Breast Cancer Resistance Protein Interacts with Various Compounds in Vitro, but Plays a Minor Role in Substrate Efflux at the Blood-Brain Barrier

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ABSTRACT:

Expression of breast cancer resistance protein (Bcrp) at the blood-brain barrier (BBB) has been revealed recently. To investigate comprehensively the potential role of Bcrp at the murine BBB, a chemically diverse set of model compounds (cimetidine, alfuzosin, dipyridamole, and LY2228820) was evaluated using a multiexperimental design. Bcrp stably transfected MDCKII cell monolayer transport studies demonstrated that each compound had affinity for Bcrp and that polarized transport by Bcrp was abolished completely by the Bcrp inhibitor chrysin. However, none of the compounds differed in brain uptake between Bcrp wild-type and knockout mice under either an in situ brain perfusion or a 24-h subcutaneous osmotic minipump continuous infusion experimental paradigm. In addition, alfuzosin and dipyridamole were shown to undergo transport by P-glycoprotein (P-gp) in an MDCKII-MDR1 cell monolayer model. Alfuzosin brain uptake was 4-fold higher in mdr1a(−/−) mice than in mdr1a(+/+) mice in situ and in vivo studies, demonstrating for the first time that it undergoes P-gp-mediated efflux at the BBB. In contrast, P-gp had no effect on dipyridamole brain penetration in situ or in vivo. In fact, in situ BBB permeability of these solutes appeared to be primarily dependent on their lipophilicity in the absence of efflux transport, and in situ brain uptake clearance correlated with the intrinsic transcellular passive permeability from in vitro transport and cellular accumulation studies. In summary, Bcrp mediates in vitro transport of various compounds, but seems to play a minimal role at the BBB in vivo.

The blood-brain barrier (BBB) is composed of brain capillary endothelial cells, which are characterized by highly developed tight junctions, a lack of fenestrations, and a paucity of pinocytic and transcytotic activities. Expression of metabolic enzymes and efflux transporters in these and associated glial cells provides additional limiting factors at the BBB. Insufficient drug exposure at the pharmacologic target within the brain, as a consequence of limited flux from blood to brain, represents a major obstacle for effective treatment of central nervous system (CNS) disorders (Begley, 2004; Pardridge, 2005). It is widely accepted that P-glycoprotein (P-gp) is expressed abundantly at the BBB and functions as an efflux pump that extrudes toxic substances and therapeutic agents in the brain-to-blood direction, representing a functional barrier to brain uptake (Schinkel, 1999). The potential role of other members of the ATP-binding cassette (ABC) efflux transporter family, such as multidrug resistance-associated proteins (Mrps) and breast cancer resistance protein (Bcrp), at the BBB is less clear, although the proteins appear to be expressed at the BBB in various species (Yousif et al., 2007).

Bcrp is a recently identified member of the ABC efflux transporter family encoded by gene Abcg2 (Doyle et al., 1998). Bcrp is widely expressed in the intestine, liver, mammary gland, and placenta. The functional efficiency of Bcrp in these organs, such as intestinal secretion, fetal penetration, and breast milk secretion of various compounds (e.g., topotecan, nitrofurantoin, and cimetidine), has been documented clearly (Jonker et al., 2000, 2005). In addition, Bcrp is expressed at the BBB in various species (Yousif et al., 2007). Bcrp is expressed abundantly at the BBB and functions as an efflux pump that extrudes toxic substances and therapeutic agents in the brain-to-blood direction, representing a functional barrier to brain uptake (Schinkel, 1999). The potential role of other members of the ATP-binding cassette (ABC) efflux transporter family, such as multidrug resistance-associated proteins (Mrps) and breast cancer resistance protein (Bcrp), at the BBB is less clear, although the proteins appear to be expressed at the BBB in various species (Yousif et al., 2007).

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Abbreviations: BBB, blood-brain barrier; CNS, central nervous system; P-gp, P-glycoprotein; ABC, ATP-binding cassette; MRP/Mrp, multidrug resistance-associated protein; BCRP/Bcrp, breast cancer resistance protein; GF120918, N-[4-[(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl]ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; MDR/mdr, multidrug resistance; LY2228820, 5-[2-tert-butyl-4-(4-fluorophenyl)-1H-imidazol-5-yl]-3-(2,2-dimethylpropyl)-3H-imidazo[4,5-b]pyridin-2-amine; MDCK, Madin-Darby canine kidney; LSN335984, (R)-4-[1a,6,10b]-1,1-dichloro-1a,6,10b-tetrahydrodibenzo[a,e]cyclopropa[c]cyclohepten-6-yl]-[5-(quinolin-4-yl)oxy]methyl]-1-piperazinethanol; LY335979, zosuquidar; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; A; apical; B, basolateral; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; BSA, bovine serum albumin; PBST, PBS containing 0.1% Tween 20; ANOVA, analysis of variance; OATP/oatp, organic anion-transporting protein; PSC833, valsaparol.
The initial brain uptake clearance (Cl_{imp}) was determined using in situ brain perfusion with Bcrp-competent (wild type) and Bcrp-deficient [Abcg2(+/−)], P-gp-competent [mdr1a(+/+)], and P-gp-deficient [mdr1a(−/−)] mouse models. In addition, the P-gp and Bcrp inhibitor GF120918 was coperoxidated with test compounds in the brain perfusion paradigm to generate a chemical knockout model. Finally, in vivo brain penetration of substrates was measured at 24 h during continuous subcutaneous infusion with an osmotic minipump.

Materials and Methods

Animals. Adult male mdr1a(+/+) and their natural mutant mdr1a(−/−) C57BL/6 mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Male Abcg2(+/+) and Abcg2(−/−) mice (25–35 g) were provided by Deltagen, Inc. (San Mateo, CA) and a gift from Eli Lilly and Company. Unengenotyped male C57BL/6 mice from Charles River Laboratories, Inc. were used as control wild-type mice, e.g., assumed to be Abcg2(+/+) or Abcg2(−/−), because spontaneously recessive mutants are not reported on day. Details regarding the background, generation, and breeding of these mice have been described elsewhere (Zamek-Gliszczynski et al., 2006). All mice were maintained on a 12-h light/dark cycle with access to water and food ad libitum. All experimental procedures were performed under full anesthesia induced with ketamine/xylazine (100/10 mg/kg i.p.). All procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill and were conducted in accordance with Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication 85-23, revised in 1985).

Materials. Cimetidine and dipyridamole were purchased from Sigma-Aldrich (St. Louis, MO). Alfuzosin was obtained from Toronto Research Chemicals Inc (North York, ON, Canada). LY2228820 and [14C]LY2228820 were kind gifts from Eli Lilly and Company. GF120918 was a kind gift from GlaxoSmithKline (Research Triangle Park, NC). [3H]Cimetidine (25.0 Ci/mmol) and [3H]Diazepam (50.0 Ci/mmol) were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). [14C]Inulin (2.1 mCi/g) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [3H]Inulin (180.0 mCi/g) and [3H]Hocolicine (80.4 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). LNS335984 is a dichloro homolog of the diffuoro-containing zosquidar (LY335979), which was developed as a third-generation, specific inhibitor of P-gp (Pfister et al., 1995; Yasuno et al., 2008). All of the other chemicals were commercially available and of reagent grade.

Cell Culture. MDCKII cells stably expressing either murine wild-type Abcg2 (Bcrp1) or human wild-type ABCB1 (P-gp) were obtained from the Netherlands Cancer Institute under a Materials Transfer Agreement. MDCKII cells were maintained at 37°C in humidified 5% CO2/95% air using Eagle’s minimal essential culture medium supplemented with 10% (v/v) fetal bovine serum, penicillin, and streptomycin. A 1:10 split was done twice per week, and cells at passages 7 to 10 (Bcrp) or 20 to 30 (P-gp) were plated at 50,000 cells/cm² in 12-well Transwell filter inserts (1.13 cm² surface area). Medium was changed on days 3 and 5, and the cell monolayers were used on day 6.

Bidirectional Flux Experiments in Vitro. Cells were rinsed twice with Dulbecco’s phosphate-buffered saline (PBS) containing 10 mM Hepes, pH 7.4. Some cells were pretreated twice for 15 min each with PBS containing either 2.5 μM LNS335984 to potently and selectively inhibit P-gp and not Bcrp, or 20 μM chrysin to specifically inhibit Bcrp and not P-gp (Zhang et al., 2005a). Transport was measured in both directions across uninhibited and inhibited cell monolayers using a substrate concentration of 5 μM diluted from a 10 mM DMSO stock solution (final DMSO concentration of 0.05%) and a single 60-min time interval (screening mode). The upper chamber (A) contained 0.5 ml of PBS and the lower chamber (B) contained 1.0 ml of PBS without and with inhibitor. The system was mixed using a Clay-Adams Nutator and kept at 37°C in room atmosphere. Solute concentration was determined for the donor and receiver solutions, and mass balance was achieved by extracting the cells with methanol. The apparent permeability coefficients (P_{app}) were estimated as the slope of the mass transported per 60 min relative to the total recovered mass according to Ho et al. (2000).B/A-A-B P_{app} ratios were calculated for each cell line. Cell monolayer integrity was monitored with percent leakage of...
Ventricles were severed immediately before perfusion at 2.5 ml/min for 60 s via Louvres, France) after ligation of the external carotid artery. The cardiac centrifuged in a fixed-angle rotor at 25,000 \( \text{g} \) for 10 min, and the supernatant was discarded. The pellet was resuspended with 4 volumes of 20% Ficoll T-400. The tissue was then homogenized with 10 strokes at approximately 500 revolutions/min. The homogenate was centrifuged in a fixed-angle rotor at 3500 \( \text{g} \) for 10 min at 4°C. The myelin fraction was determined by measuring the app of \([14\text{C}]\text{mannitol} \) in the absence and presence of solute and DMSO. The contribution of permeability at the aqueous boundary layer \( (P_{\text{anl}}) \) was measured using the method of Dallaire et al. (1991) with some modifications. In brief, 8 to 10 animals/group were euthanized by carbon dioxide inhalation, and the brains were quickly removed and placed in ice-cold capillary buffer consisting of 147 mM NaCl, 4 mM KCl, 3 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 5 mM glucose, and 15 mM HEPES, pH 7.4. The tissue was kept on ice at 4°C throughout the procedure. Large surface blood vessels were removed, and the brains from each animal group were pooled together. The tissue was then minced with a razor blade and added to a Potter-Elvehjem homogenizer (clearance 0.15–0.23 mm) with 20 strokes. The suspension was diluted 1:10,000 in PBST. Proteins were detected using the ECL system (GE Healthcare). Western Blot Analysis. Isolated capillary and whole brain homogenate samples were homogenized in SW-28, swinging bucket rotor (Beckman Coulter, Fullerton, CA) at 25,000g for 10 min at 4°C. The supernatant was aspirated, and the pellet was resuspended in capillary buffer plus 1% bovine serum albumin (BSA). This was applied to a prewetted 2.5 × 4 cm, 0.5-mm diameter glass bead column (Sartorius AG, Goettingen, Germany). The capillaries were washed with 75 ml of capillary buffer-BSA. The beads were transferred into a 200-ml beaker, and the capillaries were detached from the beads by gently swirling in 50 ml of buffer-BSA. The isolated capillaries were decanted and centrifuged at 300g for 10 min. The supernatant was discarded, and the pellet was rinsed with 50 ml of capillary buffer and centrifuged again; this process was repeated one additional time. The final pellet was resuspended in capillary buffer and stored at −70°C until further use. The purity of the capillary fraction was determined by measuring \( \gamma \)-glutamyl transferase activity according to Orlowski and Meister (1965). The activity in isolated capillaries was compared with whole brain homogenate activity levels. The enzyme activities for the capillary fractions from the three groups of animals were in excess of 20-fold greater than those for the whole brain homogenates and therefore were judged to be acceptable for Western blot analysis. Western Blot Analysis. Isolated capillary and whole brain homogenate samples were homogenized in SW-28, swinging bucket rotor (Beckman Coulter, Fullerton, CA) at 25,000g for 10 min. The supernatant was discarded, and the pellet was resuspended in capillary buffer and centrifuged again; this process was repeated one additional time. The final pellet was resuspended in capillary buffer and stored at −70°C until further use. The purity of the capillary fraction was determined by measuring \( \gamma \)-glutamyl transferase activity according to Orlowski and Meister (1965). The activity in isolated capillaries was compared with whole brain homogenate activity levels. The enzyme activities for the capillary fractions from the three groups of animals were in excess of 20-fold greater than those for the whole brain homogenates and therefore were judged to be acceptable for Western blot analysis.

**Mouse Brain Capillary Depletion**. Brain capillaries from wild-type and \( Abcg2(-/-) \) C57BL/6 mice were isolated using the method of Dallaire et al. (1991) with some modifications. In brief, 8 to 10 animals/group were euthanized by carbon dioxide inhalation, and the brains were quickly removed and placed in ice-cold capillary buffer consisting of 147 mM NaCl, 4 mM KCl, 3 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), 5 mM glucose, and 15 mM HEPES, pH 7.4. The tissue was kept on ice at 4°C throughout the procedure. Large surface blood vessels were removed, and the brains from each animal group were pooled together. The tissue was then minced with a razor blade and added to a Potter-Elvehjem homogenizer (clearance 0.15–0.23 mm) with 20 strokes. The suspension was diluted 1:10,000 in PBST. Proteins were detected using the ECL system (GE Healthcare). Western Blot Analysis. Isolated capillary and whole brain homogenate samples were homogenized in SW-28, swinging bucket rotor (Beckman Coulter, Fullerton, CA) at 25,000g for 10 min at 4°C. The supernatant was discarded, and the pellet was resuspended in capillary buffer plus 1% bovine serum albumin (BSA). This was applied to a prewetted 2.5 × 4 cm, 0.5-mm diameter glass bead column (Sartorius AG, Goettingen, Germany). The capillaries were washed with 75 ml of capillary buffer-BSA. The beads were transferred into a 200-ml beaker, and the capillaries were detached from the beads by gently swirling in 50 ml of buffer-BSA. The isolated capillaries were decanted and centrifuged at 300g for 10 min. The supernatant was discarded, and the pellet was rinsed with 50 ml of capillary buffer and centrifuged again; this process was repeated one additional time. The final pellet was resuspended in capillary buffer and stored at −70°C until further use. The purity of the capillary fraction was determined by measuring \( \gamma \)-glutamyl transferase activity according to Orlowski and Meister (1965). The activity in isolated capillaries was compared with whole brain homogenate activity levels. The enzyme activities for the capillary fractions from the three groups of animals were in excess of 20-fold greater than those for the whole brain homogenates and therefore were judged to be acceptable for Western blot analysis.

**Bcrp PLAYS A MINOR ROLE AT MURINE BBB**. A 25-\( \mu \)l aliquot of brain hemisphere homogenate or plasma was transferred to an HPLC vial, and protein was precipitated with 100 \( \mu \)l of methanol containing internal standard (10 ng/ml loperamide), followed by a 25-\( \mu \)l aliquot of DMSO. The sample was vortex-mixed and centrifuged. Three microliters of sample solutions were injected via an autosampler (Leap Technologies, Carrboro, NC). Cimetidine, alfuzosin, dipryridamole, and the internal standard, loperamide, were eluted from an Aquasil C18 column (2.1 × 50 mm, \( d_p = 5 \mu \text{m} \); Thermo Fisher Scientific, Waltham, MA) using a mobile phase gradient [A, 0.1% formic acid in water; B, 0.1% formic acid in methanol].
**TABLE 1**

Transport of [\(^{1}H\)]cimetidine, [\(^{1}H\)]alfuzosin, [\(^{1}H\)]dipyridamole, and [\(^{14}C\)]LY2228820 (all at 5 \(\mu M\)) across MDCK-II-Bcrp cell monolayers in the absence (-) or presence (+) of Bcrp inhibitor chrysin (20 \(\mu M\))

The intrinsic transmonolayer permeability coefficient (\(P_{\text{app}}\)) was calculated using a measured \(P_{\text{abl}}\) = 80 \(\times\) 10\(^{-6}\) cm/s (Ho et al., 2000). Mass recoveries were 92 to 112%.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Inhibitor</th>
<th>(P_{\text{app}}) ± S.D.</th>
<th>(P_{\text{app}}) ratio</th>
<th>(P_{\text{cell}})</th>
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<tr>
<td></td>
<td>A-B</td>
<td>B-A</td>
<td>A-B/A-B</td>
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</tr>
<tr>
<td><strong>Cimetidine</strong></td>
<td>-</td>
<td>1.4 ± 0.2</td>
<td>23 ± 2</td>
<td>16</td>
</tr>
<tr>
<td>+</td>
<td>1.8 ± 0.2</td>
<td>3.6 ± 0.3</td>
<td>31</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Alfuzosin</strong></td>
<td>-</td>
<td>1.9 ± 0.002</td>
<td>60 ± 1</td>
<td>34</td>
</tr>
<tr>
<td>+</td>
<td>25 ± 1</td>
<td>30 ± 2</td>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td><strong>Dipyridamole</strong></td>
<td>-</td>
<td>2.6 ± 10.0</td>
<td>90 ± 0.3</td>
<td>34</td>
</tr>
<tr>
<td>+</td>
<td>48 ± 1</td>
<td>70 ± 3</td>
<td>1.5</td>
<td>3.4</td>
</tr>
<tr>
<td><strong>LY2228820</strong></td>
<td>-</td>
<td>3.9 ± 0.2</td>
<td>143 ± 1</td>
<td>37</td>
</tr>
<tr>
<td>+</td>
<td>44 ± 3</td>
<td>50 ± 8</td>
<td>1.1</td>
<td>44</td>
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</table>

\(P_{\text{abl}}\) permeability coefficient for the aqueous boundary layer.

**Results**

**In Vitro Transport.** The B-A/B-A/B \(P_{\text{app}}\) ratios for each of the four compounds tested ranged from 16 to 37, suggesting that each compound underwent active efflux across MDCK-II-Bcrp cell monolayers. The positive control prazosin had a ratio of 27, which compared favorably to historical data (mean ± S.D.) of 22 ± 7 (n = 17) (G. A. Sawada and T. J. Raub, unpublished data). In addition, the B-A/B-A/B \(P_{\text{app}}\) ratios were decreased substantially (to 1.1–2.0) in the presence of the Bcrp inhibitor chrysin (Table 1). Prazosin efflux by MDCKII-Bcrp was not inhibited by the specific P-gp inhibitor LSN335984 (data not shown). Cellular substrate concentrations at equilibrium, estimated by methanol wash, of alfuzosin, dipyridamole, and [\(^{14}C\)]LY2228820, were decreased 4- to 6-fold in Bcrp-expressing cells because of active efflux, whereas cellular cimetidine concentrations were very low and unaffected by Bcrp. Alfuzosin and dipyridamole also were identified as P-gp substrates when substrate flux was evaluated in the MDCK-MDR1 cell monolayer model, with P-gp-mediated transport inhibited by the P-gp inhibitor LSN335984 (Fig. 2). In these cases, cellular concentrations at equilibrium were decreased 4- to 6-fold in the presence of P-gp-mediated efflux. Amprenavir efflux by MDCK-II-MDR1 was not inhibited by the Bcrp inhibitor chrysin (data not shown).

**Bcrp Expression at the BBB.** The expression of Bcrp in whole brain homogenate and isolated brain capillaries is shown in Fig. 3. There was no Bcrp detected in any of the whole-brain homogenate samples from the three mouse strains, whereas a protein band of ~70 kDa was present in isolated brain capillaries. This suggested that Bcrp is expressed primarily at the BBB in wild-type and Abcg2(+/-) mice, and, as expected, Bcrp is completely absent in Abcg2(-/-) mouse brain capillaries.

**In Situ Brain Perfusion.** [\(^{14}C\)]Insulin was used as a brain capillary space marker to assess BBB physical integrity. BBB integrity was not changed by knockout of the \(mdr1a\) or \(Abcg2\) gene or by coperfusion with 2 \(\mu M\) GF120918. In addition, the brain capillary volumes in wild-type and Abcg2(-/-) mice were comparable to those in CF-1 mice \((V_{\text{vasc}} = 1.69 ± 0.10 \text{ ml/100 g})\). The cerebral blood flow rates in wild-type and Abcg2(-/-) mice also were similar to that in CF-1 mice, measured using [\(^{14}C\)]diazepam as the marker \((250 ± 41 \text{ ml/min/100 g})\).

The values of the initial brain uptake clearance of [\(^{1}H\)]cimetidine and [\(^{14}C\)]LY2228820 in all four mouse strains, i.e., wild-type and Abcg2(-/-) C57BL/6 and \(mdr1a(+/-)\) and \(mdr1a(-/-)\) CF-1 mice, are presented in Table 2. Cimetidine \(C_{\text{Lapp}}\) increased by 33% but did not reach statistical difference \((1.1 ± 0.4 \text{ versus } 0.8 ± 0.3 \text{ ml/min/100 g, } p > 0.05)\) when coperfused with 2 \(\mu M\) GF120918 in wild-type mice. [\(^{14}C\)]LY2228820 is very permeable at the BBB. The initial rate of...
brain uptake in mdr1a(−/−) mice was close to the functional perfusate flow rate and was 2.3-fold higher than that in mdr1a(+/+) mice (p < 0.05). [3H]LY2228820 was also perfused in Abcg2(+/+) mice and the Clup was 120 ± 9 ml/min/100 g of brain, which did not differ significantly from that in wild-type and Abcg2(−/−) mice (136 ± 3 and 131 ± 26 ml/min/100 g, one-way ANOVA, p > 0.05).

Alfuzosin brain uptake was moderate in all mouse strains. The inhibitory effect of GF120918 on P-gp- and/or Bcrp-mediated alfuzosin efflux is illustrated in Fig. 4. Figure 4A shows that alfuzosin brain uptake is comparable in wild-type and Abcg2(−/−) mice in the absence of GF120918 (p > 0.05). Copercfusion with GF120918 significantly increased alfuzosin brain uptake in both wild-type and Abcg2(−/−) mice (p < 0.05, inhibitory effect) but to a greater extent in Abcg2(−/−) mice (p < 0.05, strain difference). The increased alfuzosin brain uptake can be ascribed to P-gp inhibition in the BBB of GF120918 (p < 0.05). Figure 4B demonstrates that alfuzosin brain uptake increased ~3.7-fold in mdr1a(−/−) mice compared with that in mdr1a(+/+) mice (p < 0.05). In a consistent manner, alfuzosin brain uptake increased approximately 4.4-fold with GF120918 copercfusion in mdr1a(+/+) mice (p < 0.05). GF120918 had no effect on alfuzosin brain uptake in mdr1a(−/−) mice.

Three concentrations (1, 2, and 5 µM) of dipyridamole were perfused in wild-type and Abcg2(−/−) mice, respectively (Fig. 5). Two-way ANOVA analysis indicated that there were no statistical differences between these two mouse strains at any of the concentrations tested or among concentrations in any mouse strain (p > 0.05).

Figure 6 depicts dipyridamole brain uptake when perfused at 2 µM in the absence or presence of 2 µM GF120918 in all four mouse strains. Dipyridamole brain uptake did not differ between wild-type and Abcg2(−/−) mice or between mdr1a(+/+) and mdr1a(−/−) mice. Figure 6A illustrates that dipyridamole brain uptake was increased by 2.2-fold in the presence of 2 µM GF120918 copercfusion in both wild-type and Abcg2(−/−) mice, respectively (two-way ANOVA, p < 0.05). Copercfusion of GF120918 increased dipyridamole brain uptake by 1.4-fold in mdr1a(+/+) and mdr1a(−/−) mice, although the differences were not statistically significant (p > 0.05; two-way ANOVA) (Fig. 6B).

**Osmotic Minipump Studies.** The vehicle, DMSO, up to 50% in water has been reported to be compatible with the minipump (www.alzet.com). The osmotic minipumps provided reliable delivery of cimetidine, alfuzosin, and dipyridamole (at a dose of 1.28 mg/kg/day). However, with the dipyridamole administration rate of 12.8 mg/kg/day, precipitation was visible around the exit hole of the device at the end of the experiment, and the plasma and brain concentrations were highly variable; thus, these data were excluded for comparison. Cimetidine brain/plasma concentration ratios were 0.024 ± 0.005 and 0.020 ± 0.017 in wild-type and Abcg2(−/−) mice, respectively (Student’s t test, p > 0.05). The brain/plasma concentration ratios of alfuzosin and dipyridamole (1.28 mg/kg/day) are shown in Fig. 7. Alfuzosin brain penetration was significantly higher (4.1-fold) in mdr1a(−/−) mice than in mdr1a(+/+) mice (Student’s t test, p < 0.05). Alfuzosin and dipyridamole brain penetration was ~3.5-fold higher in Abcg2(−/−) mice than in wild-type mice, although these differences did not achieve statistical significance. In addition, dipyridamole brain penetration was comparable between mdr1a(+/+) and mdr1a(−/−) mice (Student’s t test, p > 0.05).

**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type</th>
<th>Bcrp(−/−)</th>
<th>mdr1a(+/+)</th>
<th>mdr1a(−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clup (ml/min/100 g)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cimetidine</td>
<td>0.8 ± 0.3</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>LY2228820</td>
<td>136 ± 33</td>
<td>131 ± 26</td>
<td>104 ± 8</td>
<td>239 ± 24*</td>
</tr>
</tbody>
</table>

* Statistical difference (p < 0.05) between mdr1a(+/+) and mdr1a(−/−) mice for [3H]LY2228820 initial brain uptake.
behavior, the $P_{\text{app}}$ for each compound was corrected for the aqueous boundary layer to give the intrinsic transmonolayer permeability coefficient ($P_{\text{cel}}$) (Table 1). Consequently, the linear correlation between $Cl_{\text{up}}(-/-)$ and $P_{\text{app}}$ was markedly improved ($R^2 = 0.997$) (Fig. 8C). In addition, $Cl_{\text{up}}(-/-)$ was correlated with the in vitro cellular accumulation of compound ($R^2 = 0.952$) (Fig. 8D). These correlation analyses suggest that the cell line model may serve as a high-throughput in vitro system to predict passive permeability and contribute to improved compound selection in CNS drug discovery and development.

**Discussion**

Bcrp mediates intestinal and biliary secretion and substrate extrusion at mammary epithelia and in the placenta, and it plays an important pharmacologic and toxicologic role in the absorption and disposition of xenobiotics and xenotoxins (Jonker et al., 2000, 2005). However, the extent to which Bcrp might influence brain distribution of known Bcrp substrates has been unclear. In the current study we investigated the functional efficiency of Bcrp in vitro, in situ, and in vivo using four model compounds: cimetidine, alfuzosin, dipyridamole, and LY2228820.

Asymmetric transport of cimetidine was mediated by Bcrp in transfected MDCK cell lines (Table 1), as evidenced by a B-A/A-B $P_{\text{app}}$ ratio of 16. Under similar experimental conditions, a B-A/B $P_{\text{app}}$ ratio of ∼9 has been reported (Pavek et al., 2005). Cimetidine was transported actively by Bcrp in an MDCKII-Bcrp1 cell line as well as in rat and mouse liver and rat placenta (Merino et al., 2005; Staud et al., 2006). However, in the current study $Abcg2$ gene knockout did not change the initial rate of brain uptake or steady-state brain distribution using in situ brain perfusion and in vivo brain penetration paradigms. In addition, cimetidine brain uptake was independent of P-gp and Bcrp inhibition by GF120918. Furthermore, cimetidine brain penetration was minimal during a 24-h continuous subcutaneous infusion, and steady-state brain/plasma concentration ratios in wild-type and $Abcg2(-/-)$ mice were similar to a previously published value of 0.017 after intraperitoneal injection of cimetidine (100 mg/kg) in rats (Hough et al., 1986). The present results indicate that Bcrp does not pose a substantial barrier for cimetidine brain uptake and that the poor brain penetration of cimetidine is primarily due to low passive permeability.

This study constitutes the first investigation of alfuzosin interaction with ABC efflux transporters. Alfuzosin $Cl_{\text{up}}$ and the brain/plasma concentration ratio in $mdrla(-/-)$ mice were 4.4- and 4.1-fold higher than those in $mdrla(+/+)$, wild-type, and $Abcg2(-/-)$ mice, respectively. In addition, P-gp-mediated alfuzosin efflux was inhibited by GF120918 in $mdrla(+/-)$ mice and $Abcg2(-/-)$ mice, respectively. In contrast, alfuzosin appears to be transported efficiently by BCRP only when Bcrp is overexpressed in vitro. Alfuzosin $Cl_{\text{up}}$ and the brain/plasma concentration ratio was comparable between wild-type and $Abcg2(-/-)$ mice and copervation with GF120918 did not increase alfuzosin $Cl_{\text{up}}$ in $mdrla(-/-)$ mice. Taken together, these data indicate that alfuzosin is not transported by Bcrp at the BBB. In all mouse strains, alfuzosin did not cross the BBB substantially, and brain concentrations were much lower than plasma concentrations.

Dipyridamole has been reported to be a substrate of human BCRP in both human embryonic kidney and MDCK cell lines stably transfected with human BCRP (Zhang et al., 2005b). The current study confirms that dipyridamole interacts with murine Bcrp and human P-gp in vitro (Table 1; Fig. 2). Brain uptake of dipyridamole did not appear to be concentration-dependent in the range of 1 to 5 μM that was selected based on a reported dipyridamole mean plasma concen-
H11002 mice. Coperfusion with GF120918 did not increase brain uptake of 3.5 µM (1.77 µg/ml) in the clinic (Hervey and Goa, 1999). In addition, brain penetration did not vary between wild-type versus Abcg2(-/-) mice. Coperfusion with GF120918 did not increase brain uptake in mdr1a(-/-) mice, animals that express Bcrp, but not P-gp, at the BBB. Thus, Bcrp cannot be an important factor in determining dipyridamole brain uptake. In addition, the initial rate of dipyridamole brain uptake did not differ between mdr1a(+/-) and mdr1a(-/-) mice, also suggesting that dipyridamole is not transported by P-gp at the mouse BBB.

Efflux of [14C]LY2228820 was mediated by Bcrp in vitro. [14C]LY2228820 transport across the BBB was highly permeable, consistent with the rapid passive diffusion observed in in vitro cell monolayers. Brain uptake of [14C]LY2228820 was almost perfusion flow rate-limited in mdr1a(-/-) mice. In mdr1a(+/-) mice, [14C]LY2228820 brain uptake decreased ~60%. [14C]LY2228820 brain uptake in Abcg2(-/-) mice was comparable to that in wild-type and Abcg2(-/-) mice, indicating that [14C]LY2228820 brain uptake is not limited by Bcrp.

Taken together, all four model compounds appeared to interact with Bcrp in the MDCK-Bcrp cell line in vitro. However, none was transported by Bcrp at the mouse BBB, using the genetic knockout models, i.e., Bcrp-competent (wild type) and Bcrp-deficient [Abcg2(-/-)], as well as P-gp-competent [mdr1a(+/-)] and P-gp-deficient [mdr1a(-/-)] mouse models for comparison or chemical inhibition with GF120918, an inhibitor of P-gp and Bcrp. It is widely accepted that genetic knockout models are equivalent and essentially interchangeable with specific chemical knockout models, especially for cases in which the desired genetic knockout models are not available, as is most common in rat studies. However, the compensatory regulation of other transporter proteins after knockout of a specific gene and the specificity of inhibitors are always fundamental concerns in functional studies. Bcrp mRNA has been reported to be up-regulated in mdr1a(-/-) FVB mice and was 3-fold higher than that in mdr1a(+/-) FVB mice (Cisternino et al., 2004). Other evidence suggested that mRNA levels of mdr1a, Mrp1, Mrp4, and oatp2 were not changed in Bcrp knockout mice (Lee et al., 2005). Likewise, chemical inhibitors such as PSC833 for P-gp, probenecid for Mrps, and GF120918 for both P-gp and Bcrp are widely used in the literature. Cyclosporin A, which has been regarded as a specific P-gp inhibitor, has recently been demonstrated to inhibit Bcrp and OATPs (Xia et al., 2007). In a quercetin in situ rat brain perfusion study, coperfusion with the P-gp inhibitor PSC833 did not change Clup of quercetin, whereas coperfusion with the P-gp/Bcrp inhibitor GF120918 significantly enhanced brain uptake of quercetin (Youdim et al., 2004). The authors concluded that Bcrp was involved in quercetin brain uptake. In the absence of appropriate comparisons with a genetic knockout model or information regarding the specificity of a given inhibitor for a transporter, such a conclusion is potentially erroneous.

Western blot analysis demonstrated that Bcrp was expressed at the BBB of the wild-type mice (Fig. 3), and this finding was consistent with previous studies on the expression of Bcrp at the BBB (Cisternino et al., 2004; Lee et al., 2005). However, a recent observation demonstrated that protein expression of Bcrp in human and rat brain microvessel endothelial cells is much lower than that in the in vitro overexpressed system (Lee et al., 2007). This difference in protein expression might explain the discrepancies between the in vitro cell line model and in situ or in vivo animal models. However, as an overexpressed cell line model, good in vitro-in vivo correlation has been identified for the P-gp efflux ratio in the brain (Adachi et al., 2001), in which P-gp is abundantly expressed.

In summary, the results of the present study suggest that Bcrp plays a minor role in brain distribution of cimetidine, alfuzosin, dipyridamole, and LY2228820, although each of these compounds interacted with Bcrp in transfected cell line models. Together with previously published data on prazosin, dehydroepiandrosterone sulfate, and mitoxantrone (Cisternino et al., 2004; Lee et al., 2005), these results suggest that Bcrp does not contribute significantly to murine BBB function in most cases or from a drug screening and development standpoint. Yet, exceptions do occur as demonstrated by a recent study showing that Bcrp is rate-limiting for several phytoestrogens at murine BBB (Enokizono et al., 2007). Another speculation regarding the role of Bcrp at the BBB is that Bcrp and P-gp work together to limit the brain penetration of therapeutic agents (de Vries et al., 2007).
or CNS toxic effects (Erdilyi et al., 2008). The present results also raise the related question of the specific role of organic anion transporters in the overall barrier function at the blood-brain interface. It has been shown that inhibition of Mrp2 and Mrp4 significantly enhanced brain exposure of anti-epileptic drugs, topotecan, and nucleotides; however, the efflux effects observed were modest and all less than 2-fold (Potschka et al., 2003; Leggas et al., 2004; Belinsky et al., 2007). Our studies with phenytoin, valproic acid, and ritonavir using Mrp2-competent and Mrp2-deficient C57BL/6 mice and in situ brain perfusion demonstrated no significant Mrp2 function at the BBB (R. Zhao and G. M. Pollack, unpublished data). The spectrum of reports to date suggest that P-gp, but not other ABC efflux transporters, provides the primary transport-mediated attenuation of brain uptake in the intact BBB. Caution must be taken in the design and interpretation of in vitro and in situ experiments before assigning specific functional importance to a given efflux transport system in the in vivo situation.

In this regard, the current results with alfuzosin may serve as a useful template. These experiments demonstrated that alfuzosin is a P-gp substrate, with a 4-fold P-gp effect at the BBB. This P-gp effect was observed consistently among in vitro, in situ, and in vivo experiments. This type of information set should be available for a given substrate/transport protein pair before ascribing functional in vivo and potential pharmacologic/toxicologic importance to that transport protein in vivo.

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References

Mrp2-competent and Mrp2-deficient CF57BL/6 mice and in situ brain perfusion provided the primary transport-mediated attenuation of brain uptake in the intact BBB. Caution must be taken in the design and interpretation of in vitro and in situ experiments before assigning specific functional importance to a given efflux transport system in the in vivo situation. In this regard, the current results with alfuzosin may serve as a useful template. These experiments demonstrated that alfuzosin is a P-gp substrate, with a 4-fold P-gp effect at the BBB. This P-gp effect was observed consistently among in vitro, in situ, and in vivo experiments. This type of information set should be available for a given substrate/transport protein pair before ascribing functional in vivo and potential pharmacologic/toxicologic importance to that transport protein in vivo.