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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Running title: Bcrp plays a minor role at murine BBB

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Abbreviations: BBB, blood-brain barrier; BCRP/Bcrp, breast cancer resistance protein; BSA, bovine serum albumin; CNS, central nervous system; GGT, γ-glutamyl transpeptidase; HPLC/MS/MS, high performance liquid chromatography/tandem mass spectrometry; MDCK, Madin-Darby canine kidney; MRPs, multidrug resistance associated proteins; P-gp, P-glycoprotein; RSD, relative standard deviation; SD, standard deviation.
Abstract

Expression of breast cancer resistance protein (Bcrp) at the blood-brain barrier (BBB) has been revealed recently. In order 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 utilizing a multiexperimental design. Bcrp1 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-hr subcutaneous osmotic minipump continuous infusion experimental paradigm. In addition, alfuzosin and dipyridamole were shown to undergo transport by P-gp in an MDCKII-MDR1 cell monolayer model. Alfuzosin brain uptake was four-fold higher in mdr1a(-/-) mice than in mdr1a(+/+) mice in 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 appears to play a minimal role at the BBB in vivo.
Introduction

The blood-brain barrier (BBB) is composed of brain capillary endothelial cells, which are characterized by highly-developed tight junctions, lack of fenestrations and paucity of pinocytic and transcytotic activities. Expression of metabolic enzymes and efflux transporters in these and associated glial cells provide 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 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 ATP-binding cassette (ABC) efflux transporters, 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; Jonker et al., 2005). In addition, Bcrp is expressed on the luminal side of brain capillary endothelial cells in mice, rats, pig and human, with localization similar to that of P-gp; mRNA expression level for Bcrp also is similar to that of P-gp (Cooray et al., 2002; Cisternino et al., 2004; Aronica et al., 2005; Lee et al., 2005; Tanaka et al., 2005).
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Bcrp mediates the transport of a wide variety of substrates, from sulfoconjugated organic anions to various organic cations. The extensive overlap of Bcrp with P-gp in expression pattern (Tanaka et al., 2005) and substrate specificity leads to the hypothesis that Bcrp, like P-gp, might have an important role in the handling of substrate drugs, with subsequent regulation of pharmacologic response, in animals and humans. However, studies to date have not clarified Bcrp function at the BBB, which remains controversial. For example, brain penetration of imatinib mesylate was 2.5-fold higher in Bcrp-deficient mice as compared to wild-type animals, suggesting that Bcrp contributes to the functional barrier to brain uptake of this compound (Breedveld et al., 2005). In contrast, the initial rate of brain uptake of imatinib mesylate did not differ between wild-type and Bcrp1(-/-) mice during in situ brain perfusion, and GF120918 (elacridar), an inhibitor of P-gp and Bcrp, did not alter brain uptake of imatinib in mdr1a/1b(-/-) mice, leading to the conclusion that Bcrp was not involved in imatinib brain uptake (Bihorel et al., 2007).

Exploring the role of Bcrp at the BBB may provide important information on the design of efficient drug delivery to CNS, and may add to the understanding of CNS toxicity, for Bcrp substrates. In the present study, four compounds with different physicochemical properties, cimetidine, alfuzosin, dipyridamole, and LY2228820, were selected as model substrates based on the in vitro screening in an MDCKII-Bcrp cell monolayer transport assay to evaluate comprehensively the role of Bcrp at the murine BBB (Figure 1). Cimetidine, a prototypical histamine H2 receptor antagonist, recently was demonstrated to possess anti-tumor activity against a variety of cancers and malignant brain glioma (Lefranc et al., 2006). Cimetidine is a hydrophilic compound that has poor brain penetration (Hough et al., 1986) and is actively secreted from rat mammary gland and placenta by Bcrp (Jonker et al., 2005; Staud et al., 2006).
Alfuzosin is a clinical uroselective $\alpha_1$-adrenergic receptor antagonist with proven efficacy and safety in the treatment of benign prostatic hyperplasia (BPH). Alfuzosin is a structural analog of prazosin, which is a prototypical Bcrp substrate (Staud et al., 2006). Dipyridamole is a platelet inhibitor used for treatment of stroke. It was identified recently as a human BCRP substrate (Zhang et al., 2005b). LY2228820 is a p38 mitogen-activated protein kinase (MAPK) inhibitor discovered by Eli Lilly and Company (Mader et al., 2008). In the present study, bidirectional transport of these model compounds across the MDCKII-Bcrp and MDCKII-MDR1 cell monolayers was evaluated. The initial brain uptake clearance ($\text{Cl}_{\text{up}}$) was determined using $\textit{in situ}$ 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 co-perfused with test compounds in the brain perfusion paradigm to generate a chemical knockout model. Finally, $\textit{in vivo}$ brain penetration of substrates was measured at 24 hr during continuous subcutaneous infusion with an osmotic minipump.
Materials and Methods

Animals

Adult male mdr1a(+/+) and their natural mutant mdr1a (-/-) CF-1 mice (30-40 g) were obtained from Charles River Laboratories (Wilmington, MA). Male Abcg2(+/+) and Abcg2(-/-) mice (25-35 g) were provided by Deltagen Inc. and a gift from Eli Lilly and Company. Un-genotyped male C57BL/6 mice from Charles River Laboratories were used as control wild type, e.g., assumed Abcg2(+/-) or Abcg2(+/+) since spontaneously recessive mutants are not reported to occur. 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 “Principles with Laboratory Animal Care” (NIH publication No. 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 (New York, ON, Canada). LY2228820 and 14C-LY2228820 were kind gifts from Eli Lilly and Company (Indianapolis, IN). GF120918 [N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide] was a kind gift from GlaxoSmithKline (Research Triangle Park, NC). 3H-cimetidine (25.0 Ci/mmol) and 14C-diazepam (56.0 mCi/mmol) were purchased from Amersham Biosciences (Buckinghamshire, UK). 14C-inulin (2.1 mCi/g) was
purchased from American Radiolabeled Chemical Inc. (St Louis, MO). $^{3}$H-inulin (180.0 mCi/g) and $^{3}$H-colchicine (80.4 Ci/mmol) were purchased from PerkinElmer (Waltham, MA).

LSN335984 or ((R)-4-[(1a,6,10b)-1,1-dichloro-1,1a,6,10b-tetrahydrodibenzo[a,e]cyclopropa[c]cyclohepten-6-yl]-[(5-quinolinyloxy)methyl]-1-piperazineethanol) is a dichloro-homolog of the difluoro-containing zosuquidar (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**

Madin-Darby canine kidney (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. MDCK cells were maintained at 37°C in humidified 5% CO$_{2}$/95% air using Eagle’s MEM 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 passage 7-10 (Bcrp) or 20-30 (P-gp) were plated at 50,000 cells/cm$^{2}$ in 12-well Transwell filter inserts (1.13 cm$^{2}$ surface area). Medium was changed on days 3 and 5 and the cell monolayers used on day 6.

**Bi-directional 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 of LSN335984 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 PBS and the lower chamber (B) contained 1.0 mL 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). BA/AB P_app ratios were calculated for each cell line. Cell monolayer integrity was monitored with percent leakage of 14C-mannitol in the absence and presence of solute and DMSO. The contribution of the aqueous boundary layer (PABL) was measured using the P_app of 14C-testosterone (Ho et al., 2000). Positive controls run periodically included amprenavir for P-gp and prazosin for Bcrp with triamterene as control for a non-substrate. Experiments were done in duplicate at least twice. All test solutes were quantified by reverse-phase high performance liquid chromatography with detection by tandem mass spectrometry (HPLC/MS/MS) as detailed below.

**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. Briefly, 8-10 animals/group were euthanized by CO₂ inhalation and the brains were quickly removed and placed into ice-cold capillary buffer consisting of 147 mM NaCl, 4 mM KCl, 3 mM CaCl₂, 1.2 mM MgCl₂, 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) along with 4 volumes of capillary buffer. Brains were homogenized with 10 strokes at approximately 500 rev/min. The homogenate was centrifuged in a fixed-angle rotor at 3,500 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 a loose-fitting Teflon pestle (>0.23 mm) with 20 strokes. The suspension was centrifuged in a fixed-angle rotor for 10 min at 25,000 g at 4°C. The myelin layer floating at the top was carefully removed along with the remaining supernatant. The pellet was resuspended in 15 ml of 15% dextran T-500 (Sigma-Aldrich Inc., St. Louis, MO) and layered onto 5 ml of 20% dextran T-500. The gradient was centrifuged in a SW-28 swinging bucket rotor (Beckman Coulter, Fullerton, CA) at 25,000 g 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 pre-wetted 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 300 g for 10 min. The supernatant was discarded, the pellet rinsed with 50 ml capillary buffer, and centrifuged again; this 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 γ-glutamyl transpeptidase (GGT) activity according to Orlowski and Meister (1965). The activity in isolated capillaries was compared to 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 the whole brain homogenates, and therefore judged to be acceptable for Western blot analysis.
Western Blot Analysis

Isolated capillary and whole brain homogenate samples were lysed for 30 min at 4°C in a buffer containing 150 mM NaCl, 100 mM Tris-HCl, pH 7.5, 1% Triton X-100 and protease inhibitors. Protein concentrations were determined using a BCA assay kit (Sigma, St. Louis, MO). The lysates were electrophoresed on a 4-12% SDS-polyacrylamide gel and then transferred to PVDF membranes (Invitrogen, Carlsbad, CA). The membrane was blocked in Dulbecco’s phosphate buffered saline containing 0.1% Tween 20 (PBST) and 5% non-fat dry milk (BioRad, Hercules, CA) for 2 hr at room temperature. The membrane was briefly washed with PBST and then incubated overnight at 4°C with a 1:25 dilution of anti-mouse Bcrp in PBST (BXP-53 clone, Kamiya Biomedical Company, Seattle, WA). The membrane was then washed three times for 10 min each and incubated with an HRP-conjugated goat anti-rat secondary antibody (Jackson ImmunoResearch, West Grove, PA), diluted 1:10,000 in PBST. Proteins were detected using the ECL system (Amersham Biosciences Inc, Arlington Heights, IL).

In Situ Mouse Brain Perfusion

The details of the *in situ* mouse brain perfusion have been described elsewhere Dagenais et al. (2000). Briefly, mice were anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.). The right common carotid artery was cannulated with polyethylene tubing (0.30-mm inner diameter × 0.70-mm outer diameter; Biotrol Diagnostic, Chennevières-les-Louvres, France) following ligation of the external carotid artery. The cardiac ventricles were severed immediately before perfusion at 2.5 mL/min for 60 s via a syringe pump (Harvard Apparatus, Holliston, MA). The perfusion buffer (Krebs-bicarbonate buffer, with 9 mM of D-glucose, pH 7.4) was oxygenated with 95% O\textsubscript{2} and 5% CO\textsubscript{2} and maintained at 37°C. \textsuperscript{14}C-diazepam and \textsuperscript{3}H- or \textsuperscript{14}C-inulin were
used as blood flow rate and vascular space markers, respectively. The test compounds, \( ^3 \)H-cimetidine (1.6 \( \mu \)Ci/mL), \( ^{14} \)C-LY2228820 (0.1 \( \mu \)Ci/mL), alfuzosin (2 \( \mu \)M) or dipyridamole (1, 2, 5 \( \mu \)M) was added to the perfusate to achieve appropriate concentrations. GF120918 and dipyridamole were dissolved in DMSO. The final concentration of DMSO in the perfusate was less than 2%. GF120918 (2 \( \mu \)M) was co-perfused with alfuzosin and dipyridamole to inhibit P-gp and Bcrp. The experiment was terminated by decapitation. The brain was carefully removed from the skull, cleaned of meninges and choroids plexus, the cerebellum was excised and the right brain hemisphere was collected. Aliquots of perfusate were collected from the catheter and weighed for determination of perfusate concentration.

All non-radioactive samples were analyzed by HPLC/MS/MS. Radioactive brain samples were digested in 0.7 mL Solvable\textsuperscript{®} (Packard, Boston, MA) at 50°C overnight. Five mL of UltimaGold\textsuperscript{®} scintillation cocktail (PerkinElmer, Wellesley, MA) was added and vortex-mixed. Total radioactivity (\( ^3 \)H and/or \( ^{14} \)C) was determined in a Packard Tri-carb TR 1900 liquid scintillation analyzer (Packard, Boston, MA). Parameters related to the \textit{in situ} mice brain perfusion, i.e., the cerebral vascular volume (mL/100 g brain) was calculated using equation:

\[
V_{\text{vasc}} = \frac{X_{\text{inulin}}}{C_{\text{inulin}}},
\]

the initial brain uptake clearance (\( Cl_{\text{up}} \), mL/min/100 g brain) were calculated as:

\[
Cl_{\text{up}} = \frac{X_{\text{brain}}/T}{C_{\text{perf}}},
\]

where the amount of substrate in brain \( X_{\text{brain}} \) was corrected for residual blood contamination (Dagenais et al., 2000).

\textbf{Osmotic Minipump Studies}

\( Mdr1a(+/+), mdr1a(-/-), \) wild type, and \( Abcg2(-/-) \) mice (n=3) were anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.). An Alzet 2001D\textsuperscript{®} osmotic minipump (Alza Corporation,
Palo Alto, CA) was selected to release the drug continuously over 24 hr to achieve steady-state condition at a flow rate of 8 μl/h. The concentration of dosing solution was adjusted to the average animal body weight and mean pump rate to deliver an appropriate dose. Cimetidine, alfuzosin and dipyridamole were dissolved in 50% DMSO and loaded into the minipumps. Cimetidine (200 μg/pump, approximately 6.4 mg/kg/day) was implanted subcutaneously in the back of wild type and Abcg2(-/-) mice. Alfuzosin (200 μg/pump, approximately 6.4 mg/kg/day) and two doses of dipyridamole (40 and 400 μg/pump, approximately 1.28 and 12.8 mg/kg/day, respectively, with the higher dose dissolved in 80% DMSO to ensure adequate solubility) were implanted subcutaneously in mdr1a(+/-), mdr1a(-/-), wild type, and Abcg2(-/-) mice. The experiments were terminated at 24 hr by decapitation. The brain was removed carefully from the skull and weighed. Trunk blood was collected in heparinized 1.5-mL microcentrifuge tubes. Plasma was harvested following centrifugation at 3,000 rpm for 5 minutes. The plasma and brain samples were stored at -20°C until analysis by HPLC/MS/MS. To ensure a reliable drug release during the dosing period, the minipump was removed from the body and the residual volume was measured.

**HPLC/MS/MS Assay**

Two volumes of distilled water were added to brain samples and homogenized with brief probe sonication. Plasma, brain homogenate and perfusate samples were analyzed by HPLC/MS/MS (API 4000 triple quadrupole with TurboIonSpray interface; Applied Biosystems/MDS Sciex, Concord, ON, Canada). A 25-μL aliquot of brain hemisphere homogenate or plasma was transferred to an HPLC vial, and protein was precipitated with 100 μl of methanol containing internal standard (10 ng/mL loperamide), followed by a 25-μl aliquot of DMSO. The sample was vortex-mixed and centrifuged. Three microliters of sample solutions were injected via an
autosampler (Leap, Carrboro, NC). Cimetidine, alfuzosin, dipyridamole and the internal standard, loperamide, were eluted from an Aquasil C18 column (2.1 x 50 mm, d_p = 5 µm; Thermo Electron Corporation, Waltham, MA) using a mobile phase gradient (A, 0.1% formic acid in water; B, 0.1% formic acid in methanol; 0-0.70-min hold at 0% B, 0.70-3.12-min linear gradient to 90% B, 3.12-4.10 min hold at 90% B, 4.10-4.20-min linear gradient to 0% B, 4.20-4.90-min hold at 0% B; solvent delivery system (Shimadzu); flow rate = 0.75 ml/min; 0.8-4 min directed to mass spectrometer) and were detected in positive ion mode using multiple reaction monitoring: cimetidine: 253.1→117.0 m/z, alfuzosin, 390.2→235.2 m/z; dipyridamole, 505.5→429.3 m/z. All analytes were quantified with standard curves (0.05-5000 ng/ml) prepared in the appropriate matrix. The lower limit of detection was 0.1 ng/ml for all analytes; inter- and intraday RSDs were <15%.

Statistical Analysis

Data are reported as mean ± standard deviation (SD) for 3 mice per condition (n=3). Two-tailed Student’s t-test, one-way or two-way ANOVA, where appropriate, was used to determine the statistical significance of differences among two or more groups. The level of significance was corrected for multiple comparisons (e.g., Bonferroni test) or adjusted for unequal variance when necessary. In all cases, p<0.05 was considered to be statistically significant.
Results

In Vitro Transport

The basolateral-to-apical / apical-to-basolateral (B-A / A-B) P_{app} ratios for each of the four compounds tested ranged from 16 to 37, suggesting that each compound underwent active efflux across MDCKII-Bcrp cell monolayers. The positive control prazosin had a ratio of 27, which compared favorably to our assay’s historical data (mean ± SD) of 22 ± 7 (n=17) (Sawada and Raub, unpublished data). In addition, the B-A / A-B P_{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-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 (Figure 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 MDCKII-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 Figure 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 kD 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-inulin 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 co-perfusion with 2 µM GF120918. In addition, the brain capillary volumes in wild type and Abcg2(-/-) mice were comparable to those in CF-1 mice ($V_{\text{vasc}} = 1.69\pm0.10 \text{ mL/100 g}$). 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 mL/min/100 g).

The values of initial brain uptake clearance of $^3$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 does not cross the BBB to an appreciable extent. Cimetidine $Cl_{\text{up}}$ increased by 33%, but did not reach statistical difference (1.1 ± 0.4 vs. 0.8 ± 0.3 mL/min/100 g, $p>0.05$) when co-perfused with 2 µM 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 ($p<0.05$). $^{14}$C-LY2228820 was also perfused in Abcg2(+/+) mice and the $Cl_{\text{up}}$ was 120 ± 9 mL/min/100g 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 Figure 4. Figure 4A showed alfuzosin brain uptake is comparable in wild type and Abcg2(-/-) mice in the absence of
GF120918 ($p>0.05$). Co-perfusion 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 by GF120918. Figure 4B demonstrates that alfuzosin brain uptake increased ~3.7-fold in $mdr1a(-/-)$ mice compared to $mdr1a(+/+)$ mice ($p<0.05$). Consistently, alfuzosin brain uptake increased about 4.4-fold with GF120918 co-perfusion 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 (Figure 5). Two-way ANOVA analysis indicated that there was 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 dipyridamole brain uptake was increased by 2.2-fold in the presence of 2 µM GF120918 co-perfusion in both wild type and $Abcg2(-/-)$, respectively (two-way ANOVA, $p<0.05$). Co-perfusion 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, Figure 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 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 highly variable, thus these data were excluded for comparison. Cimetidine brain-to-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-to-plasma concentration ratios of alfuzosin and dipyridamole (1.28 mg/kg/day) are shown in Figure 7. Alfuzosin brain penetration was significantly higher (4.1-fold) in mdr1a(-/-) mice compared to mdr1a(+/+) mice (Student’s t-test, p<0.05). Alfuzosin and dipyridamole brain penetration was ~3.5-fold higher in Abcg2(-/-) mice compared to 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).

**BBB Penetration, In Vitro Prediction, and Physicochemical Relationship**

It has been demonstrated that BBB permeability is primarily dependent on the lipophilicity for compounds that undergo solely passive diffusion at the BBB (Takasato et al., 1984). Figure 8A demonstrates that in the absence of P-gp, the Cl_up values for cimetidine, alfuzosin, dipyridamole and LY2228820 in mdr1a(-/-) mice were correlated (R²=0.956) with clogD7.4, the calculated logarithm of octanol water partition coefficient at pH 7.4. The values of clogD7.4 were 0.12, 0.56, 3, 6.3 for cimetidine, alfuzosin, dipyridamole and LY2228820, respectively, and were obtained using Marvin and calculator plugin freeware (www.chemaxon.com, ChemAxon Kft, Budapest, Hungary). In addition, the in situ brain permeability has been found to be correlated with in vitro apparent permeability (Summerfield et al., 2007). There is a curvilinear relationship between the Cl_up in Abcg2(-/-) mice [Cl_up(-/-)] and the P_app,AB or P_app,BA in MDCKII-Bcrp cell line when Bcrp was completely inhibited by chrys, in Figure 8B). This curvilinearity was attributed to the
aqueous boundary layer affecting the more lipophilic compounds in monolayer transport studies (Ho et al., 2000). To demonstrate this, the P<sub>app</sub> for each compound was corrected for the aqueous boundary layer to give the intrinsic transmonolayer permeability coefficient (P<sub>cell</sub>) (Table 1). Consequently, the linear correlation between Cl<sub>up</sub><sup>(i−)</sup> and P<sub>cell</sub> was markedly improved (R<sup>2</sup>=0.997, Figure 8C). In addition, Cl<sub>up</sub><sup>(i−)</sup> was correlated with the in vitro cellular accumulation of compound (R<sup>2</sup>=0.952, Figure 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.
Bcrp mediates intestinal and biliary secretion, substrate extrusion at mammary epithelia and in the placenta, and plays an important pharmacologic and toxicologic role in the absorption and disposition of xenobiotics and xenotoxins (Jonker et al., 2000; Jonker et al., 2005). However, the extent to which Bcrp might influence brain distribution of known Bcrp substrates has been unclear. The current study 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 $P_{app}$ ratio of 16. Under similar experimental conditions, a B-A / A-B $P_{app}$ 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-hr continuous subcutaneous infusion, and steady-state brain-to-plasma concentration ratios in wild type and $Abcg2(-/-)$ mice were similar to a previously published value of 0.017 after i.p. 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_{up} and brain-to-plasma concentration ratio in mdr1a(-/-) mice were 4.4- and 4.1-fold higher than those in mdr1a(+/+) mice, respectively. In addition, P-gp-mediated alfuzosin efflux was inhibited by GF120918 in mdr1a(+/-), wild type and Abcg2(-/-) mice, but not in mdr1a(-/-) mice, which confirmed that alfuzosin is a P-gp substrate at the BBB. In contrast, alfuzosin appears to be transported efficiently by Bcrp only when Bcrp is overexpressed in vitro. Alfuzosin Cl_{up} and brain-to-plasma concentration ratio was comparable between wild type and Abcg2(-/-) mice and co-perfusion with GF120918 did not increase alfuzosin Cl_{up} in mdr1a(-/-) 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 HEK 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 and Figure 2). Brain uptake of dipyridamole did not appear to be concentration-dependent in the range of 1-5 µM selected based on a reported dipyridamole mean plasma concentration 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 vs. Abcg2(-/-) mice. Co-perfusion 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 $^{14}$C-LY2228820 was mediated by Bcrp in vitro. $^{14}$C-LY2228820 transport across the BBB was highly permeable, consistent with the rapid passive diffusion observed in in vitro cell monolayers. Brain uptake of $^{14}$C-LY2228820 was almost perfusion flow rate-limited in mdr1a(-/-) mice. In mdr1a(+/+) mice, $^{14}$C-LY2228820 brain uptake decreased ~60%. $^{14}$C-LY2228820 brain uptake in Abcg2(+/+) mice was comparable to wild type and Abcg2(-/-) mice, indicating that $^{14}$C-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 following knockout of specific gene, as well as the specificity of inhibitors, are always fundamental concerns in the functional studies. Bcrp mRNA has been reported to be upregulated 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). Similarly, 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. Cyclosporine A which has long 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, co-perfusion with the P-gp inhibitor PSC833 did not
change Cl\text{up} of quercetin, while co-perfusion of 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 (Figure 3) and this 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 the protein expression of Bcrp in human and rat brain microvessel endothelial cells is much lower than in the \textit{in vitro} overexpressed system (Lee et al., 2007). This difference in protein expression might explain the discrepancies between the \textit{in vitro} cell line model and \textit{in situ} or \textit{in vivo} animal models. However, as an overexpressed cell line model, good \textit{in vitro-in vivo} correlation has been identified for 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, DHEAS and mitoxantrone (Cisternino et al., 2004; Lee et al., 2005), these results strongly 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 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 (Erdelyi et al., 2007). 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 antiepileptic 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 CF57BL/6 mice and in situ brain perfusion demonstrated no significant Mrp2 function at the BBB (Zhao et al., unpublished data). The spectrum of reports to date suggest that P-gp, and 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 prior to 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 prior to ascribing functional in vivo, and potential pharmacologic/toxicologic importance, to that transport protein in vivo.
References


Zhang S, Wang X, Sagawa K and Morris ME (2005a) Flavonoids chrysin and benzoflavone, potent breast cancer resistance protein inhibitors, have no significant effect on topotecan pharmacokinetics in rats or mdr1a/1b (-/-) mice. *Drug Metab Dispos* **33**:341-348.
Footnotes

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Figure Legends

**Figure 1.** Chemical structures of (A) cimetidine, (B) alfuzosin, (C) dipyridamole and (D) LY2228820.

**Figure 2.** Apparent permeability ($P_{\text{app}}$, $\times 10^{-6}$ cm/s) of alfuzosin (AL) and dipyridamole (DP) across the MDCKII-MDR1 cell monolayer in apical-to-basolateral (A-B, solid bar) and basolateral-to-apical (B-A, open bar) directions in the absence (AL, DP) or presence (AL+I, DP+I) of P-gp inhibitor LSN335984 (2.5 µM).

**Figure 3.** BBB Bcrp expression in whole brain homogenates (H) and isolated capillaries (C). An ~70-kD band was present in the enriched capillary fraction from both wild-type (lane 2) and Abcg2(+/+)) (lane 4) mice, but expression was not detected in Abcg2(-/-) mouse capillaries (lane 6). There was no Bcrp detected in any of the whole brain homogenate samples from wild-type (lane 1), Abcg2(+/+)) (lane 3) or Abcg2(-/-) (lane 5) mice.

**Figure 4.** Initial brain uptake clearance ($Cl_{\text{up}}$, mL/min/100 g brain) of alfuzosin in the absence (solid bar) and presence (open bar) of co-perfusion with P-gp and Bcrp inhibitor GF120918 in (A) wild type and Abcg2(-/-) mice, (B) mdr1a(+/+) and mdr1a(-/-) mice. Two-way ANOVA analysis was used to determine the statistical significance of GF120918 inhibitory effect and Bcrp effect [wild type vs. Abcg2(-/-)] or P-gp effect [mdr1a(+/+) vs. mdr1a(-/-)]. Asterisk (*) represents a statistical difference ($p<0.05$) with GF120918 inhibition; dagger (†) represents a statistical difference ($p<0.05$) in the initial rate of alfuzosin brain uptake between mdr1a(+/+) and mdr1a(-/-) mice.
Figure 5. Initial brain uptake clearance (Cl<sub>up</sub>, mL/min/100 g) for dipyridamole at different concentrations (1, 2, and 5 µM) in wild type and Abcg2(-/-) mice. Two-way ANOVA analysis was used to determine the statistical significance of concentration-dependence and Bcrp effect (p>0.05).

Figure 6. Initial brain uptake clearance (Cl<sub>up</sub>, mL/min/100 g brain) of dipyridamole in the absence (solid bar) and presence (open bar) of co-perfusion with P-gp and Bcrp inhibitor GF120918 in (A) wild type and Abcg2(-/-) mice, (B) mdr1a(+/+) and mdr1a(-/-) mice. Two-way ANOVA analysis was used to determine the statistical significance of GF120918 inhibitory effect and Bcrp effect [wild type vs. Abcg2(-/-)] or P-gp effect [mdr1a(+/+) vs. mdr1a(-/-)]. Asterisk (*) represents a statistical difference (p<0.05) with GF120918 inhibition.

Figure 7. Brain-to-plasma concentration ratios for alfuzosin and dipyridamole in wild type and Abcg2(-/-) mice, mdr1a(+/+) and mdr1a(-/-) mice following 24 hr of osmotic minipump administration. Data are presented as mean ± SD (n=3). Two tailed Student’s t-tests were used to compare brain-to-plasma concentration ratios between wild type and Abcg2(-/-) mice, and between mdr1a(+/+) and mdr1a(-/-) mice. Asterisk (*) represents a statistical difference (p<0.05) between mdr1a(+/+) and mdr1a(-/-) mice for alfuzosin brain penetration.

Figure 8. Relationship between the initial uptake clearance of substrates in mdr1a(-/-) mice [Cl<sub>up</sub>(-/-)], and (A) calculated octanol-water partition coefficient at pH 7.4 (clogD<sub>7.4</sub>); and (B) the in vitro P<sub>app</sub> in A-B (circles) or in B-A (triangles) direction; (C) the in vitro P<sub>cel</sub> in A-B (circles) direction; and (D) the percent dose of cellular accumulation [log(%Ccell)] in MDCKII-Bcrp cells incubated with 20 µM of chrysin.
DMD #25064

Table 1. Transport of ³H-cimetidine, alfuzosin, dipyridamole and ¹⁴C-LY2228820 (all at 5 µM) across MDCKII-Bcrp cell monolayer in the absence (-) or presence (+) of Bcrp inhibitor chrysin (20 µM). The intrinsic transmonolayer permeability coefficient (Pcell) was calculated using a measured $P_{ABL} = 80 \times 10^{-6}$ cm/s (Ho et al., 2000). Mass recoveries were 92 to 112%.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Inhibitor</th>
<th>$P_{app} \pm SD$ ($\times 10^{-6}$ cm/s)</th>
<th>B-A/A-B</th>
<th>$P_{cell}$ ($\times 10^{-6}$ cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A-B</td>
<td>B-A</td>
<td>$P_{app}$ ratio</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>-</td>
<td>1.4 ± 0.2</td>
<td>23 ± 2</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.8 ± 0.2</td>
<td>3.6 ± 0.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Alfuzosin</td>
<td>-</td>
<td>1.9 ± 0.002</td>
<td>60 ± 1</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>25 ± 1</td>
<td>30 ± 2</td>
<td>1.2</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>-</td>
<td>2.6 ± 1.0</td>
<td>90 ± 0.3</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>48 ± 1</td>
<td>70 ± 3</td>
<td>1.5</td>
</tr>
<tr>
<td>LY2228820</td>
<td>-</td>
<td>3.9 ± 0.2</td>
<td>143 ± 1</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>44 ± 3</td>
<td>50 ± 8</td>
<td>1.1</td>
</tr>
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Table 2. Initial brain uptake clearance (Cl<sub>up</sub>, mL/min/100 g) for <sup>3</sup>H-cimetidine and <sup>14</sup>C-LY2228820 in wild type and Abcg2(-/-) C57BL/6 mice and mdr1a(+/-) and mdr1a(-/-) CF-1 mice. Data are presented as mean ± SD (n=3). Two-tailed Student’s t-tests were used to determine the statistical significance of the Bcrp effect between wild type and Abcg2(-/-) mice, and P-gp effect between mdr1a(+/-) and mdr1a(-/-) mice. Asterisk (*) represents a statistical difference (p<0.05) between mdr1a(+/-) and mdr1a(-/-) mice for <sup>14</sup>C-LY2228820 initial brain uptake.

<table>
<thead>
<tr>
<th>Cl&lt;sub&gt;up&lt;/sub&gt; (mL/min/100g)</th>
<th>wild type</th>
<th>Bcrp(-/-)</th>
<th>mdr1a(+/-)</th>
<th>mdr1a(-/-)</th>
</tr>
</thead>
<tbody>
<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>

Asterisk (*) represents a statistical difference (p<0.05) between mdr1a(+/-) and mdr1a(-/-) mice for <sup>14</sup>C-LY2228820 initial brain uptake.
Figure 1
Figure 2

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Figure 3

Abcg2(+/-)  Abcg2(+/+)  Abcg2(-/-)
Figure 4

A

Alfuzosin Cl\textsubscript{up} (mL/min/100 g brain)

- no inhibitor
- with inhibitor

C57BL/6 Abcg2(-/-)

B

Alfuzosin Cl\textsubscript{up} (mL/min/100 g brain)

- no inhibitor
- with inhibitor

mdr1a(+/+) mdr1a(-/-)

* indicates a significant difference.
† indicates a trend towards significance.
Figure 5

Dipyridamole $\text{Cl}_{\text{up}}$ (ml/min/100g brain)

- 1 µM
- 2 µM
- 5 µM

C57BL/6  Abcg2(-/-)

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Figure 6
Figure 7

![Brain-to-Plasma Ratio Graph]

- **C57BL/6**
- **Abcg2(-/-)**
- **mdr1a(+/+)**
- **mdr1a(-/-)**

**Comparison of Brain-to-Plasma Ratios for Alfuzosin and Dipyridamole**
Figure 8

A

B

C

D

R²=0.956

R²=0.952

R²=0.997