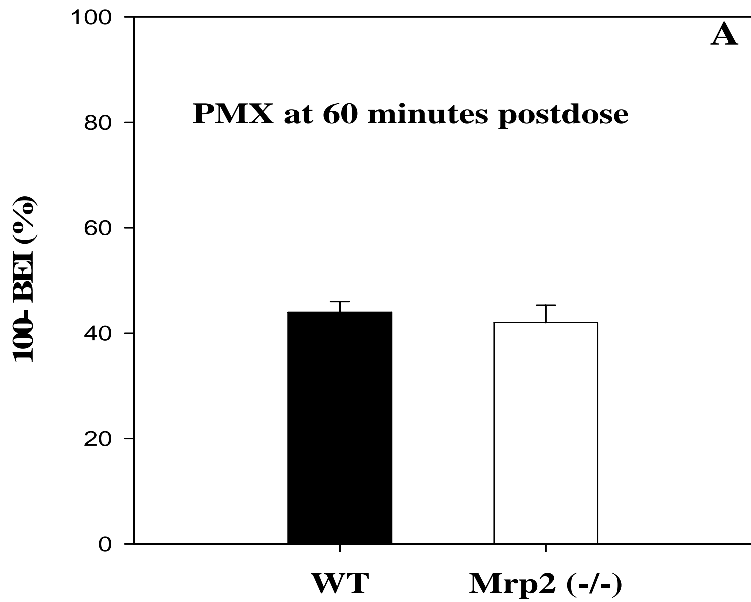


Figure 3-3. Inhibitory effect of unlabeled PMX (1 or 50 mM in the injectate) on the remaining percentage of [^{3}H]-PMX in the brain of wild-type mice at 30 minutes. Data are mean ± S.D. (n=3~4). (**, $p < 0.01$ compared with WT control; ***, $p < 0.001$ compared with WT control)



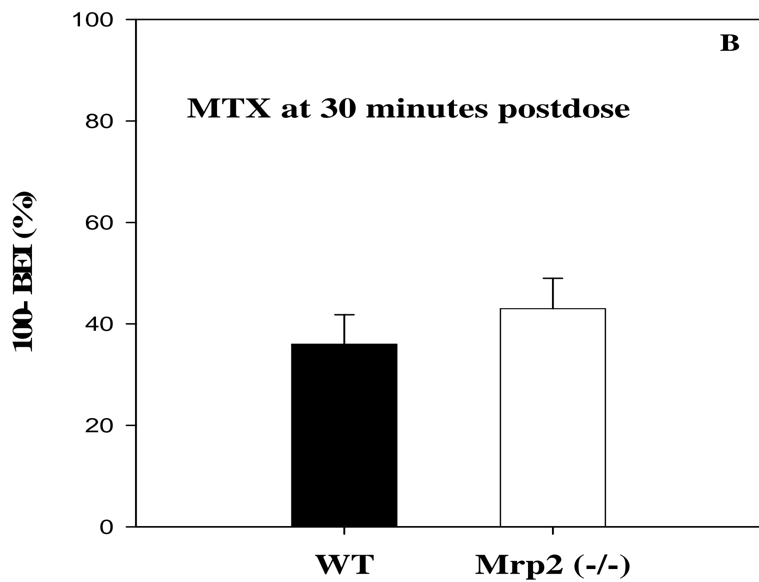


Figure 3-4 The remaining percentage of [^{3}H]-PMX and [^{3}H]-MTX in the brain of wild-type mice and MRP2 deficient (*Mrp2* (-/-)) mice at 60 or 30 minutes after administration.

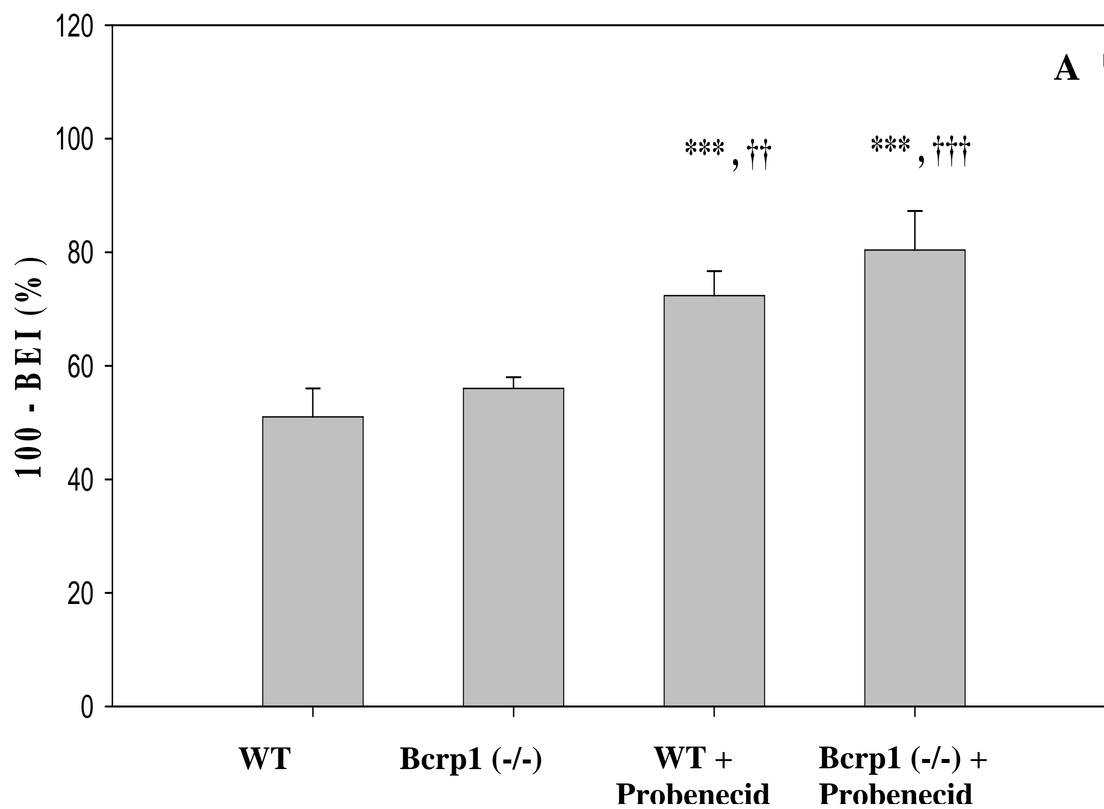


Figure 3-5A. Inhibitory effect of probenecid (100 mM in the injectate) on the remaining percentage of [^{3}H]- PMX in the brain of the wild-type and *Bcrp1(-/-)* mice at 30 minutes. Data are mean ± S.D. (n=3~4). (***, p< 0.001 compared with WT control; †††, P< 0.001, compared with *Bcrp1*(-/-) control; ††, P< 0.01, compared with *Bcrp1*(-/-) control)

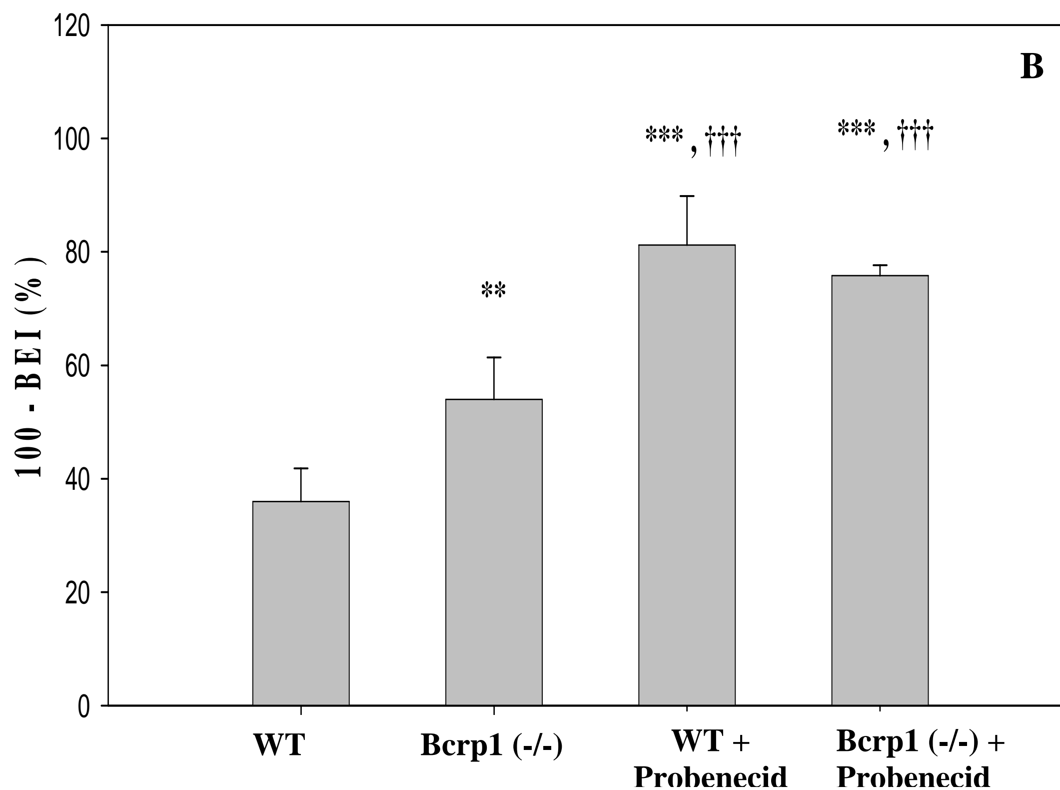
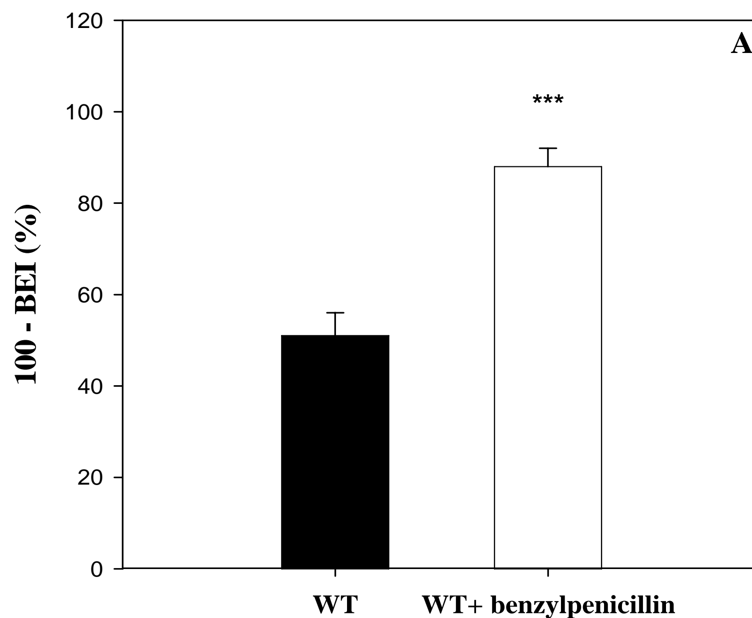


Figure 3-5B. Inhibitory effect of probenecid (100mM in the injectate) on the remaining percentage of [^{3}H]-MTX in the brain of the wild-type and *Bcrp1(-/-)* mice at 30 minutes. Data are mean ± S.D. (n=3~4) (***, p< 0.001 compared with WT control; †††, P< 0.001, compared with *Bcrp1* (-/-) control; **, P< 0.01, compared with WT control)



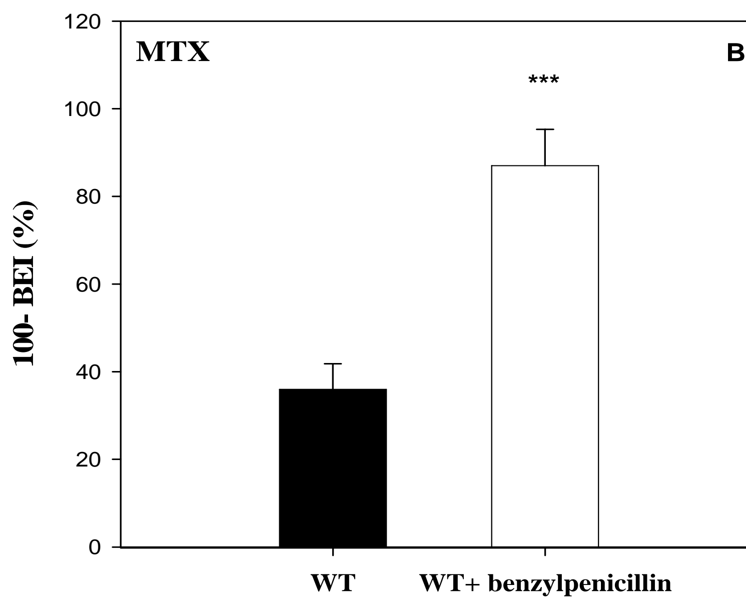


Figure 3-6 Inhibitory effect of benzlpenecillin (100mM in the injectate) on the remaining percentages of [^{3}H]-PMX and [^{3}H]-MTX in the brain of the wild-type mice at 30 minutes. Data are mean ± S.D. (n=3~4) (***, $P < 0.001$)

CHAPTER 4

pH-DEPENDENT TRANSPORT ACTIVITY OF BCRP

Breast Cancer Resistance Protein (BCRP), an ATP-dependent efflux transporter, confers drug resistance to many chemotherapy agents. Given the fact that BCRP is expressed in tumors and physiological sites such as the epithelial lining of the intestine and kidney tubules that have an acidic pH, we examined the effect of pH on BCRP transport activity using pemetrexed (PMX), methothrexate (MTX), and estrone sulfate (ES) as model compounds. The vesicular uptake assay at pH 5.5 showed about 40-fold increase in BCRP-mediated transport activity for PMX and MTX than at pH 7.4. In contrast, only a 1.7-fold increase was observed for estrone sulfate, which has no alteration in the state of ionization within the applied pH. The results suggest the major contributing factor associated with this dramatic increase in the transport activity at low pH is governed by the change of ionization state of substrate molecules. Previous mutation studies have demonstrated the importance of R482 for substrate recognition of ionized substrates at physiological pH. Based on a recent homology model of BCRP, we propose a mode of binding involving direct interactions between the negatively charged D477 with the positive charged moiety of MTX and PMX at low pH, thereby, leading to the dramatic increased in the transport activity at low pH. This study sheds light into the importance of electrostatic interactions in determining the binding affinity of substrates to the transporter protein and has important implications in the design of ionizable chemotherapy drug molecules for tumors in the acidic environment.

4.1 Introduction

Multi-drug resistance (MDR) in tumor cells is a significant obstacle to the success of chemotherapy in cancers. One of the major drug resistance mechanisms for chemotherapy is the active drug efflux from the cell. Several members of these transporters belonging to the ATP-binding cassette (ABC) family have been identified (Diestra et al., 2003; Gatti et al., 2009). Clinically important representatives of this family include P-glycoprotein (P-gp), multidrug resistance-related proteins (MRPs) and Breast Cancer Resistance Protein (BCRP) (Diestra et al., 2003). BCRP is a plasma membrane-bound protein that is genetically encoded by the ABCG2 gene (Doyle et al., 1998; Allen and Schinkel, 2002). It is present in many human tissues including placenta, blood-brain barrier, prostate, small intestine, testis, liver, adrenal gland and stem cells (Doyle et al., 1998; Fetsch et al., 2006). BCRP has been reported to confer drug resistance to many anti-cancer agents such as mitoxantrone, flavopiridol, topotecan and irinotecan (Robey et al., 2001). More recently, it was found that some tyrosine kinase inhibitors (imatinib, gefitinib and erlotinib) and antifolates (methotrexate, tomudex and GW 1843) are also substrates of BCRP (Volk and Schneider, 2003; Shafran et al., 2005; Bram et al., 2006). The implication of BCRP with variety of anticancer agents in various human solid tumors makes BCRP a clinically relevant target for mechanism of drug resistance studies.

Solid tumors exist in a more acidic microenvironment than normal tissues with extracellular pH as low as 5.8 (Tannock and Rotin, 1989; Ojugo et al., 1999). The

causes for the acidic pH environment in tumors are not well-understood. It has been linked to the increased use of the glycolytic pathways and the compromised vasculature of tumor which may lead to the poor removal of lactic acid (Vaupel, 2004). Physiological barriers such as intestinal epithelium and kidney tubule also have an acidic extracellular environment (Shiau et al., 1985; Jaramillo-Juarez et al., 1990). Moreover, it has been reported that the pH of the interstitial fluid in ischemic brain cortex can be as low as pH 6.4 during hypoxia-ischemia with associated hyperglycemia in vivo due to the accumulation of lactic acid (Nedergaard et al., 1991).

Given the information above, it is essential to establish the effect of low pH on the transport activity with respect to drug penetration in tumors, oral absorption, renal excretion, and brain under hypoxia-ischemia. Recently, it has been reported that the transport activities of BCRP for several compounds are greater at the acidic pH (Breedveld et al., 2007). To further examine whether this is a general property of the transporter protein, methothrexate (MTX), pemetrexed (PMX) and estrone sulfate (ES) (**Figure 4-1**) were used as model compounds to explore the effect pH on BCRP transport activity. In addition, the molecular basis of the pH-dependency in BCRP-mediated transport activity was examined based on the recent homology model of BCRP (to be published).

4.2 Materials and Methods

4.2.1 Chemicals

[^{3}H]-pemetrexed, [^{3}H]-methotrexate and [^{3}H]-estrone sulfate were obtained from Moravek Biochemicals (Brea, CA). GF120918 (N-[4-[2-(6, 7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)ethyl]phenyl]-5-methoxy-9-oxo-10H-acridine-4-carboxamide) was a gift from GlaxoSmithKline (Research Triangle, NC). Ko143 (a fumitremorgin C analog) was kindly provided by Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). GF120918 and Ko143 were dissolved in DMSO and diluted to the desired concentration with assay buffer (25 mM $NaHCO_3$, 122 mM NaCl,.10 mM Glucose, 10 mM HEPES, 1.2 mM $MgSO_4$, 3 mM KCl, 1.4 mM $CaCl_2$, and 0.4. mM K_2HPO_4, pH 7.4). The final concentration of DMSO in all reaction solutions was less than 0.1%. All other chemicals used were HPLC or reagent grade.

4.2.2 Cell lines

HEK293 cells transfected with ABCG2-R482, ABCG2-R482G, ABCG2-R482T and plasmid vector were obtained from Dr. Susan E. Bates (National Cancer Institute, Bethesda, MD). Stable transfectants were maintained in Dulbecco's modified Eagle's medium (Mediatech, Inc., Herndon, VA) fortified with 10% heat-deactivated fetal bovine serum (SeraCare Life Sciences, Inc., Oceanside, CA), 100 U/ml penicillin and

100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO) at 37°C under humidity and 5% CO_2 tension. The medium for ABCG2 transfected cells was supplemented with geneticin (G418) at a concentration of 2 mg/ml to maintain positive selection pressure for BCRP expression.

4.2.3 Membrane vesicle preparation.

Vector control or BCRP transfected HEK293 cells were cultured in 500 cm^2 dishes (Corning 431110). When 75% confluence was reached, the cells were washed twice with PBS and scraped into residue PBS. After centrifugation at 1700 rpm at 4°C for 5 min, the cell pellet was diluted with hypotonic buffer containing 1mM sodium bicarbonate at pH 9 and 1% EDTA free protease inhibitors. The cells were allowed to sit and swelled on ice for 5 minutes followed by vigorous shake for 20 times. The resultant cell lysate was centrifuged at 1700 rpm at 4°C for 5 min to remove nuclei, mitochondria and whole cells. The supernatant (crude membrane fraction) was layered over 40% (w/v) sucrose solution and centrifuged at 25000 rpm at 4°C for 30 min. The turbid layer at the interface was collected and suspended in Tris/sucrose buffer (250 mM sucrose containing 50 mM Tris/HCl, pH 7.4), and centrifuged at 25000 rpm for 40 min. The membrane fraction was collected and re-suspended in a small volume (150–250 μl) of Tris-sucrose buffer. The membrane vesicles were made by slowly passing the suspension through 27 gauge needle 20 times. The vesicle containing solution was aliquoted into 100 uL fractions and frozen at -80 ºC. The amount of membrane vesicles was quantified by measuring membrane protein using the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL).

4.2.4 Vesicular uptake study

The vesicular uptake study was performed using rapid filtration method. The uptake was started by addition of 20 μg inside-out membrane vesicles (BCRP expressing vesicles or pc-DNA vector control vesicles) to 20 μL Tris-HCl sucrose buffer containing [^{3}H]-PMX, 20 mmol/L $MgCl_2$, 4 mmol/L ATP, 10mM phosphocreatine, and 100 μg /mL creatine kinase. Reactions were carried out at 37 °C for 60 minutes and stopped by addition of 200 μL ice-cold Tris-HCl sucrose buffer (pH 7.4)]. Vesicles were separated from free drug by passing through 0.22 μm Durapore membrane filters with a 96 channel cell harvester (PerkinElmer, Boston, MA). The filters were washed three times with ice-cold Tris-HCl sucrose buffer and dried at room temperature overnight. Radioactivity was measured by a liquid scintillation counter (TOPCOUNT, PerkinElmer, Boston, MA).

*Time course assays***.** BCRP (ABCG-R) overexpressing vesicles and pc-DNA vector control vesicles were incubated with [3H] PMX for 0, 5, 10, 15, 30, 45 and 60 minutes at 37ºC.

Inhibitor assays. PMX uptake into BCRP or control vesicles was measured in the presence or absence of BCRP inhibitors (200 nM Ko143 or 5 μM GF120918).

Kinetic studies. PMX uptake into BCRP or control vesicles was measured after 15 minutes exposure to 0, 100, 250, 500, 900, 2000, 3350 μM PMX. The same batch of membrane vesicles was used and the vesicular transport assay buffer was adjusted to

pH 5.5 or 6.5. Kinetic parameters (K_m and V_{max}) were determined by nonlinear regression analysis using Sigma Plot (version 9.0.1, SYSTAT software).

*Calculation***.** ATP-dependent uptake was calculated by subtracting uptake measured in the absence of ATP from uptake measured in the presence of ATP. BCRP-mediated transport was calculated by subtracting ATP-dependent uptake into vector control vesicles from the ATP-dependent uptake into BCRP overexpressing vesicles.

4.2.5 Molecular modeling

All molecular modeling studies were carried out using Schrödinger modeling suite package (Schrodinger, LLC., New York, NY). The homology model of BCRP based on the recently solved X-ray structure of p-glycoprotein (PDB 3G5U) (Aller et al., 2009) was a generous gift of Dr. Zsolt Bikadi (to be published). The modeled BCRP structure was energy minimized using generalized Born implicit solvent model with OPLS 2005 forcefield (Jorgensen, 1996) with restraint heavy atoms. The electrostatic potential surfaces of MTX and PMX were evaluated based on the protonated and deprotonated forms of both compounds at pH 5.5 and physiological pH. The pK_a values of PMX, MTX and ES were estimated by ACD pK_a predictor software (Advanced Chemistry Development, Inc., Toronto, Canada).

4.2.6 Statistical analysis

Statistical comparison between two groups was made using the two-sample t-test. Differences were considered to be statistically significant when $P < 0.05$. Multiple groups were compared using ANOVA with Holm-Sidak post-hoc test at $P < 0.05$ level of significance.

4.3 Results

4.3.1 Effect of pH on ATP-dependent BCRP transport activity

At the physiological pH of 7.4, the uptake of PMX in BCRP membrane vesicles was two-fold higher than that of control vesicles. In contrast, the uptake of PMX in BCRP vesicles increased by 20-fold relative to the control vesicles at pH 5.5 (**Figure 4-2A**). By subtracting the uptake in BCRP vesicles from that in control vesicles, BCRP-mediated transport of PMX was 43-fold higher at pH 5.5 than at physiological pH, indicating a dramatically enhanced BCRP transport activity for PMX at lower pH. MTX was used here as a positive control for method validation. Consistent with previous report (Breedveld et al., 2007), a marked increase in BCRP transport of MTX was observed when the pH was decreased from 7.4 to 5.5 (**Figure 4-2B**). The apparent pH sensitivity of BCRP might be due to the change in the protein activity at different pH or reflect the ionization state of the substrate molecules. To elucidate the mechanism of the pH dependency of BCRP, the effect of pH on BCRP-mediated transport of estrone sulfate, which has a constant ionization status at applied pH, was studied (**Figure 4-3**). As pH decreased, a slight increase in the uptake of ES by BCRP was observed. However, the relative change in BCRP transport activity of estrone sulfate at pH 7.4 and pH 5.5 was only 1.7-fold, which was quite mild as compared to the 40-fold increase as observed in the BCRP transport of PMX and MTX.

4.3.2 Effect of BCRP inhibitors on PMX and MTX accumulation into vector control and BCRP expressing membrane vesicles at low pH

In order to confirm that the markedly increased uptake of PMX and MTX into BCRP vesicles at low pH was mainly associated with BCRP-mediated transport, inhibition studies of BCRP were carried out (**Figure 4-4**) (**Figure 4-5**). Specific BCRP inhibitors Ko143 (200nM) significantly decreased the uptake of both PMX and MTX into BCRP membrane vesicles, strongly indicating that at lower pH, the uptake into the vesicles is a BCRP-mediated process. BCRP inhibitor GF120918 (5µM) was also able to attenuate the difference between uptake in BCRP vesicles and control vesicles, suggesting the higher level of uptake at lower pH was mainly attributed to the increased BCRP transport activity for PMX and MTX.

4.3.3 Effect of pH on PMX uptake kinetics

To characterize the effect of pH on BCRP mediated transport kinetics of PMX, a concentration dependent study was performed at pH 5.2 versus pH 6.5. From the time course of PMX uptake determined at pH 5.5 (**Figure 4-6**), a 15 minute uptake time was chosen to provide an estimate of the linear uptake rate and was used to determine the concentration-dependent uptake of PMX. As shown in **Figure 4-7**, BCRP uptake of PMX in concentration range from 0 to 5 mM follows Michaelis-Menten kinetics, and the parameters $\mathbf{K_m}$ and V_{max} are shown in **Table 4-1**. The BCRP-mediated transport of PMX followed Michaelis-Menten kinetics that were pH-dependent with a $\mathbf{K_m}$ of 0.39 ± 0.08 mM and $\mathbf{V_{max}}$ of 4725 ± 279 pmol/mg/min at pH 5.2 and a $\mathbf{K_m}$ of 1.47 ± 0.35 mM and $\mathbf{V_{max}}$ of 1993 ± 213 pmol/mg/min at pH 6.5.

4.3.4 Effect of R482 mutation on pH-dependent BCRP transport

To address the mechanism of pH-dependency, the effect of pH on the transport activity of BCRP mutants was examined (**Figure 4-8**). At physiological pH, wild-type BCRP (R482) vesicles exhibited significant PMX uptake compared to control vesicles. In contrast, the uptake of PMX in mutant BCRP (R482G, R482T) vesicles was not significantly different from that in control vesicles, indicating that R482G and R482T mutations lead to abolished BCRP transport activity for PMX. Interestingly, at pH 5.5, both mutant forms of BCRP exhibited significant higher uptake of PMX relative to the control vesicles, suggesting the transport activity of mutant BCRP for PMX at lower pH (**Figure 4-8A**). The extent of increase, calculated as the ratio of uptake at pH 5.5 over the uptake at pH 7.4, was about 61, 12 and 6 for R482, R482G and R482T, respectively. A similar trend was observed for MTX, i.e., stimulated transport activity of mutant BCRP(R482G, R482T) for MTX at lower pH, and the greatest extent of pH-dependency was observed in wild-type BCRP (**Figure 4-8B**). In contrast, wild-type and mutant BCRP exhibited similar extent (1.5-fold) of increase in transport activity for estrone sulfate as pH decreased from 7.4 to 5.5 (**Figure 4-9**).

4.3.5 BCRP R482 binding site

BCRP is a homodimeric transport membrane protein consisting of an extracellular neocleotide binding (NB) domian and a transmembrane helical (TM) domain in each of its monomeric subunit. The TM domain consists of six transmembrane helices

with R482 located in the helix III (H3) region along the transport channel of BCRP (**Figure 4-11A**). To understand molecular basis for the increase in the binding selectivity of PMX and MTX, the BCRP binding site consisting of R482 was closely examined for possible key residues that can provide significant interaction with the charged carboxylate and pteridinium groups of PMX and MTX. Residues D477, R465 and H630 were identified to be the only ionizable residues within 10Å radius of R482 located within the transport channel of BCRP (**Figure 4-11C**). The nearest interatomic distance between R482 and R465 guanidinium groups was 6.9Å similar to the interatomic distance of 6.0Å of the carboxylate atoms in energy minimize conformation of PMX (**Figure 4-11B**). H630, which was located on the helix VI region further inside along the BCRP transport channel, has a nearest interatomic side-chain distances to R482 and R465 of 6.0Å and 9.5Å. D477, which was located on the transmembrane helix III region at the interface between the TM and NB domains, has a nearest interatomic side-chain distances to R482 and R465 of 6.9Å and 8.7Å. The nearest interatomic side-chain distances between the arginines to H630 and D477 were found to be similar to the interatomic distances of pteridine and carboxylate groups in PMX.

4.4 Discussion

pH-dependent transporters and the mechanisms of pH-dependency

It is well known that pH plays an important role in modulating the functional activity of proteins. This fact applies to many transporters as well. Studies of Nozawa, et. al. have shown that human organic anion transporting polypeptide OATP-B (SLC21A9) exhibits pH-sensitive transport activity for various organic anions (Nozawa et al., 2004). Human oligopeptide transporter 1 (hPEPT 1) exhibits a bell-shaped activity with an optimal pH of 5.5 (Fujisawa et al., 2006). The pH-dependent transport was also reported for proton-coupled folate transporter (PCFT, SLC46A1) and organic cation transporters such as OCT1 (SLC22A1) and OCT2 (SLC22A2) (Urakami et al., 1998; Fujita et al., 2006; Zhao and Goldman, 2007). There are different mechanisms for the pH-dependent transport activity, including proton coupled transport, membrane potential dependent transport and the pH-dependent ionization of key amino acid residues at the active binding sites. The latter is most often observed for the amino acid histidine. With an intrinsic pKa of 6.1, hisitidine can interact differently with substrate molecules depending on its protonation state at various pHs, thereby playing an important role in pH-sensitive transporters such as PCFT and PEPT. (Said and Mohammadkhani, 1993; Fei et al., 1997; Metzner et al., 2008; Unal et al., 2009).

Characterization of pH-dependent transport using uptake assays

The objective of current study was to examine the effect of pH on BCRP transport activity and to further characterize the possible mechanism of pH sensitivity of BCRP. Toward this end, we examined the transport activity of BCRP at different pH's using PMX, MTX and ES as model BCRP substrates. PMX (Alimta®) is a novel antifolate and has been demonstrated to be a BCRP substrate from our previous studies (manuscript submitted, see chapter two). The vesicular uptake assays showed lowering the pH from 6.5 to 5.2 led to a significantly increased affinity of PMX for BCRP as shown by K_m values, and also a markedly increased V_{max}. In addition, BCRP-mediated transport of PMX was 43-fold higher at acidic pH (5.5) as compared to at physiological pH (7.4). However, for estrone sulfate, the pH-dependence of transport behavior was quite different. The results from uptake studies showed that lowering pH from 7.4 to 5.5 led to only 1.7-fold increase in BCRP transport of estrone sulfate.

Effect of ionization on the transport activity for PMX at lower pH

With a pK_a of 2, estrone sulfate is predominantly present in its anionic form with a constant negative charge from pH 7.4 to pH 5.5 (**Figure 4-10**). As such, any change in the uptake of estrone sulfate from pH 7.4 to pH 5.5 should be attributable to a pH-induced change in the transporter protein. Therefore, the very mild pH-effect observed for the transport of estrone sulfate indicates that, unlike other pH-sensitive transporters, the general transport activity of BCRP is not significantly affected by pH. Conversely, the dramatic pH-dependent transport observed in MTX and PMX may be

mainly due to the change in the ionization state of the substrate and its subsequent interaction with BCRP.

PMX is a polyelectrolyte carrying two carboxyl groups, with pK_a of 3.46 (α-carboxyl) and 4.77 (γ-carboxyl), and the guanidinic N-1 on the pterine ring (pKa 5.27) (predicted using ACD software). As shown in **Figure 4-10**, at physiological pH, PMX exists in a predominantly negatively charged form due to the deprotonation of the two carboxyl groups. When the pH is lowered from 7.4 to 5.5, there is a negligible change in the ionization of carboxyl groups (i.e., from 99% to 97%), but a marked increase in ionization of nitrogen group (i.e., from 0% to 30%). It is possible that the enhanced transport of PMX at low pH is due to the increased positive charge in the drug molecule, which may lead to the stronger electrostatic interaction with BCRP.

Importance of R482 in the pH-dependent transport of PMX, MTX and estrone sulfate

The amino acid at position 482 has been reported to be a hot spot for substrate specificity of BCRP and plays a crucial role in MTX transport (Honjo et al., 2001; Allen et al., 2002a). Results from the current uptake studies showed that R482 was also important for the recognition of PMX and estrone sulfate. To characterize the role of R482 in the observed pH-dependency of BCRP, we measured the effect of low pH on the transport activity of polymorphic BCRP variants at 482 for PMX and estrone sulfate. For estrone sulfate, both mutant (R482G, R482T) and wild-type

(R482) BCRP exhibited a similar extent of increase in the transport activity, suggesting that R482 did not involve in the pH-dependent transport of estrone sulfate. This is reasonable based on the fact that the intrinsic pK_a of arginine is 12. Therefore, with constant ionization state through the applied pH, R482 should not produce any change in the electrostatic property of BCRP and the subsequent interaction with estrone sulfate. Surprisely, for PMX, although both wild-type and mutant forms of BCRP exhibited stimulated transport activity at lower pH, the extent of increase was greatest in wild-type BCRP as compared to BCRP mutants, suggesting the importance of R482 in the pH-dependent transport of PMX.

Electrostatic interactions and potential mode of binding for MTX and PMX

Electrostatic interactions play a critical role in many molecular recognition processes. Substrate molecules can interact electrostatically with the protein via hydrogen bonding, ion-pairing to ionized amino acids as well as $\pi - \pi$ stacking with aromatic residues within the active-site. Based on the homology model of BCRP, residues D477, R465 and H630 were identified to be the only ionizable residues within 10Å radiues of R482 located within the transport channel of BCRP. In the presence of two positive charged R482 and R465 residues, the pK_a's of D477 and H630 were expected to be significantly perturbed and will remain predominantly negatively and neutrally charged respectively at the applied pH of 7.4 and 5.5. This will result in an overall hydrophilic environment for the recognition of ionizable substrates. With the

presence of the doubly negatively charged carboxylate groups as well as an ionizable aromatic pteridine group, electrostatic interactions were expected to play a crucial role in the molecular recognition of PMX and MTX within the binding site. The decrease in Km for PMX from 1.47 mM to 0.39 mM from pH 7.4 to 5.5 further support an overall increase in binding selectivity as PMX undergo protonation at lower pH. The presence of R482 and R465 along the BCRP transport channel suggested a unique molecular recognition mechanism of the doubly negatively charged MTX and PMX substrate which were not observed in ES. The presence of D477 and H630 at distances similar to the distances of the folded PMX conformation suggested possible interaction between PMX's pteridine ring with H630 and D477 side-chain during the transport process resulting in a molecular recognition triad. During the protonation of PMX and MTX at lowered pH, it is expected the electrostatic interaction between the positively charged pteridliinium group with either D477 or H630 would result in enhancement in the binding selectivity. Conversely, when R482 was replaced by either G or T, such unique molecular recognition mechanism of selecting the doubly negatively charged group was abolished leading to only moderate increase in the transport activity of MTX and PMX compare to that of Wild-type BCRP (R482) at lower pH.

4.5 Conclusion

In summary, BCRP-mediated transport of MTX and PMX was markedly enhanced at an acidic pH. For estrone sulfate, only a very mild change in the transport activity was observed. We conclude that the marked difference in the transport activity between these two classes of substrate molecules was primarily due to the change in the ionization state of the substrates at the lower pH. Mutation studies indicated the critical role of R482 in the transport activity of both PMX and MTX. As MTX and PMX were expected to undergo protonation at the lower pH, a possible mechanism for MTX and PMX binding to BCRP was proposed involving R482, R465, D477 and H630. The proposed model provided insights into the charged interactions between the substrate and the transporter proteins and demonstrated the importance of electrostatic interactions involved in the BCRP transport process. This study may have important implications in the handling of PMX and other chemotherapy drug molecules in the acidic environment of tumors and in the distribution and elimination of the drug molecules.

4.6 Acknowledgements

We want to thank Yuk Yin Sham (Center for Drug Design, University of Minnesota) for his help in homology modeling and scientific discussion on this research project. We also would like to thank Susan E. Bates (National Cancer Institute, NIH) for kindly providing HEK293 cells, Dr. Alfred H. Schinkel (Netherlands Cancer Institute) for generously providing Kol43, and GlaxoSmithKline for their gift of GF120918. We also appreciate the help from the scientists at Eli Lilly, in particular, Kate Hillgren's lab (Youngeen Anne Pak) and Anne Dantzig's lab (Susan Pratt) for technical training on membrane vesicle studies.

The University of Minnesota Supercomputing Institute provided all the necessary computational resources for the modeling studies.

Table 4-1. Kinetic parameters for BCRP-mediated uptake of PMX at pH 5.2 and 6.5.

pH	**Km (mM)**	**V_{max}(pmol•min/mg)**
5.2	0.39 ± 0.08	4725 ± 279
6.5	1.47 ± 0.35	1993 ± 213

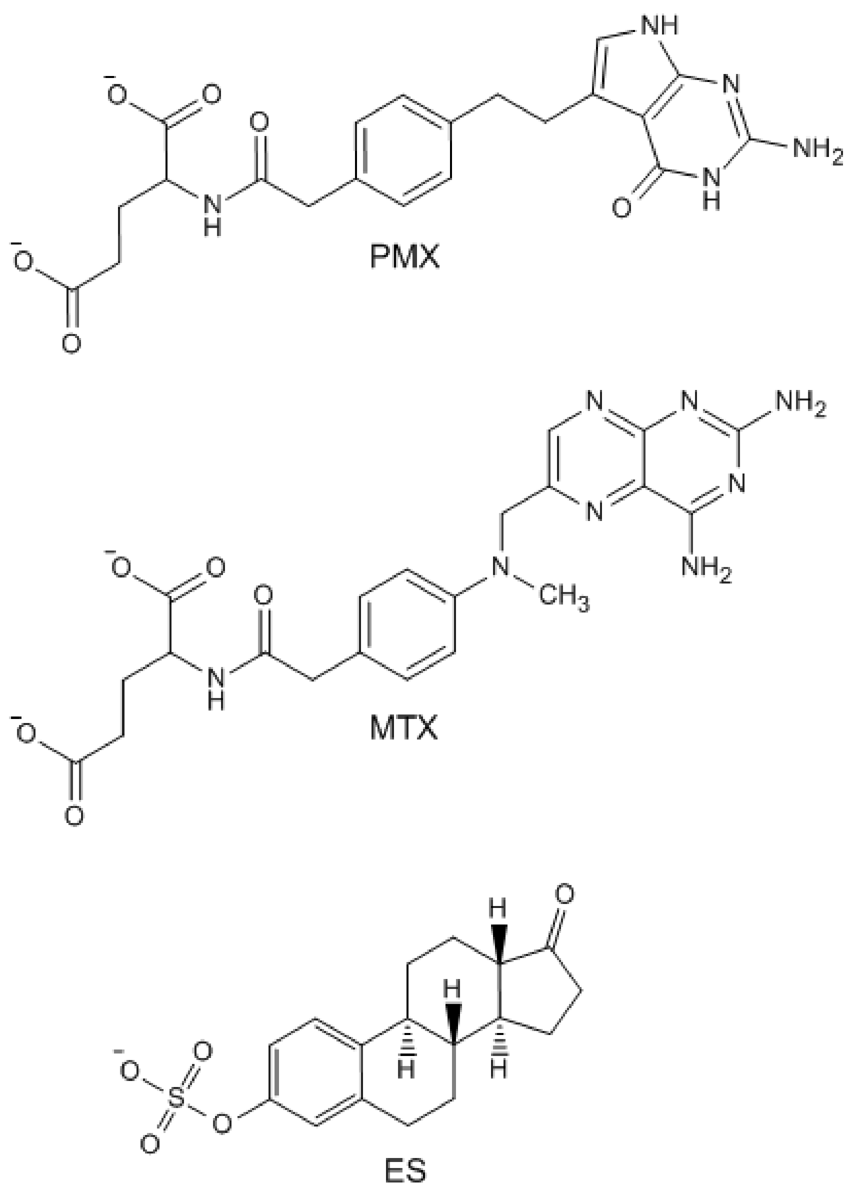


Figure 4-1. Structure of PMX, MTX and estrone sulfate (ES) at pH 7.0

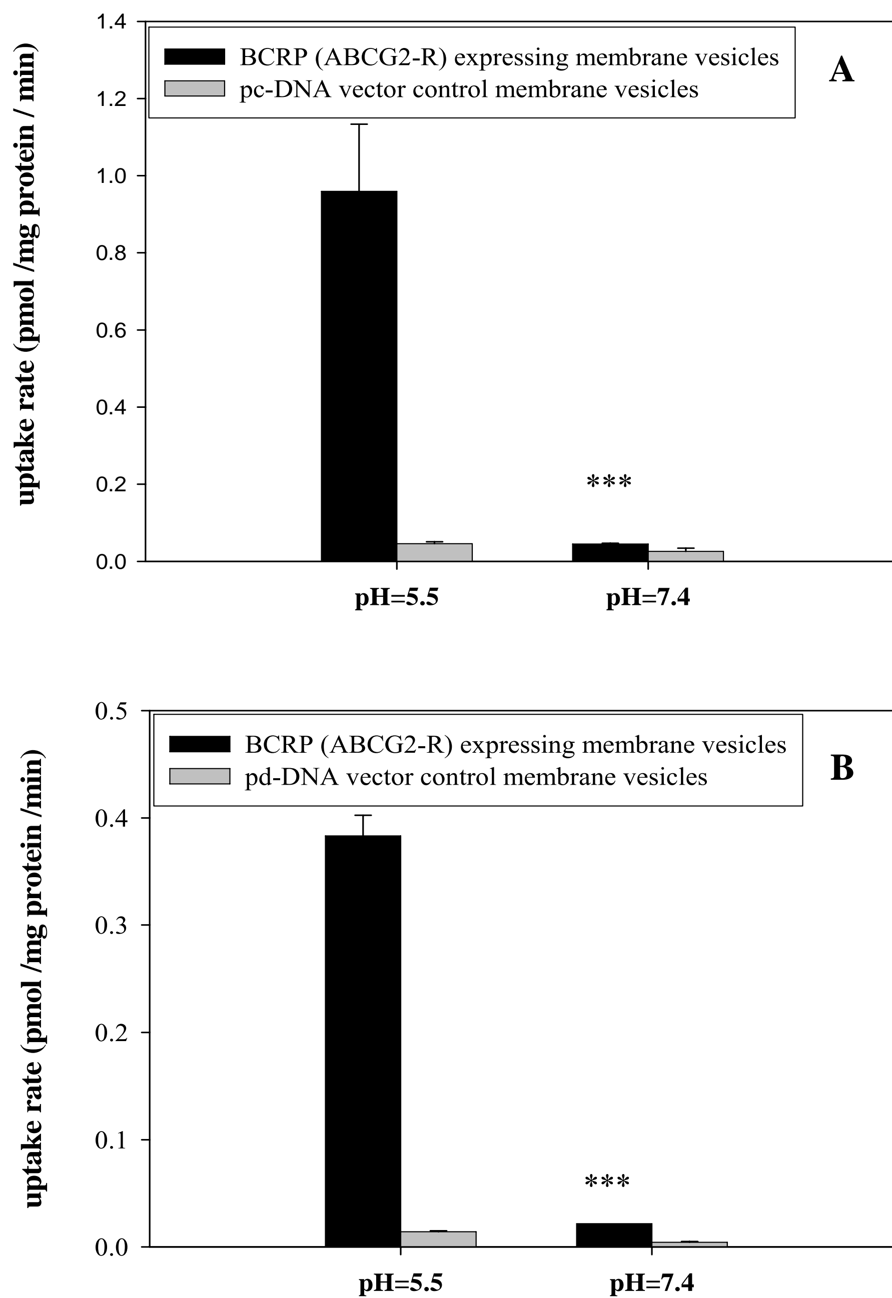


Figure 4-2. Effect of pH on ATP-dependent uptake of PMX (**A**) and MTX (**B**) into BCRP expressing vesicles and control vesicles. HEK293-BCRP membrane vesicles and pc-DNA (wild type) membrane vesicles were incubated with 0.3μM [^{3}H]-PMX or [^{3}H]-MTX at pH 5.5 at 37°C for 60 min in the presence or absence of 4 mM ATP. Values shown are mean ± S.D. of each experiment (n=4). (***, $p<0.001$ compared to the BCRP uptake at pH 5.5.)

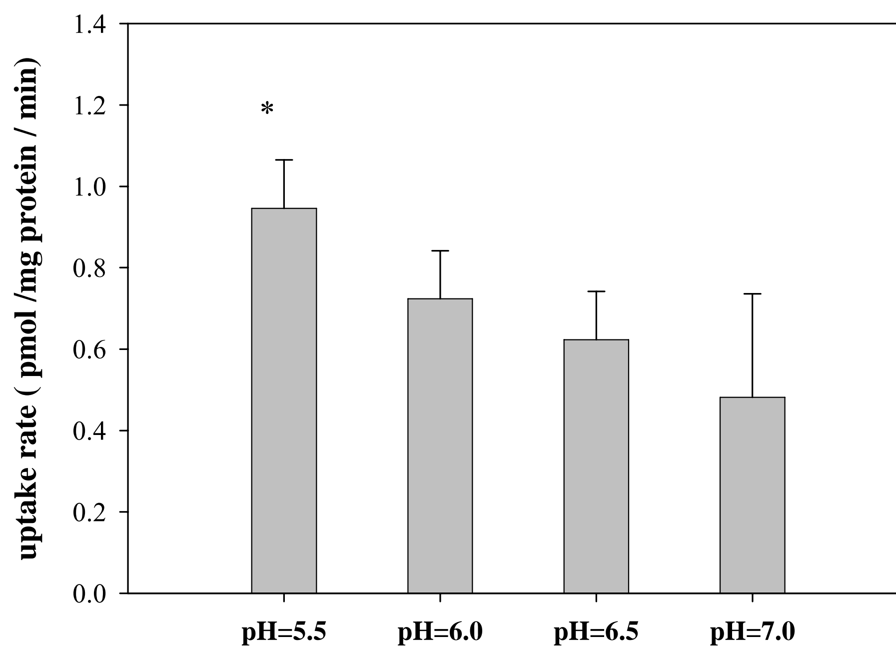


Figure 4-3 Effect of pH on ATP-dependent uptake of estrone sulfate into BCRP expressing vesicles and control vesicles. HEK293-BCRP membrane vesicles and pc-DNA (wild type) membrane vesicles were incubated with 0.03μM [^{3}H]-estrone sulfate at pH 5.5, 6.0, 6.5and 7.0 at 37°C for 10 min in the presence or absence of 4 mM ATP. Values shown are mean ± S.D. of each experiment (n=4) (*, $p<0.05$ compared to the BCRP uptake at pH 7.0.)

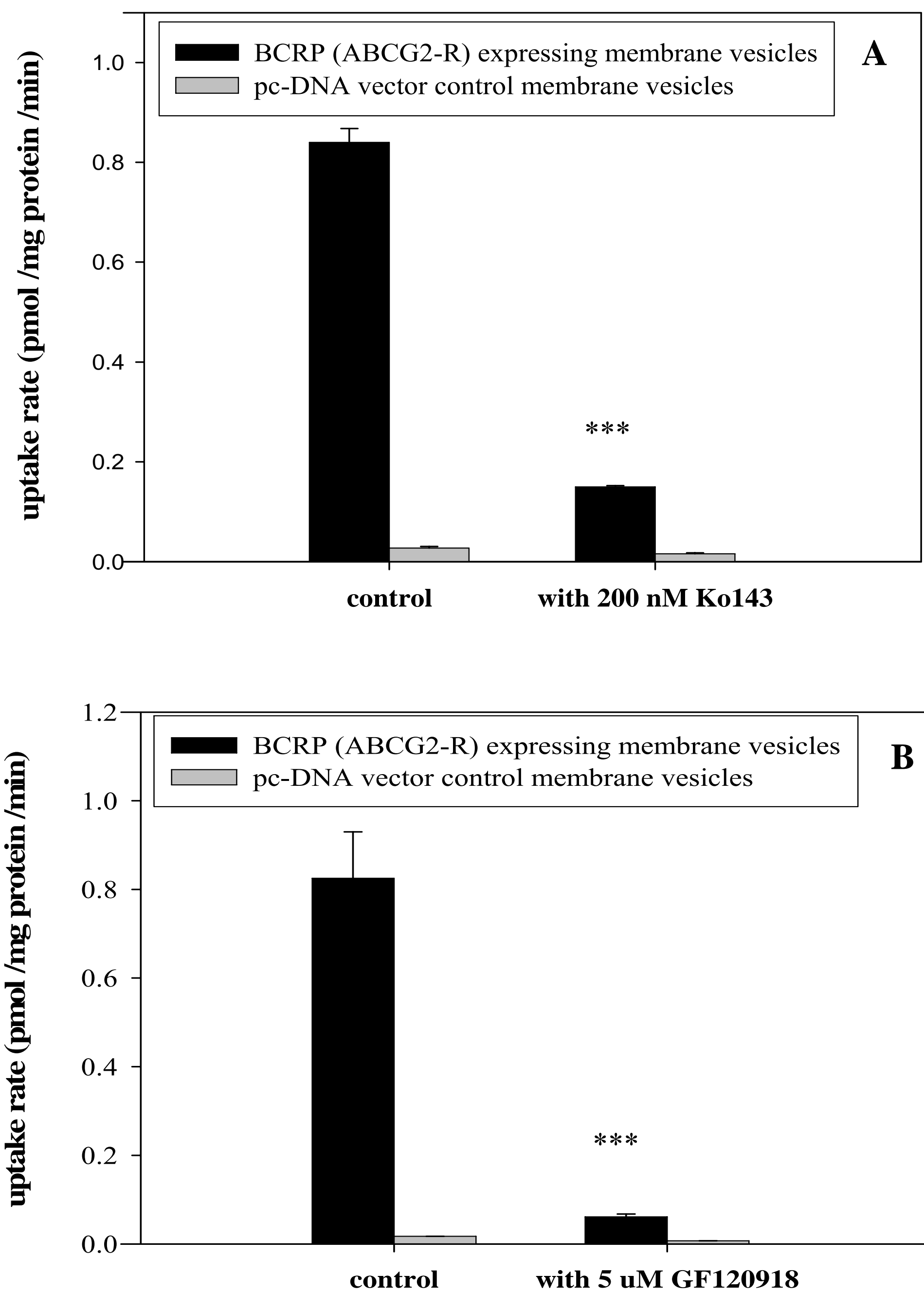


Figure 4-4 ATP-dependent uptake of PMX with and without BCRP inhibitors: 200nM Ko143 (**A**) and 5 μM GF120918 (**B**). HEK293-BCRP membrane vesicles and pc-DNA (wild type) membrane vesicles were incubated with 0.11μM [^{3}H]-PMX at pH 5.5 at 37°C for 60 min in the presence or absence of 4 mM ATP. Values shown are mean ± S.D. of each experiment (n=4) (***, p<0.001 compared to the BCRP uptake without the treatment of BCRP inhibitors.)

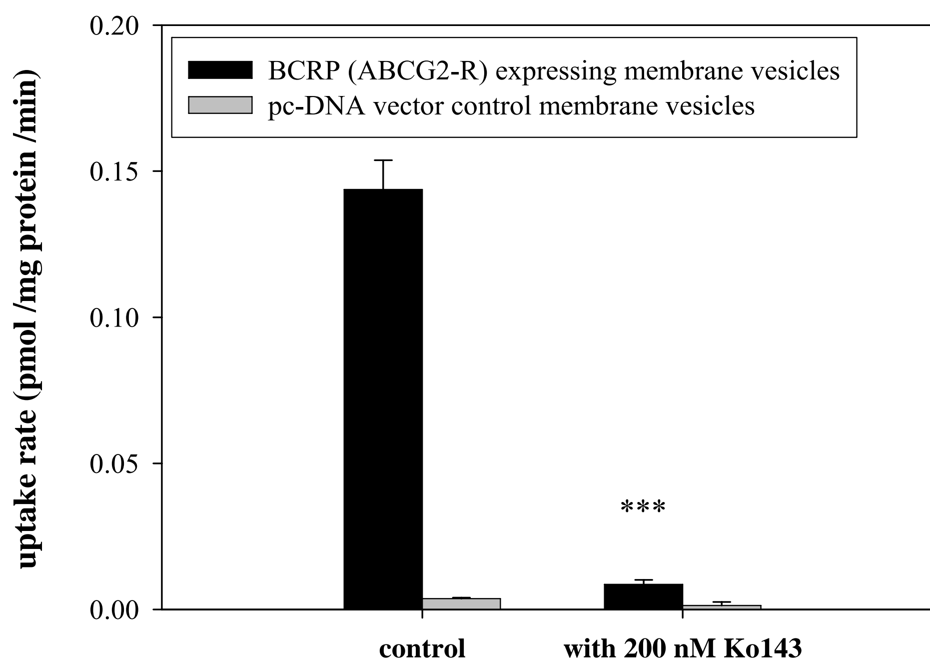


Figure 4-5 ATP-dependent uptake of MTX with and without 200nM Ko143. HEK293-BCRP membrane vesicles and pc-DNA (wild type) membrane vesicles were incubated with 0.02 μM [^{3}H]-MTX at pH 5.5 at 37°C for 60 min in the presence or absence of 4 mM ATP. Values shown are mean ± S.D. of each experiment (n=4) (***, $p<0.001$ compared with BCRP uptake without Ko143.

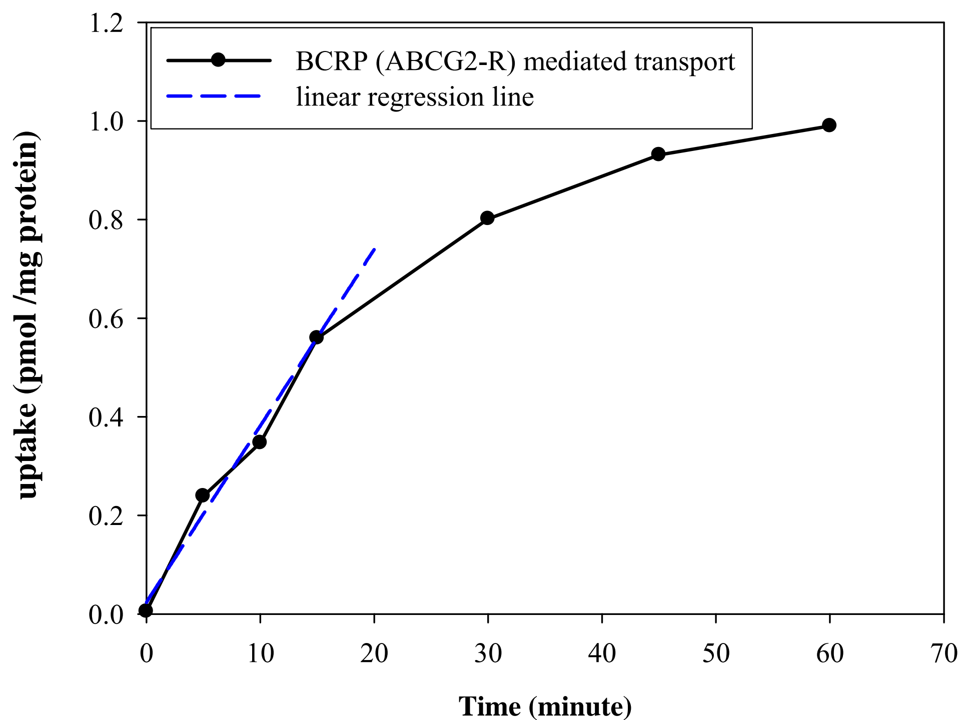


Figure 4-6 Time-dependent uptake study of PMX. HEK293-BCRP membrane vesicles and pc-DNA (wild type) membrane vesicles were incubated with 0.02 μM [^{3}H]-PMX at pH 5.5 at 37°C for 60 min in the presence or absence of 4 mM ATP. The uptake was measured at 0, 5, 10, 15, 30, 45, 60 minutes. Values shown are mean ± S.D. of each experiment (n=2)

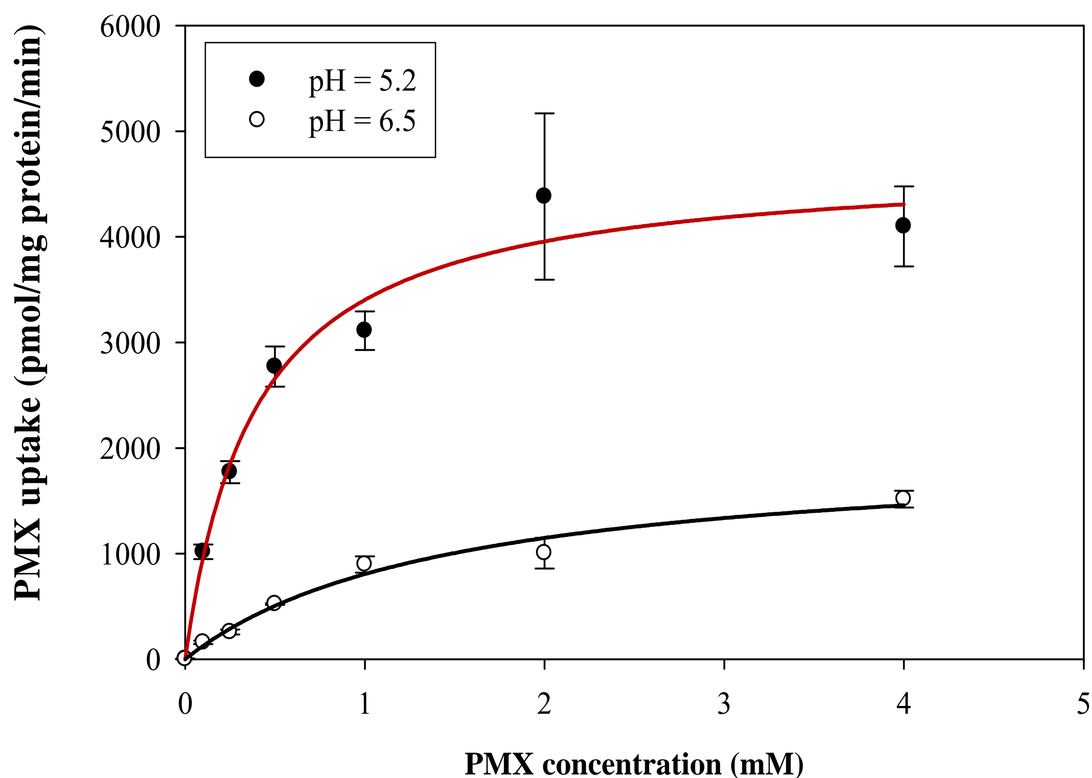


Figure 4-7 Concentration dependent study of PMX. BCRP expressing vesicles and control vesicles were exposed for 15minutes to various concentrations of [^{3}H]-PMX at pH 5.2 and pH 6.5, ranging from 0 to 4 mM, in the presence or absence of ATP. Values shown are mean ± S.D. of each experiment (n=4). The solid line represents a best fit Michaelis-Menton plot of the net initial velocity relative to increasing substrate concentrations.

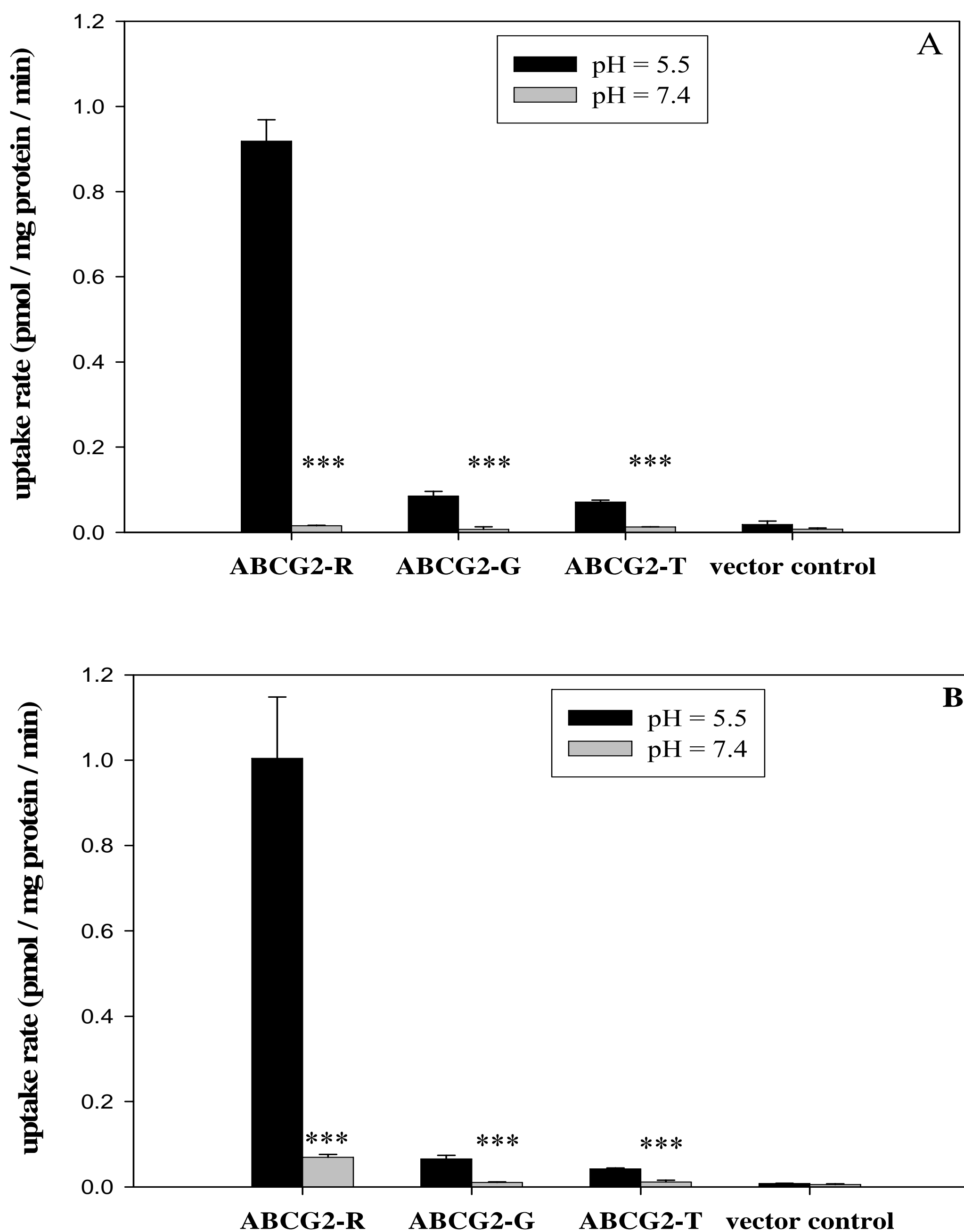


Figure 4-8 Effect of pH on ATP-dependent transport of [^{3}H]-PMX (**A**) and [^{3}H]-MTX (**B**) into BCRP expressing vesicles (wild type BCRP: ABCG2-R and two mutant BCRP: ABCG2-G, ABCG2-T) and control vesicles. HEK293-BCRP membrane vesicles and pc-DNA membrane vesicles were incubated with [^{3}H] PMX or [^{3}H]-MTX at pH 7.4 and 5.5 at 37°C for 60 min in the presence or absence of 4 mM ATP**.** Values shown are mean ± S.D. of each experiment (n=4) (*** represents $p<0.001$, compared with the uptake at pH 5.5)

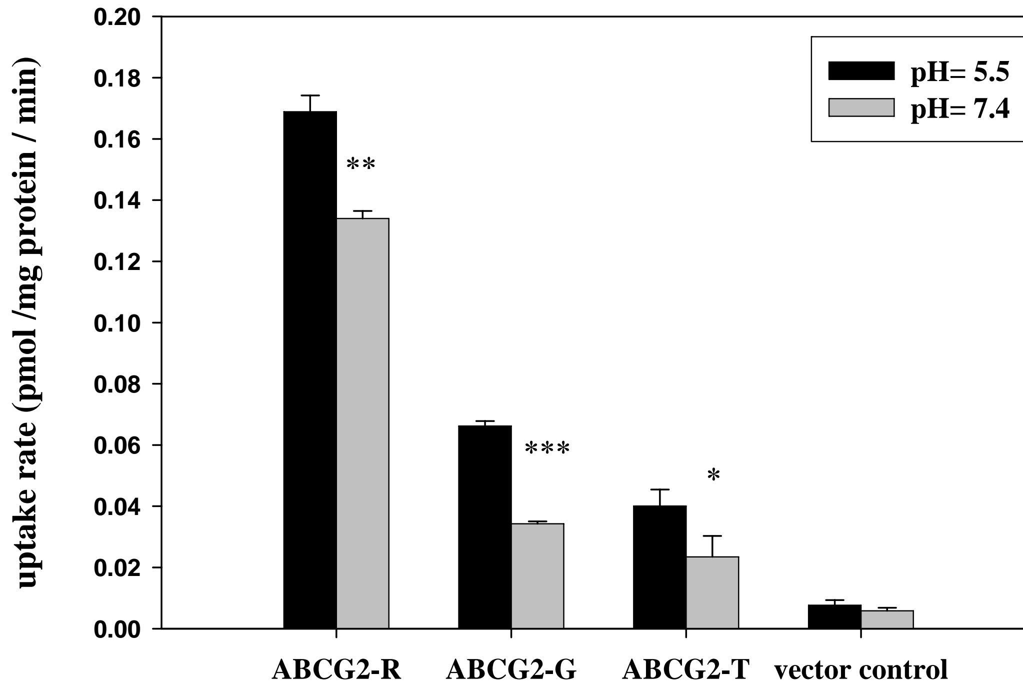


Figure 4-9 ATP-dependent estrone sulfate (ES) accumulation into BCRP expressing vesicles (wild type BCRP: ABCG2-R and two BCRP mutants: ABCG2-G, ABCG2-T) and control vesicles. HEK293-BCRP membrane vesicles and pc-DNA membrane vesicles were incubated with 0.03μM [^{3}H]-estrone sulfate at pH 7.4 and 5.2 at 37°C for 10 min in the presence or absence of 4 mM ATP. Values shown are mean ± S.D. of each experiment (n=4) (* $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ compared with the uptake at pH 5.5)

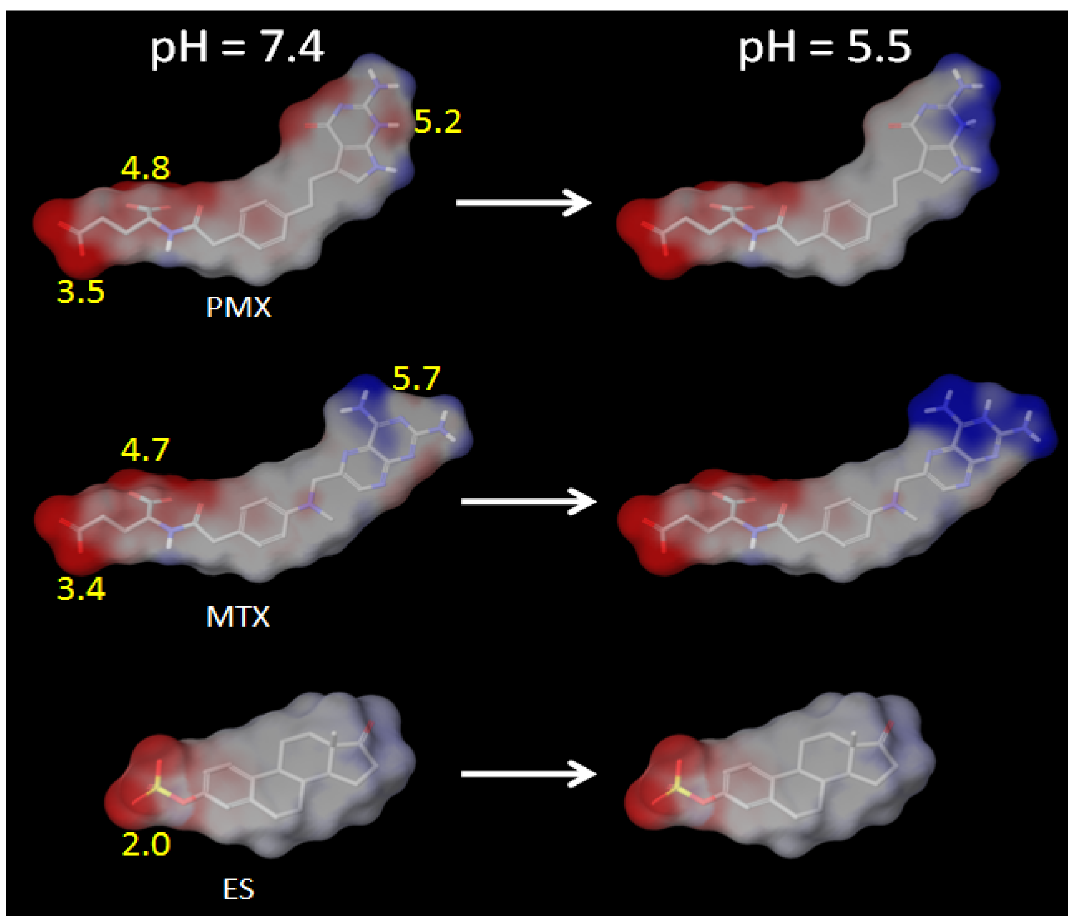


Figure 4-10 Electrostatic potential surface of PMX, MTX and ES at pH 7.4 and 5.5 based on the predicted pK_a's of each ionization functional groups (yellow).

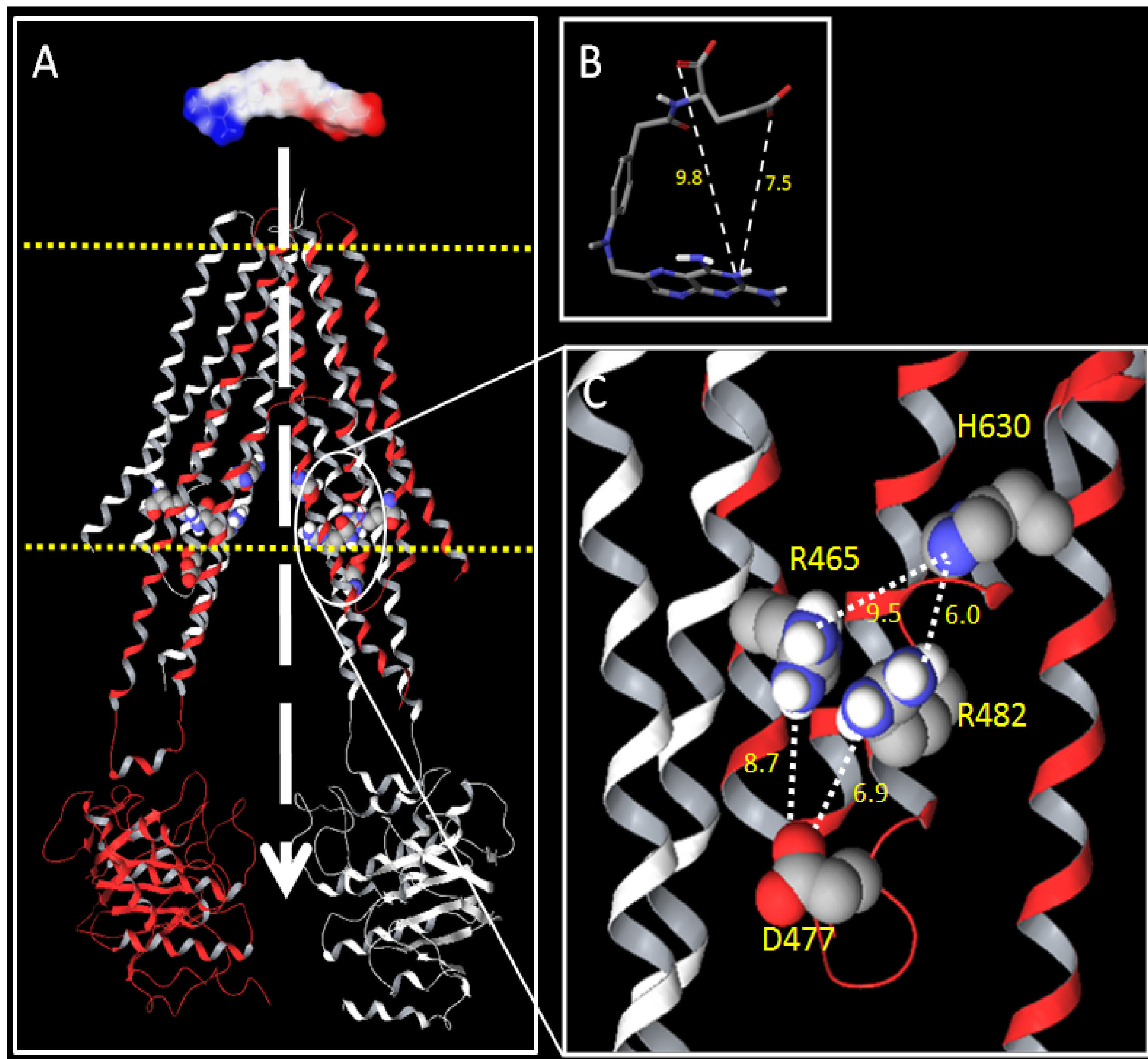


Figure 4-11 (**A**) Homology model of BCRP with its transmembrane helical domains embedded within the phospholipid bilayers (illustrated by the yellow dotted lines). (**B**) An energy minimized folded conformation of PMX. (**C**) BCRP molecular recognition site involving R482 shown to be critical for the selectivity of PMX and MTX at low pH.

CHAPTER 5

RECAPITULATION

Over the last several decades, significant research efforts have led to improved treatment of cancer. However, in spite of overall advance in cancer treatment, the prognosis for patients with malignant primary or metastatic brain tumors remains extremely poor. This led to the opinion that the brain is a pharmacological sanctuary for chemotherapy and the relapse in the CNS may be an important factor for the failure in the treatment of metastatic tumors (Palmieri et al., 2007; Laquintana et al., 2009).

The classic antifolate, methotrexate (MTX) is the most commonly used chemotherapy agent for the treatment of primary CNS lymphoma. During the last decade, a novel multi-targeted antifolate, pemetrexed (PMX, Alimta®) has been approved for the treatment of malignant pleural mesothelioma and non-small cell lung cancer (NSCLC). Given the spectrum of activity of MTX, it can be expected that PMX may have useful applications in the treatment of primary CNS tumor as well with a possible benefit of less acquired resistance. This is also important for the treatment of brain metastases, considering that CNS metastases are a common complication of NSCLC, occurring in approximately 30% to 50% of patients (Taimur and Edelman, 2003). Similar to MTX, the brain distribution of PMX is very limited with steady-state brain-to-plasma unbound concentration ratio of 0.106 ± 0.054 (Devineni et al., 1996; Dai et al., 2005). One important determinant of CNS distribution is the efflux transport of BBB transporters.

The overall objective of this study was to characterize the brain-to-blood efflux transport of PMX and to examine role of efflux transporters such as BCRP, MRP2 and organic anion transporters, in brain distribution of PMX.

To evaluate the specific role of BCRP in the CNS penetration of PMX, we examined the interaction of PMX with BCRP using *in vitro* and *in vivo* methods (**chapter 2**). Using an *in vitro* inside-out membrane vesicle system, significantly higher amount of PMX was found in BCRP overexpressing vesicles as compared to vector control vesicles, indicating BCRP can actively transport PMX. In addition, this active transport was ATP-dependent, osmotically sensitive, and inhibited by BCRP inhibitors Ko143 (200nM) and GF120918 (5µM). Kinetic studies revealed BCRP-mediated transport of PMX followed Michaelis-Menten model, the kinetic parameters Km and V_{max} were determined to be 1.52 ± 0.27 mM and 678 ± 40.68 pmol/ mg protein/ min, respectively. Compared with the classic antifolate MTX, which is a prototypical substrate for BCRP (MTX: Km; 1.76 ± 0.48 mM, V_{max}; 1840 ± 221 pmol/ mg protein/min), BCRP has similar affinity for PMX as MTX, but the capacity for PMX is less than that of MTX (in this cell model).

It is well established that the amino acid at position 482 is a polymorphic hot spot for BCRP activity that can be associated with altered substrate specificities and transport activities. Vesicular uptake studies showed that the mutant forms of BCRP (ABCG2-

R482G and ABCG2-R482T) did not transport PMX, indicating that amino acid 482 is also an important site for BCRP-mediated transport of PMX.

Intracellular polyglutamation of antifolates has a great impact on cellular retention and drug potency. It is of great interest to know if BCRP is able to transport these active metabolites. Towards this end, the ability of BCRP to export polyglutamate derivatives of PMX was examined using vesicular uptake study via a competition assay. We found that tri- and penta- glutamated PMX (500 μM) were unable to inhibit the BCRP transport of the parent drug, indicating that BCRP may not be able to transport the active metabolites of PMX. This is further confirmed by the growth inhibition assay that showed BCRP-transfected cells were resistant to PMX only after 4 hours of drug exposure but lost most of this resistance after continuous (72 hours) drug exposure. We hypothesize that this is because after the long term incubation (72 hours), the majority of intracellular PMX has been converted to active polyglutamated metabolites that can no longer be effluxed by BCRP.

Based on the information gathered from *in vitro* study that PMX is a substrate of BCRP, the effect of BCRP on the brain penetration of PMX was studied by using an *in vivo* mouse model. *In vivo* brain distribution studies in wild-type mice showed that CNS distribution of PMX is limited, with a steady-state brain-to-plasma concentration ratio of 0.086 ± 0.032. No significant difference was found in brain-to-plasma ratios between wild-type mice and Bcrpl *(-/-)* mice, implying that the presence of BCRP alone may not limit PMX brain distribution. Our study underscores the fact

that the relevant physiological function of one active drug transporter needs to be evaluated relative all other transporters present in the same tissue.

Using brain efflux index method (intracerebral microinjection technique), we further examined the mechanism responsible for brain efflux of PMX and MTX (**chapter 3**). The apparent brain elimination rate constant (k_{eff}) of PMX was determined to be 0.0143 ± 0.0043 min^{-1} from the kinetic analysis study. Although MTX and PMX are structural analogs with similar physicochemical properties, MTX was cleared from the brain more rapidly than PMX after intracerebral administration. The k_{eff} value for MTX was 0.0271 ± 0.0184 min^{-1} with half-life of 32 minutes. The brain-to-blood efflux of [^{3}H]-PMX was inhibited by unlabelled PMX in a concentration-dependent manner, indicating that PMX undergoes carrier-mediated efflux transport across the BBB. In addition, with 50 mM unlabelled PMX in the injectate, the elimination of [^{3}H]-PMX from the brain was almost completely inhibited, suggesting that the contribution of passive diffusion to PMX brain efflux transport is very limited and active transport is the major driving force of brain elimination of PMX.

Using gene knockout mice, the role of MRP2 and BCRP on brain efflux of PMX and MTX were examined. The remaining percentage of [^{3}H]-PMX and [^{3}H]-MTX in the brain of *Mrp2(-/-)* mice was not significantly different from that of wild-type mice, suggesting that MRP2 does not play a role in the brain distribution of both antifolates. Significantly higher amount of [^{3}H]-MTX was found in the brain of *Bcrp1(-/-)* mice

than that in wild-type mice, indicating BCRP makes a significant contribution to the brain elimination of MTX. Consistent with the results obtained from chapter 2, no significant change in PMX brain elimination was observed in *Bcrp1(-/-)* mice compared to wild-type mice. Organic anions, probenecid and benzylpenicillin, markedly reduced the brain efflux of PMX and MTX, suggesting the involvement of organic anion transporters, possibly OAT3, in the brain elimination of antifolates. These results revealed that both PMX and MTX undergo saturable efflux transport across the BBB and inhibition of the transporter-mediated efflux may be a clinically viable strategy to improve brain delivery of these antifolates.

It is well known that solid tumors and some physiological barriers such as intestinal epithelium and kidney tubule have an acidic extracellular environment. The effect of pH on transport activity of BCRP may be important with respect to distribution and accumulation of PMX in tumors. In **chapter 4**, we examined the effect of pH on BCRP-mediated transport of PMX and MTX. In addition, the molecular basis of the pH-dependency in BCRP transport activity was explored based on the recent homology model of BCRP.

The vesicular transport of PMX by wild-type BCRP was 50-fold higher at pH 5.5 that at pH 7.4. This transport activity was diminished significantly in the presence of BCRP inhibitors Ko143 (200nM) and GF120918 (5μM). BCRP-mediated uptake of PMX followed Michaelis-Menten kinetics that were pH-dependent; with a $\mathbf{K}_m$ of 0.39

± 0.08 mM and $\mathbf{V}_{max}$ of 4725 ± 279 pmol/mg/min at pH 5.2; and a $\mathbf{K}_m$ of 1.47 ± 0.35 mM and $\mathbf{V}_{max}$ of 1993 ± 213 pmol/mg/min at pH 6.5. To address the mechanism of pH-dependency, we examined the effect of pH on BCRP transport of estrone sulfate, whose ionization state is constant within the applied pH. Interestingly, the relative change in BCRP transport activity of estrone sulfate at pH 7.4 and pH 5.5 was only 1.7-fold. The results suggest the major contributing factor associated with the dramatic increase in the transport activity at low pH is governed by the change of ionization state of substrate molecules.

Previous mutation studies have demonstrated the importance of R482 for substrate recognition. Results from the current uptake studies showed that R482 was also important for the recognition of PMX and estrone sulfate. To characterize the role of R482 on the pH-dependent transport of PMX, we measured the effect of low pH on the transport activity of polymorphic BCRP variants at 482 for estrone sulfate and PMX. Both mutant (ABCG2-R482G, ABCG2-R482T) and wild-type (ABCG2-R482) BCRP exhibited a similar extent of increase in the transport activity of estrone sulfate at low pH, suggesting that R482 did not involved in the pH-dependent transport of estrone sulfate. In contrast, for PMX, greatest extent of pH-dependency was observed in wild-type BCRP compared to the mutant BCRP.

Based on a recent homology model of BCRP, a negatively charged amino acid Asp-477 (D477) is localized within 7 and 9 angstrom from the arginine's (R482) facing

inside the channel, and the distances are similar to the extended and folded distances of PMX. We propose a mode of binding involving direct interactions between the negatively charged D477 with increased portion of the positive charged moiety of PMX at low pH, thereby, leading to the dramatic increased in the transport activity at low pH. This study sheds light into the importance of electrostatic interactions in determining the binding affinity of substrates to the transporter protein and may have important implications in the handling of PMX and other chemotherapy drug molecules in the acidic environment of tumors and in the distribution and elimination of the drug molecules.

In conclusion, the brain elimination of PMX was mainly mediated by active efflux transport. Of the efflux transporters expressed at the BBB, BCRP or MRP2 alone did not make a significant contribution to the brain penetration of PMX and organic anion transporters may play an important role in brain elimination of PMX, as suggested by the evidence from the BEI studies. The dramatically stimulated BCRP transport activity for PMX at low pH may have clinical implications with respect to the delivery of PMX to solid tumors in the acidic environment.

Future work in this field would involve the evaluation of the role of OAT3 and MRP4 in limiting the brain penetration of PMX and MTX by using gene knockout mice. Identifying the specific mechanisms involved in limiting the brain penetration of

PMX and MTX would help in formulating strategies to improve the delivery of these antifolates to the brain and resulting in more successful treatment of primary and secondary CNS tumors.

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CHAPTER 5

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