pH-Dependent Transport of Pemetrexed by Breast Cancer Resistance Protein

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ABSTRACT:

Breast cancer resistance protein (BCRP), an ATP-dependent efflux transporter, confers drug resistance to many chemotherapy agents. BCRP is overexpressed in tumors exposed to an acidic environment; therefore, it is important to establish the effect of low pH on BCRP transport activity. It has recently been reported that BCRP transports substrates more efficiently in an acidic microenvironment. In the study presented here, we examine the pH dependence of BCRP using methotrexate (MTX), pemetrexed (PMX), and estrone sulfate (ES) as model substrates. Our study revealed an increase of approximately 40-fold in the BCRP-mediated transport of PMX and MTX when the pH was decreased from 7.4 to 5.5. In contrast, only a 2-fold increase was observed for ES. These results indicate a mechanism of transport that is directly dependent on the effective ionization state of the substrates and BCRP. For ES, which retains a constant ionization state throughout the applied pH, the observed mild increase in activity is attributable to the overall changes in the effective ionization state and conformation of BCRP. For MTX and PMX, the marked increase in BCRP transport activity was likely due to the change in ionization state of MTX and PMX at lowered pH and their intermolecular interactions with BCRP. To further rationalize the molecular basis of the pH dependence, molecular modeling and docking studies were carried out using a homology model of BCRP, which has previously been closely examined in structural and site-directed mutagenesis studies (Am J Physiol Cell Physiol 299:C1100–C1109, 2010). On the basis of docking studies, all model compounds were found to associate with arginine 482 (Arg482) by direct salt-bridge interactions via their negatively charged carboxylate or sulfate groups. However, at lower pH, protonated MTX and PMX formed an additional salt-bridge interaction between their positively charged moieties and the nearby negatively charged aspartic acid 477 (Asp477) carboxylate side chain. The formation of this “salt-bridge triad” is expected to increase the overall electrostatic interactions between MTX and PMX with BCRP, which can form a rational basis for the pH dependence of the observed enhanced binding selectivity and transport activity. Removal of Arg482 in site-directed mutagenesis studies eliminated this pH dependence, which lends further support to our binding model. These results shed light on the importance of electrostatic interactions in transport activity and may have important implications in the design of ionizable chemotherapeutics intended for tumors in the acidic microenvironment.

Introduction

Multidrug resistance in tumors represents a significant obstacle in chemotherapeutic treatment of cancers. Drug efflux is one major drug resistance mechanism through which chemotherapeutics are actively removed from tumor cells. Clinically relevant efflux transporters include P-glycoprotein, multidrug resistance-related proteins, and breast cancer resistance protein (BCRP) (Diestra et al., 2003). BCRP (ABCG2) is a membrane-bound protein and belongs to the ATP-binding cassette (ABC) family (Doyle et al., 1998; Allen and Schinkel, 2002). BCRP is present in many solid tumors and normal tissues including placenta, blood-brain barrier, small intestine, testis, liver, adrenal gland, and stem cells (Doyle et al., 1998; Fetsch et al., 2006). BCRP confers drug resistance to many anticancer agents including mitoxantrone, topotecan, irinotecan, tyrosine kinase inhibitors (imatinib, gefitinib, and erlotinib), and antifolates [methotrexate, tomudex, and (S)-2-[(1,2-dihydro-3-methyl-1-oxobenzof[quinazolin-9-yl]methyl]amino-1-oxo-2-isindolyl]-glutaric acid (GW 18433)] (Robey et al., 2001; Volk and Schneider, 2003; Shafran et al., 2005; Bram et al., 2006). The implication of BCRP with various anticancer agents makes BCRP a clinically relevant target for studying drug resistance mechanisms.

One of the intriguing characteristics of tumors is the acidic microenvironment (i.e., an extracellular pH as low as 5.8), compared with normal tissues (Tamock and Rotin, 1989; Ojugo et al., 1999). The causes for the acidic pH environment in tumors are not well understood, but it has been closely examined in structural and site-directed mutagenesis studies (Am J Physiol Cell Physiol 299:C1100–C1109, 2010). One of the most important implications of tumor acidity is that it provides an acidic microenvironment for BCRP, which confers drug resistance to many drugs. BCRP is overexpressed in tumors exposed to an acidic microenvironment; therefore, it is important to establish the effect of low pH on BCRP transport activity.
understood. It has been linked to the increased use of the glycolytic pathways and the compromised vasculature of tumors, which may lead to the poor removal of lactic acid (Vaupel, 2004). On the basis of the observation that the activities of many transporters are affected by the environmental pH, it is likely that BCRP acts differently under acidic pH, which further affects its drug resistance profile.

Breedveld et al. (2007) recently demonstrated that BCRP transports substrates more efficiently under acidic pH using intact cell-based and membrane vesicle-based assays. In the study presented here, we explored the possible mechanisms for the pH-dependent BCRP transport using methotrexate (MTX), pemetrexed (PMX), and estrone sulfate (ES) as model compounds (Fig. 1). In addition, the molecular basis of the observed pH dependence was examined using docking studies with the recent inward-facing conformation homology model of BCRP (Rosenberg et al., 2010).

Materials and Methods

Chemicals. [3H]Pemetrexed, [3H]methotrexate, and [3H]estrone sulfate were obtained from Moravek Biochemicals (Brea, CA). GF120918 (of BCRP (Rosenberg et al., 2010). Studies with the recent inward-facing conformation homology model of BCRP (Rosenberg et al., 2010).

Materials and Methods

Chemicals. [3H]Pemetrexed, [3H]methotrexate, and [3H]estrone sulfate were obtained from Moravek Biochemicals (Brea, CA). GF120918 (N-[4-[2-[[1-(2,6-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). 3-(6-Isohuty-9-oxo-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydropyrazino[1’,2’:1,6]pyrido[3,4-b]indol-3-yl)-propionic acid tert-butyl ester ([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 dimethyl sulfoxide and diluted to the desired concentration with assay buffer (25 mM NaHCO3, 122 mM NaCl, 10 mM glucose, 10 mM HEPES, 1.2 mM MgSO4, 3 mM KCl, 1.4 mM CaCl2, and 0.4 mM KH2PO4, pH 7.4). The final concentration of dimethyl sulfoxide in all reaction solutions was less than 0.1%. All other chemicals used were of high-quality liquid chromatography or reagent grade.

Cell Lines. HEK293 cells transfected with ABCG2-Arg482, ABCG2-R482G, and ABCG2-R482T and plasmid vectors 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% CO2 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. Comparable protein expression in the wild-type (Arg482) and mutant (R482G, R482T) ABCG2-transfected cells has been demonstrated by the Bates group (Robey et al., 2003).

Membrane Vesicle Preparation. Vesicle preparation was adapted from Tabas and Dantzig (2002). Vector control or BCRP-transfected HEK293 cells were cultured in 500-cm2 plates (Corning Life Sciences, Lowell, MA). When 75% confluence was reached, the cells were washed twice with phosphate-buffered saline and scrapped into a residue of phosphate-buffered saline. After centrifugation at 1700 rpm at 4°C for 5 min, the cell pellet was diluted with a hypotonic buffer containing 1 mM sodium bicarbonate at pH 9 and 1% EDTA-free protease inhibitors. The cells were allowed to sit and swell on ice for 5 min, followed by vigorous shaking 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 layerd over 40% buffered saline and scraped into a residue of phosphate-buffered saline. After centrifugation at 1700 rpm at 4°C for 5 min, the cell pellet was diluted with a

Inhibitor Assays. PMX uptake into BCRP or control vesicles was measured in the presence or absence of BCRP inhibitors at maximal inhibitory concentrations (200 nM Ko143).

Kinetic Studies. PMX uptake into BCRP or control vesicles was measured after a 15-min exposure to 0, 100, 250, 500, 1000, 2000, and 4000 μM PMX. The same batch of membrane vesicles was used in all experiments to reduce variability, and the vesicular transport assay buffer was adjusted to pH 5.5 or 6.5. Kinetic parameters (Km and Vmax) were determined by nonlinear regression analysis using SigmaPlot, version 9.0.1 (Systat Software, Inc., San Jose, CA).

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.

Molecular Modeling. All molecular modeling studies were carried out using the Schrödinger modeling suite package (Schrödinger, LLC, New York,
The homology model of BCRP in the inward-facing closed conformation was previously reported (Rosenberg et al., 2010) and was based on the recently solved X-ray structure of mouse P-glycoprotein (Protein Data Bank code 3GSU) (Aller et al., 2009). This model has been previously shown to be consistent with experimentally observed conformation change upon substrate binding (Rosenberg et al., 2010) and for the identification of important residues for drug transport (Ni et al., 2010). The homology-modeled BCRP structure was refined by restraint energy minimization using the OPLS 2005 force field (Jorgensen et al., 1996) with the generalized born solvent accessible implicit solvent model (Still et al., 1990). Docking of all compounds was carried out using Glide at Standard Precision (Fröster et al., 2004). For each ligand, the top-ranked docking geometry based on G-score scoring function is reported. 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 calculated by ACD $pK_a$ predictor software (Advanced Chemistry Development, Inc., Toronto, Canada; http://www.acdlabs.com/products/phys_chem_lab/pka/), a reliable knowledge-based $pK_a$ calculator using Hammett equations derived from a database of over 30,000 experimental $pK_a$ values (Balogh et al., 2009).

**Statistical Analysis.** Statistical comparisons between the two groups were made using the two-sample t test. Differences were considered to be statistically significant when $P < 0.05$. Multiple groups were compared using analysis of variance with Holm–Sidak post hoc test at $P < 0.05$ level of significance.

**Results**

**Effect of pH on ATP-Dependent BCRP Transport Activity of PMX, MTX, and ES.** At pH 7.4, PMX uptake in BCRP membrane vesicles was 2-fold higher than that of control vesicles ($P < 0.05$). In contrast, PMX uptake in BCRP vesicles was approximately 20-fold higher than that in the control vesicles at pH 5.5 (Fig. 2A). By subtracting the uptake in BCRP vesicles from that in control vesicles, BCRP-mediated transport of PMX increased by 43-fold at pH 5.5 compared with the physiological pH. MTX was used here as a positive control for method validation. Consistent with a previous report (Breedveld et al., 2007), a marked increase in BCRP transport of MTX was observed when the pH decreased from 7.4 to 5.5 (Fig. 2B). The observed pH-sensitive BCRP-mediated transport may be a consequence of changed intrinsic protein activity at different pH and/or altered ionization state of the substrate molecules and thus different substrate-transporter protein interaction. To elucidate the mechanism of BCRP pH dependence, we investigated the BCRP-mediated transport of estrone sulfate, a compound with constant ionization status at the applied pH range (Fig. 3). As the pH decreased from 7.4 to 5.5, we observed a 1.7-fold increase in the BCRP uptake of estrone sulfate, a finding that was quite similar with the 40-fold increase observed for PMX and MTX. No optimal pH was observed for maximal transport activity for BCRP.

**Effect of BCRP Inhibitor on PMX and MTX Uptake into BCRP-Expressing and Control Vesicles at Low pH.** To confirm that the markedly increased PMX and MTX transport at low pH was a BCRP-mediated process, we determined the effect of BCRP-specific inhibitor on the pH-dependent BCRP transport of PMX and MTX. As shown in Fig. 4, 200 nM K0143 significantly decreased the uptake of PMX and MTX in BCRP-expressing membrane vesicles at pH 5.5, suggesting that the enhanced uptake at the low pH was mainly a result of stimulated transport by BCRP.

**pH-Dependent BCRP Transport Kinetics of PMX.** To characterize the influence of pH on the BCRP-mediated transport kinetics of PMX, concentration-dependent uptake studies were performed at pH 5.2 and 6.5. From the time course of PMX uptake at pH 5.5, a 15-min time point was within the linear uptake range and was used to determine the concentration-dependent uptake of PMX (data not shown). As shown in Fig. 5, the BCRP-mediated uptake of PMX was saturable and followed Michaelis–Menten kinetics, with $K_m$ of $0.39 \pm 0.08$ mM and $V_{\max}$ of $4725 \pm 279$ pmol $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$ at pH 5.2 and a $K_m$ of $1.47 \pm 0.35$ mM and $V_{\max}$ of $1993 \pm 213$ pmol $\cdot$ mg$^{-1}$ $\cdot$ min$^{-1}$ at pH 6.5.

The observed pH-sensitive BCRP-mediated transport may be a consequence of changed intrinsic protein activity at different pH and/or altered ionization state of the substrate molecules and thus different substrate-transporter protein interaction. To elucidate the mechanism of BCRP pH dependence, we investigated the BCRP-mediated transport of estrone sulfate, a compound with constant ionization status at the applied pH range (Fig. 3). As the pH decreased from 7.4 to 5.5, we observed a 1.7-fold increase in the BCRP uptake of estrone sulfate, a finding that was quite similar with the 40-fold increase observed for PMX and MTX. No optimal pH was observed for maximal transport activity for BCRP.

**Fig. 2. Effect of pH on ATP-dependent uptake of $[^{3}H]$PMX (A) and $[^{3}H]$MTX (B) into BCRP-expressing vesicles and control vesicles. HEK293-BCRP membrane vesicles and pc-DNA membrane vesicles were incubated with 0.3 $\mu$M $[^{3}H]$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 $\pm$ S.D. of each experiment ($n = 4$) ($**$, $P < 0.01$ compared with the BCRP uptake at pH 7.4; *, $P < 0.05$ compared with uptake into control vesicles at pH 7.4).**
The amino acid residue 482 is a reported hot spot for BCRP substrate specificity, and it plays a crucial role in the transport of MTX (Honjo et al., 2001; Allen et al., 2002). Studies from our laboratory showed that Arg482 was also important for the recognition of PMX and estrone sulfate. To understand the molecular basis for the increased binding selectivity of PMX and MTX at low pH, the BCRP binding site consisting of Arg482 was closely examined for possible key residues that can provide significant interaction with the charged carboxylate and pteridinium groups of PMX and MTX in the recently published homology model.

**BCRP Arg482 Binding Site.** The amino acid residue 482 is a reported hot spot for BCRP substrate specificity, and it plays a crucial role in the transport of MTX (Honjo et al., 2001; Allen et al., 2002). Studies from our laboratory showed that Arg482 was also important for the recognition of PMX and estrone sulfate. To understand the molecular basis for the increased binding selectivity of PMX and MTX at low pH, the BCRP binding site consisting of Arg482 was closely examined for possible key residues that can provide significant interaction with the charged carboxylate and pteridinium groups of PMX and MTX in the recently published homology model.

BCRP is a homodimeric transport membrane protein consisting of an extracellular nucleotide binding domain and a transmembrane helical (TM) domain in each of its monomeric subunits. The TM domains (Wang et al., 2008) consist of six transmembrane helices with Arg482 located in the helix III region along the transport channel of BCRP (Fig. 7A). In the model, residues Asp477, Arg465, and His630 were calculated to be the only ionizable residues within an 18-Å radius of Arg482 located within the transport channel of BCRP (Fig. 7C). The nearest interatomic distance between the Arg482 and Arg465 guanidinium groups was 6.9 Å, which is similar to the interatomic distance of 6.0 Å of the carboxylate atoms in the energy-minimized conformation of PMX (Fig. 7B). His630, which was located in the helix VI region further inside along the BCRP transport channel, has the nearest interatomic side-chain distances to Arg482 and Arg465 of 6.0 and 9.5 Å, respectively. Asp477, which was located in the transmembrane helix III region at the interface between the TM and nucleotide binding domains, has the nearest interatomic side-chain distances to Arg482 and Arg465 of 6.9 and 8.7 Å, respectively. The nearest interatomic side-chain distances between the arginines to His630 and Asp477 were found to be similar to the interatomic distances of pteridine and carboxylate groups in the energy-minimized structure of PMX. The nearest interatomic side-chain distance of the guanidinium group between the two Arg482s on each of the monomers was 11.2 Å. With the interatomic distance of 6.0 Å between the dicarboxylic acids in MTX and PMX, the homology model also suggests a possible mode of binding involving the direct interaction between each of the negatively charged carboxylate groups in PMX and MTX with the positively charged guanidinium group of Arg482 in each of the monomeric units.

**Potential Mode of Binding.** Docking studies were carried out within the Arg482 binding site to identify the potential mode of binding. Under acidic pH, His630 is expected to become positively ionized. Our docking study did not identify any reasonable mode of binding involving His630 and Arg482. At physiological pH when substrate and His630 are neutralized, the mode of binding involving Asp477 was consistently ranked higher than His630. As a result, His630’s involvement was excluded as a potential contributor toward the observed pH-dependent activity of BCRP. With the defined constraints of Arg482 and Asp477 side chains as potential hydrogen bond

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**Fig. 3.** Effect of pH on BCRP-mediated uptake of \([\text{H}]\)ES. HEK293-BCRP membrane vesicles and pc-DNA membrane vesicles were incubated with 0.03 μM \([\text{H}]\)ES at pH 5.5, 6.0, 6.5, and 7.0 at 37°C for 10 min in the presence or absence of 4 mM ATP. The BCRP-mediated uptake was calculated by subtracting ATP-dependent uptake into vector control vesicles from the ATP-dependent uptake into BCRP-overexpressing vesicles. Values shown are mean ± S.D. of each experiment (n = 4) (*, P < 0.05 compared with the BCRP uptake at pH 7).

**Fig. 4.** Effect of BCRP-specific inhibitor Ko143 (200 nM) on ATP-dependent uptake of \([\text{H}]\)PMX (A) and \([\text{H}]\)MTX (B) at pH 5.5. HEK293-BCRP membrane vesicles and pc-DNA membrane vesicles were incubated with 0.11 μM \([\text{H}]\)PMX and 0.02 μM \([\text{H}]\)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 the BCRP uptake without Ko143).
donor and acceptor, two common modes of binding were observed for MTX and PMX (Fig. 8). With the farthest interatomic distance of 6 Å between the oxygen atoms of the dicarboxylic acid group, each of the carboxylate groups of PMX and MTX has the potential to form direct salt bridges with the guanidinium side chains of Arg482 and Arg465 within each of the monomeric subunits or with the guanidinium side chains of Arg482 between the two monomeric units. With the dicarboxylic acid anchored in place, the positively charged base of PMX and MTX at low pH was found to be within the distal constraint to form a third salt bridge with the negatively charged side chain of Asp477. On the basis of the calculated $pK_a$, ES was expected to retain its negatively charged state at the applied pH. The only mode of binding observed for ES involved a single salt-bridge interaction between the negatively charged sulfate and the positively charged guanidinium group of Arg482.

The Role of Arg482 in pH-Dependent BCRP Transport. The results from docking studies indicate that the formation of a “salt-bridge triad” between PMX/MTX molecules and BCRP at amino acid residues Arg482, Arg465, and Asp477 may contribute to the greatly enhanced transport activity of BCRP for PMX and MTX at low pH. To confirm the role of the salt-bridge triad in the observed pH-dependent transport, we determined the influence of polymorphic BCRP mutation at 482 on the pH-dependent transport of PMX, MTX, and estrone sulfate. As shown in Fig. 9A, the extent of pH dependence, calculated as the ratio of uptake at pH 5.5 to the uptake at pH 7.4, was approximately 61, 12, and 6 for Arg482 (wild-type BCRP), R482G (mutant BCRP), and Asp477 may contribute to the greatly enhanced transport activity of BCRP for PMX and MTX at low pH. To confirm the role of the salt-bridge triad in the observed pH-dependent transport of PMX, MTX, and estrone sulfate. As shown in Fig. 9A, the extent of pH dependence, calculated as the ratio of uptake at pH 5.5 to the uptake at pH 7.4, was approximately 61, 12, and 6 for Arg482 (wild-type BCRP), R482G (mutant BCRP), and R482T (mutant BCRP), respectively. A similar trend was observed for MTX; that is, pH-dependent transport was significantly disturbed when positively charged arginine at 482 (Arg482) was replaced by nonionized amino acids (R482G and R482T) at lower pH (Fig. 9B). In contrast, the transport of estrone sulfate by wild-type and mutant BCRP were similarly enhanced (1.5-fold) when the pH changed from 7.4 to 5.5 (Fig. 10).

Discussion

It is well known that pH plays an important role in modulating the functional activity of proteins. This phenomenon also applies to many transporters. Studies by Nozawa et al. (2004) have shown that human organic anion transporting polypeptide-B (SLC21A9) exhibits pH-sensitive transport activity for various organic anions. Likewise, human oligopeptide transporter 1 exhibits a bell-shaped transport activity with an optimal pH of 5.5 (Fujisawa et al., 2006). pH-dependent transport has also been reported for proton-coupled folate transporter (SLC46A1) and organic cation transporters 1 (SLC22A1) and 2 (SLC22A2) (Urakami et al., 1998; Fujita et al., 2006; Zhao and Goldman, 2007). Various mechanisms are responsible for the pH-sensitive transport activity, including proton-coupled transport, membrane potential-dependent transport, and the pH-dependent ionization of key amino acid residues at active binding sites. In particular, it has
been documented that histidine plays an important role in pH-sensitive transporters such as proton-coupled folate transporter and peptide transporter 1 because histidine can interact differently with substrate molecules depending on its protonation state at various pH values (Said and Mohammadkhani, 1993; Fei et al., 1997; Metzner et al., 2008; Unal et al., 2009).

In the study presented here, we examined the influence of pH on BCRP transport activity using BCRP substrates PMX and estrone sulfate and characterized the possible mechanism for the observed pH dependence. Vesicular uptake assays revealed that BCRP-mediated transport of PMX was 43-fold higher at acidic pH (5.5) compared with the transport at physiological pH (7.4). In addition, the increased uptake at lower pH associated with an increased affinity of PMX for BCRP (as indicated by decreased $K_m$ values) and a markedly increased BCRP transport capacity ($V_{max}$). The pH-dependent BCRP transport was also observed for estrone sulfate. However, extent of pH dependence was mild, with a pH alteration from 7.4 to 5.5 only leading to a 2-fold increase in BCRP-mediated transport of estrone sulfate. This result is consistent with an observation from Breedveld et al. (2007) that BCRP transports substrate drugs more efficiently at low pH. However, our data further suggest that the extent of pH-dependent transport activity can vary significantly depending on the ionization status of the substrates.

With a $pK_a$ of 2, the ionization state of estrone sulfate is constant through the applied pH (pH 7.4 and 5.5) (Fig. 6). Therefore, any changes in estrone sulfate uptake from pH 7.4 to 5.5 should be a result of the pH-induced conformational and/or ionization changes associated with the transporter protein. The mild pH effect observed for

![Fig. 8](image_url)

**Fig. 8.** The two common modes of binding observed are shown in A and B with PMX binding to BCRP. The models suggest the importance of Asp477 in the formation of the third salt bridge with the protonated aromatic base of PMX and MTX at low pH. The binding of ES to BCRP is shown in C involving a single salt bridge.

![Fig. 9](image_url)

**Fig. 9.** 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 and ABCG2-T) and control vesicles. HEK293-BCRP membrane vesicles and pcDNA 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) (***, P < 0.001, compared with the BCRP-mediated uptake at pH 7.4).
estrogen sulfate indicates that the intrinsic activity of BCRP increased at lower pH, but only to a moderate extent. In contrast, the significant pH-dependent transport observed for MTX and PMX may be mainly due to the change in the ionization state of the substrate and the subsequent interaction with BCRP. PMX is a polyelectrolyte carrying two carboxyl groups, with $pK_a$ values of 3.46 (α-carboxyl) and 4.77 (γ-carboxyl), and the guanidinic $N$-1 on the pterine ring ($pK_a$ 5.27) (predicted using ACD software) (Fig. 6). At physiological pH, PMX exists in a predominantly negatively charged form because of the deprotonation of the two carboxyl groups. When the pH decreased 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 the calculated ionization of the nitrogen group (i.e., from 0 to 30%) in water. It is possible that the enhanced transport of PMX at low pH is due to the increased positive charge in the PMX molecule, which in turn leads to the stronger electrostatic interaction with BCRP.

To understand molecular basis for the increased BCRP transport of PMX and MTX at low pH, molecular modeling and docking studies were carried out. On the basis of the homology model of BCRP, residues Asp477, Arg465, and His630 were identified to be the only ionizable residues within an 18-Å radius of Arg482 located within the transport channel of BCRP. In the presence of two positively charged Arg482 and Arg465 residues, the $pK_a$ values of Asp477 and His630 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 and a positively charged aromatic 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 $K_m$ for PMX from 1.47 to 0.39 mM with pH changing from 6.5 to 5 further supports an overall increase in binding selectivity as PMX undergoes protonation at lower pH. The two observed modes of binding along the BCRP transport channel suggested two potential molecular recognition mechanisms of the doubly negatively charged MTX and PMX substrates that were not observed in ES. Although Asp477 and His630 locate at similar distance to that of the folded PMX conformation, the docking study showed consistently that the mode of binding favors the formation of a third salt bridge between the protonated base of PMX and MTX with the negatively charged side chain of Asp477, resulting in a salt-bridge triad for molecular recognition. The formation of the third salt bridge is expected to increase the electrostatic interaction between PMX/MTX with BCRP, which would lead to an enhancement in the binding selectivity. This was confirmed by site-directed mutagenesis study in which Arg482 was replaced with nonionized amino acid residuals to abolish the salt-bridge triad. The results showed that the enhancement in PMX and MTX transport at acidic pH was much less in mutant BCRP compared with that in the wild-type BCRP, suggesting the importance of Arg482 in the pH-dependent transport of PMX. For estrone sulfate, mutant (R482G, R482T) and wild-type (Arg482) BCRP exhibited a similar extent of increase in the transport activity, suggesting that Arg482 was not involved in the pH-dependent transport of estrone sulfate. This is reasonable because the intrinsic $pK_a$ of arginine is approximately 12. Therefore, with a constant ionization state throughout the applied pH, Arg482 should not produce any change in the electrostatic properties of BCRP and subsequent interaction with estrone sulfate.

In summary, the BCRP-mediated transport of PMX was markedly enhanced under acidic pH conditions. For estrone sulfate, a mild increase in the BCRP transport was observed. On the basis of docking studies with the BCRP homology model, at low pH, PMX formed an additional salt-bridge interaction between their positively charged moieties and the negatively charged Asp477 carboxylate side chain. The formation of a salt-bridge triad is expected to increase the electrostatic interaction of PMX with BCRP and lead to a dramatic enhancement in the binding selectivity at acidic pH. The proposed model provided insights into the charge interactions between the substrate and the transporter proteins and furthermore demonstrated the importance of electrostatic interactions in the BCRP transport process. This study may have important implications in the handling of PMX and other relevant chemotherapeutic drugs that are exposed to the acidic environment of tumors.

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Authorship Contributions
Participated in research design: Li, Sham, and Elmquist.
Conducted experiments: Li.
Contributed new reagents or analytic tools: Sham and Bikadi.
Performed data analysis: Li, Sham, and Elmquist.
Wrote or contributed to the writing of the manuscript: Li, Sham, and Elmquist.

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References


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