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

Rong Zhao,¹ Thomas J. Raub, Geri A. Sawada, Steven C. Kasper, James A. Bacon, Arlene S. Bridges, and Gary M. Pollack

Department of Pharmacotherapy and Experimental Therapeutics, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina (R.Z., G.M.P.); Drug Disposition, Eli Lilly and Company, Indianapolis, Indiana (T.J.R., G.A.S., S.C.K., J.A.B.); and Division of Molecular Pharmaceutics, School of Pharmacy, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina (A.S.B.)

Received October 9, 2008; accepted March 6, 2009

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, dipyrindamole, and LY2228820) was evaluated using 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-h subcutaneous osmotic minipump continuous infusion experimental paradigm. In addition, alfuzosin and dipyrindamole 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 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 dipyrindamole 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 pinocytotic 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

This study was supported in part by the National Institutes of Health National Institute of General Medical Sciences [Grant GM61191]; and Eli Lilly and Company.

¹ Current affiliation: Roche R&D Center (China) Ltd., Shanghai, China.

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

doi:10.1124/dmd.108.025064.

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-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinoliny)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; MDR/mdr, multidrug resistance; LY2228820, 5-[2-*tert*-butyl-4-(4-fluorophenyl)-1*H*-imidazol-5-yl]-3-(2,2-dimethylpropyl)-3*H*-imidazo[4,5-*b*]pyridin-2-amine; MDCK, Madin-Darby canine kidney; LSN335984, (*R*)-4-[(1*a*,6,10*b*)-1,1-dichloro-1,1*a*,6,10*b*-tetrahydrodibenzo[*a,e*]cyclopropa[*c*]cyclohepten-6-yl]-[(5-quinolinyl)oxy)methyl]-1-piperazineethanol; 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, valsopodar.

FIG. 1. Chemical structures of cimetidine (A), alfuzosin (B), dipyrindamole (C), and LY2228820 (D).

expressed on the luminal side of brain capillary endothelial cells in mice, rats, pigs, and humans, with localization similar to that of P-gp; the 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).

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 responses, in animals and humans. However, studies to date have not clarified Bcrp function at the BBB, which remains controversial. For example, the brain penetration of imatinib mesylate was 2.5-fold higher in Bcrp-deficient mice compared with that in 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, 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 the 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, dipyrindamole, 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 (Fig. 1). Cimetidine, a prototypical histamine H₂ receptor antagonist, recently was demonstrated to possess antitumor 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 α_1 -adrenergic receptor antagonist with proven efficacy and safety in the treatment of benign prostatic hyperplasia. Alfuzosin is a structural analog of prazosin, which is a prototypical Bcrp substrate (Staud et al., 2006). Dipyrindamole 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 inhibitor discovered by Eli Lilly and Company (Indianapolis, IN) (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 (Cl_{up}) 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 coperfused 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*($-/-$) CF-1 mice (30–40 g) 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. Ungenotyped 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 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 *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication 85-23, revised in 1985).

Materials. Cimetidine and dipyrindamole 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 [¹⁴C]diazepam (56.0 mCi/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]colchicine (80.4 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). LSN335984 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. 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% 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 μ M LSN335984 to potently and selectively inhibit P-gp and not Bcrp, or 20 μ M 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 μ M 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

[^{14}C]mannitol in the absence and presence of solute and DMSO. The contribution of permeability at the aqueous boundary layer (P_{ABL}) was measured using the P_{app} of [^{14}C]testosterone (Ho et al., 2000). Positive controls run periodically included amprenavir for P-gp and prazosin for Bcrp with triamterene as control for a nonsubstrate. 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. In brief, 8 to 10 animals/group were euthanized by CO_2 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) along with 4 volumes of capillary buffer. Brains were homogenized with 10 strokes at approximately 500 revolutions/min. The homogenate was centrifuged in a fixed-angle rotor at 3500g 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 at 25,000g for 10 min 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) 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 25,000g 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 γ -glutamyl transpeptidase 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 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-Aldrich). The lysates were electrophoresed on a 4 to 12% SDS-polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA). The membrane was blocked in Dulbecco's phosphate-buffered saline containing 0.1% Tween 20 (PBST) and 5% nonfat dry milk (Bio-Rad, Hercules, CA) for 2 h 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 horseradish peroxidase-conjugated goat anti-rat secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), diluted 1:10,000 in PBST. Proteins were detected using the ECL system (GE Healthcare).

In Situ Mouse Brain Perfusion. The details of the in situ mouse brain perfusion have been described elsewhere (Dagenais et al., 2000). In brief, 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 $\times$ 0.70-mm outer diameter; Biotrol Diagnostic, Chennevières-les-Louvres, France) after 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 Inc., Holliston, MA). The perfusion buffer (Krebs-bicarbonate buffer, with 9 mM D-glucose, pH 7.4) was oxygenated with 95% O_2 and 5% CO_2 and maintained at 37°C . [^{14}C]Diazepam and [^3H]- or [^{14}C]inulin were used as blood flow rate and vascular space markers, respectively. The test compound, [^3H]cimetidine (1.6 $\mu\text{Ci/ml}$), [^{14}C]LY2228820 (0.1 $\mu\text{Ci/ml}$), alfuzosin (2 μM), or dipyrindamole (1, 2, and 5 μM), was added to the perfusate to achieve appropriate concentrations. GF120918 and dipyrindamole were dissolved in DMSO. The final concentration of DMSO in the perfusate was less than 2%. GF120918 (2 μM) was coperfused with alfuzosin and dipyrindamole to inhibit P-gp and Bcrp. The experiment was terminated by decapitation. The brain was removed carefully from the skull and cleaned of meninges and choroid 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 nonradioactive samples were analyzed by HPLC-MS/MS. Radioactive brain samples were digested in 0.7 ml of Solvable (PerkinElmer Life and Analytical Sciences, Waltham, MA) at 50°C overnight. Five milliliters of UltimaGold scintillation cocktail (PerkinElmer Life and Analytical Sciences, Waltham, MA) was added and vortex-mixed. Total radioactivity (^3H and/or ^{14}C) was determined in a Packard Tri-Carb TR 1900 liquid scintillation analyzer (PerkinElmer Life and Analytical Sciences). Parameters related to the in situ mice brain perfusion, i.e., the cerebral vascular volume (milliliters per 100 g of brain) were calculated using the following equation:

$$V_{\text{vasc}} = \frac{X_{\text{inulin}}}{C_{\text{inulin}}}$$

The initial brain uptake clearance (Cl_{up} , milliliters per minute per 100 g of brain) was calculated as

$$\text{Cl}_{\text{up}} = \frac{X_{\text{brain}}/T}{C_{\text{perf}}}$$

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

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 osmotic minipump (Alza, Palo Alto, CA) was selected to release the drug continuously over 24 h to achieve a steady-state condition at a flow rate of 8 $\mu\text{l/h}$. The concentration of the dosing solution was adjusted to the average animal body weight and mean pump rate to deliver an appropriate dose. Cimetidine, alfuzosin, and dipyrindamole were dissolved in 50% DMSO and loaded into the minipumps. Cimetidine (200 $\mu\text{g/pump}$, approximately 6.4 mg/kg/day) was implanted subcutaneously in the back of wild-type and *Abcg2*($-/-$) mice. Alfuzosin (200 $\mu\text{g/pump}$, approximately 6.4 mg/kg/day) and two doses of dipyrindamole (40 and 400 $\mu\text{g/pump}$, 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 h 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 after centrifugation at 3000 rpm for 5 min. The plasma and brain samples were stored at -20°C until analysis by HPLC-MS/MS. To ensure 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 Technologies, Carrboro, NC). Cimetidine, alfuzosin, dipyrindamole, and the internal standard, loperamide, were eluted from an Aquasil C18 column (2.1 $\times$ 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 meth-

TABLE 1

Transport of [^3H]cimetidine, [^3H]alfuzosin, [^3H]dipyridamole, and [^{14}C]LY2228820 (all at 5 μM) across MDCKII-Bcrp cell monolayers in the absence (–) or presence (+) of Bcrp inhibitor chrysin (20 μM)

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

Compound Name	Inhibitor	$P_{\text{app}} \pm \text{S.D.}$		B-A/A-B P_{app} ratio	P_{cell}
		A-B	B-A		
$\times 10^{-6} \text{ cm/s}$					
Cimetidine	—	1.4 ± 0.2	23 ± 2	16	0.2
	+	1.8 ± 0.2	3.6 ± 0.3	2.0	
Alfuzosin	—	1.9 ± 0.002	60 ± 1	31	1.6
	+	25 ± 1	30 ± 2	1.2	
Dipyridamole	—	2.6 ± 10.0	90 ± 0.3	34	3.4
	+	48 ± 1	70 ± 3	1.5	
LY2228820	—	3.9 ± 0.2	143 ± 1	37	44
	+	44 ± 3	50 ± 8	1.1	

P_{ABL} , permeability coefficient for the aqueous boundary layer.

anol; 0- to 0.70-min hold at 0% B, 0.70- to 3.12-min linear gradient to 90% B, 3.12- to 4.10-min hold at 90% B, 4.10- to 4.20-min linear gradient to 0% B, 4.20- to 4.90-min hold at 0% B; Shimadzu (Kyoto, Japan) solvent delivery system; 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, $253.1 \rightarrow 117.0$ m/z ; alfuzosin, $390.2 \rightarrow 235.2$ m/z ; and dipyridamole, $505.5 \rightarrow 429.3$ 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 relative standard deviations were <15%.

Statistical Analysis. Data are reported as mean $\pm$ S.D. for three mice per condition ($n = 3$). A two-tailed Student's t test or one-way or two-way analysis of variance (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 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 historical data (mean $\pm$ S.D.) of 22 ± 7 ($n = 17$) (G. A. Sawada and T. J. 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 (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 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 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.

Fig. 2. Apparent permeability (P_{app} , $\times 10^{-6}$ centimeters per second) of alfuzosin (AL) and dipyridamole (DP) across the MDCKII-MDR1 cell monolayer in A-B (■) and B-A (□) directions in the absence (AL and DP) or presence (AL+I and DP+I) of the P-gp inhibitor LSN335984 (2.5 μM).

Fig. 3. BBB Bcrp expression in whole brain homogenates (H) and isolated capillaries (C). An ~70-kDa 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.

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 coperfusion with 2 μM GF120918. In addition, the brain capillary volumes in wild-type and *Abcg2*(–/–) mice were comparable to those in CF-1 mice ($V_{\text{vase}} = 1.69 \pm 0.10$ 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 the initial brain uptake clearance of [^3H]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_{up} increased by 33% but did not reach statistical difference (1.1 ± 0.4 versus 0.8 ± 0.3 ml/min/100 g, $p > 0.05$) when coperfused with 2 μM GF120918 in wild-type mice. [^{14}C]LY2228820 is very permeable at the BBB. The initial rate of

TABLE 2

Initial brain Cl_{up} for [3H]cimetidine and [^{14}C]LY2228820 in wild-type and *Abcg2*(-/-) C57BL/6 mice and *mdr1a*(+/+) and *mdr1a*(-/-) CF-1 miceData are presented as mean $\pm$ S.D. ($n = 3$). Two-tailed Student's t tests were used to determine the statistical significance of the Bcrp effect between wild-type and *Abcg2*(-/-) mice and the P-gp effect between *mdr1a*(+/+) and *mdr1a*(-/-) mice.

	Cl_{up}			
	Wild-Type	<i>Bcrp</i> (-/-)	<i>mdr1a</i> (+/+)	<i>mdr1a</i> (-/-)
			ml/min/100 g	
Cimetidine	0.8 $\pm$ 0.3	0.5 $\pm$ 0.1	0.7 $\pm$ 0.2	1.1 $\pm$ 0.4
LY2228820	136 $\pm$ 33	131 $\pm$ 26	104 $\pm$ 8	239 $\pm$ 24*

* Statistical difference ($p < 0.05$) between *mdr1a*(+/+) and *mdr1a*(-/-) mice for [^{14}C]LY2228820 initial brain uptake.

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_{up} 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 ($p > 0.05$). Coperfusion 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 with that in *mdr1a*(+/+) mice ($p < 0.05$). In a consistent manner, alfuzosin brain uptake increased approximately 4.4-fold with GF120918 coperfusion in *mdr1a*(+/+) mice ($p < 0.05$). GF120918 had no effect on alfuzosin brain uptake in *mdr1a*(-/-) mice.

Three concentrations (1, 2, and 5 μ M) 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 μ M in the absence or presence of 2 μ M 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 μ M GF120918 coperfusion in both wild-type and *Abcg2*(-/-) mice, respectively (two-way ANOVA, $p < 0.05$). Coperfusion 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

FIG. 4. Initial brain Cl_{up} (milliliters per minute per 100 g of brain) of alfuzosin in the absence (■) and presence (□) of coperfusion with P-gp and the Bcrp inhibitor GF120918 in wild-type and *Abcg2*(-/-) mice (A) and in *mdr1a*(+/+) and *mdr1a*(-/-) mice (B). Two-way ANOVA analysis was used to determine the statistical significance of the GF120918 inhibitory effect and Bcrp effect [wild-type versus *Abcg2*(-/-)] or P-gp effect [*mdr1a*(+/+) versus *mdr1a*(-/-)]. *, statistical difference ($p < 0.05$) with GF120918 inhibition; †, statistical difference ($p < 0.05$) in the initial rate of alfuzosin brain uptake between *mdr1a*(+/+) and *mdr1a*(-/-) mice.

FIG. 5. Initial brain Cl_{up} (milliliters per minute per 100 g of brain) for dipyridamole at different concentrations (1, 2, and 5 μ M) in wild-type and *Abcg2*(-/-) mice. Two-way ANOVA was used to determine the statistical significance of concentration dependence and the Bcrp effect ($p > 0.05$).

(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, $p > 0.05$).

FIG. 6. Initial brain Cl_{up} (milliliters per minute per 100 g of brain) of dipyrindamole in the absence (■) and presence (□) of copfusion with the P-gp and Bcrp inhibitor GF120918 in wild-type and *Abcg2*(-/-) mice (A) and *mdr1a*(+/+) and *mdr1a*(-/-) mice (B). Two-way ANOVA was used to determine the statistical significance of the GF120918 inhibitory effect and Bcrp effect [wild-type versus *Abcg2*(-/-)] or P-gp effect [*mdr1a*(+/+) versus *mdr1a*(-/-)]. *, statistical difference ($p < 0.05$) with GF120918 inhibition.

FIG. 7. Brain/plasma concentration ratios for alfuzosin and dipyrindamole in wild-type and *Abcg2*(-/-) mice, *mdr1a*(+/+), and *mdr1a*(-/-) mice after 24 h of osmotic minipump administration. Data are presented as mean $\pm$ S.D. ($n = 3$). Two-tailed Student's t tests were used to compare brain/plasma concentration ratios between wild-type and *Abcg2*(-/-) mice and between *mdr1a*(+/+) and *mdr1a*(-/-) mice. *, statistical difference ($p < 0.05$) between *mdr1a*(+/+) and *mdr1a*(-/-) mice for alfuzosin brain penetration.

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, dipyrindamole, and LY2228820 in *mdr1a*(-/-) mice were correlated ($R^2 = 0.956$) with $\log D_{7.4}$, the calculated logarithm of the octanol-water partition coefficient at pH 7.4. The values of $\log D_{7.4}$ were 0.12, 0.56, 3, and 6.3 for cimetidine, alfuzosin, dipyrindamole, and LY2228820, respectively, and were obtained using Marvin and calculator plug-in freeware (ChemAxon Kft, Budapest, Hungary; www.chemaxon.com). 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 the MDCKII-Bcrp cell line when Bcrp was completely inhibited by chrysin (Fig. 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

behavior, the P_{app} for each compound was corrected for the aqueous boundary layer to give the intrinsic transmonolayer permeability coefficient (P_{cell}) (Table 1). Consequently, the linear correlation between $Cl_{up}(-/-)$ and P_{cell} was markedly improved ($R^2 = 0.997$) (Fig. 8C). In addition, $Cl_{up}(-/-)$ 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, dipyrindamole, 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 knock-out 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_{up} and the brain/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 the brain/plasma concentration ratio was comparable between wild-type and *Abcg2*(-/-) mice and copfusion 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.

Dipyrindamole 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 dipyrindamole interacts with murine Bcrp and human P-gp in vitro (Table 1; Fig. 2). Brain uptake of dipyrindamole did not appear to be concentration-dependent in the range of 1 to 5 μM that was selected based on a reported dipyrindamole mean plasma concen-

FIG. 8. Relationship between the initial uptake clearance of substrates in *mdr1a*($-/-$) mice [$Cl_{up}^{(-/-)}$] and calculated octanol-water partition coefficient at pH 7.4 ($\log D_{7.4}$) (A) and the in vitro P_{app} in A-B (●) or in B-A (▲) direction (B), the in vitro P_{cell} in A-B (●) direction (C), and the percent dose of cellular accumulation [$\log(\%C_{cell})$] in MDCKII-Bcrp cells incubated with 20 μ M chrysin (D).

tration of 3.5 μ M (1.77 μ g/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 dipyrindamole brain uptake. In addition, the initial rate of dipyrindamole brain uptake did not differ between *mdr1a*($+/+$) and *mdr1a*($-/-$) mice, also suggesting that dipyrindamole 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 $\sim 60\%$. [14 C]LY2228820 brain uptake in *Abcg2*($+/+$) mice was comparable to that in 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 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 Cl_{up} 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, dipyrindamole, 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 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 (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.

Acknowledgments. We thank Dr. Chris Wiesen (Odum Institute for Research in Social Science, University of North Carolina at Chapel Hill) for statistical consultation.

References

- Adachi Y, Suzuki H, and Sugiyama Y (2001) Comparative studies on in vitro methods for evaluating in vivo function of MDR1 P-glycoprotein. *Pharm Res* **18**:1660–1668.
- Aronica E, Gorter JA, Redeker S, van Vliet EA, Ramkema M, Scheffer GL, Schep RJ, van der Valk P, Leenstra S, Baayen JC, et al. (2005) Localization of breast cancer resistance protein (BCRP) in microvessel endothelium of human control and epileptic brain. *Epilepsia* **46**:849–857.
- Begley DJ (2004) Delivery of therapeutic agents to the central nervous system: the problems and the possibilities. *Pharmacol Ther* **104**:29–45.
- Belinsky MG, Guo P, Lee K, Zhou F, Kotova E, Grinberg A, Westphal H, Shchaveleva I, Klein-Szanto A, Gallo JM, et al. (2007) Multidrug resistance protein 4 protects bone marrow, thymus, spleen, and intestine from nucleotide analogue-induced damage. *Cancer Res* **67**:262–268.
- Bihorel S, Camenisch G, Lemaire M, and Scherrmann JM (2007) Influence of breast cancer resistance protein (Abcg2) and p-glycoprotein (Abcb1a) on the transport of imatinib mesylate (Gleevec®) across the mouse blood-brain barrier. *J Neurochem* **102**:1749–1757.
- Breedveld P, Pluim D, Cipriani G, Wielinga P, van Tellingen O, Schinkel AH, and Schellens JH (2005) The effect of Bcrp1 (Abcg2) on the in vivo pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. *Cancer Res* **65**:2577–2582.
- Cisternino S, Mercier C, Bourasset F, Roux F, and Scherrmann JM (2004) Expression, up-regulation, and transport activity of the multidrug-resistance protein Abcg2 at the mouse blood-brain barrier. *Cancer Res* **64**:3296–3301.
- Cooray HC, Blackmore CG, Maskell L, and Barrand MA (2002) Localisation of breast cancer resistance protein in microvessel endothelium of human brain. *Neuroreport* **13**:2059–2063.
- Dagenais C, Rousselle C, Pollack GM, and Scherrmann JM (2000) Development of an in situ mouse brain perfusion model and its application to mdr1a P-glycoprotein-deficient mice. *J Cereb Blood Flow Metab* **20**:381–386.
- Dallaire L, Tremblay L, and Beliveau R (1991) Purification and characterization of metabolically active capillaries of the blood-brain barrier. *Biochem J* **276** (Pt 3):745–752.
- de Vries NA, Zhao J, Kroon E, Buckle T, Beijnen JH, and van Tellingen O (2007) P-glycoprotein and breast cancer resistance protein: two dominant transporters working together in limiting the brain penetration of topotecan. *Clin Cancer Res* **13**:6440–6449.
- Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, and Ross DD (1998) A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A* **95**:15665–15670.
- Enokizono J, Kusuhashi H, and Sugiyama Y (2007) Effect of breast cancer resistance protein (Bcrp/Abcg2) on the disposition of phytoestrogens. *Mol Pharmacol* **72**:967–975.
- Erdilyi DJ, Kámory E, Csókay B, Andrikovics H, Tordai A, Kiss C, Filni-Semsei A, Janszky I, Zalka A, Fekete G, et al. (2008) Synergistic interaction of ABCB1 and ABCG2 polymorphisms predicts the prevalence of toxic encephalopathy during anticancer chemotherapy. *Pharmacogenomics J* **8**:321–327.
- Hervey PS and Goa KL (1999). Extended-release dipyridamole/aspirin. *Drugs* **58**:469–477.
- Ho NFH, Raub TJ, Burton PS, Barsuhn CL, Adson A, Audus KL, and Borchardt RT (2000) Quantitative approaches to delineate passive transport mechanisms in cell culture monolayers, in *Transport Processes in Pharmaceutical Systems* (Gordon GL, Lee IP, and Topp EM eds) pp 219–316, Marcel Dekker, New York.
- Hough LB, Glick SD, and Su K (1986) Cimetidine penetrates brain and inhibits non-opiate footshock-induced analgesia. *Pharmacol Biochem Behav* **24**:1257–1261.
- Jonker JW, Merino G, Musters S, van Herwaarden AE, Bolscher E, Wagenaar E, Mesman E, Dale TC, and Schinkel AH (2005) The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. *Nat Med* **11**:127–129.
- Jonker JW, Smit JW, Brinkhuis RF, Maliepaard M, Beijnen JH, Schellens JH, and Schinkel AH (2000) Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. *J Natl Cancer Inst* **92**:1651–1656.
- Lee G, Babakanian K, Ramaswamy M, Prat A, Wosik K, and Bendayan R (2007) Expression of the ATP-binding cassette membrane transporter, ABCG2, in human and rodent brain microvessel endothelial and glial cell culture systems. *Pharm Res* **24**:1262–1274.
- Lee YJ, Kusuhashi H, Jonker JW, Schinkel AH, and Sugiyama Y (2005) Investigation of efflux transport of dehydroepiandrosterone sulfate and mitoxantrone at the mouse blood-brain barrier: a minor role of breast cancer resistance protein. *J Pharmacol Exp Ther* **312**:44–52.
- Lefranc F, Yeaton P, Brothi J, and Kiss R (2006) Cimetidine, an unexpected anti-tumor agent, and its potential for the treatment of glioblastoma (review). *Int J Oncol* **28**:1021–1030.
- Leggas M, Adachi M, Scheffer GL, Sun D, Wielinga P, Du G, Mercer KE, Zhuang Y, Panetta JC, Johnston B, et al. (2004) Mrp4 confers resistance to topotecan and protects the brain from chemotherapy. *Mol Cell Biol* **24**:7612–7621.
- Mader M, de Dios A, Shih C, Bonjoukian R, Li T, White W, López de Uralde B, Sánchez-Martínez C, del Prado M, Jaramillo C, et al. (2008) Imidazolyl benzimidazoles and imidazo[4,5-b]pyridines as potent p38alpha MAP kinase inhibitors with excellent in vivo anti-inflammatory properties. *Bioorg Med Chem Lett* **18**:179–183.
- Merino G, van Herwaarden AE, Wagenaar E, Jonker JW, and Schinkel AH (2005) Sex-dependent expression and activity of the ATP-binding cassette transporter breast cancer resistance protein (BCRP/ABCG2) in liver. *Mol Pharmacol* **67**:1765–1771.
- Orlowski M and Meister A (1965) Isolation of γ -glutamyl transpeptidase from hog kidney. *J Biol Chem* **240**:338–347.
- Pardridge WM (2005) The blood-brain barrier: bottleneck in brain drug development. *NeuroRx* **2**:3–14.
- Pavek P, Merino G, Wagenaar E, Bolscher E, Novotna M, Jonker JW, and Schinkel AH (2005) Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. *J Pharmacol Exp Ther* **312**:144–152.
- Pfister JR, Makra F, and Muehldorf AV (1995) Methanodibenzosuberilypiperazines as potent multidrug resistance reversal agents. *Bioorg Med Chem Lett* **5**:2473–2476.
- Potschka H, Fedorowitz M, and Löscher W (2003) Multidrug resistance protein MRP2 contributes to blood-brain barrier function and restricts antiepileptic drug activity. *J Pharmacol Exp Ther* **306**:124–131.
- Schinkel AH (1999) P-Glycoprotein, a gatekeeper in the blood-brain barrier. *Adv Drug Deliv Rev* **36**:179–194.
- Staud F, Vackova Z, Pospechova K, Pavek P, Ceckova M, Libra A, Cygalova L, Nachtigal P, and Fendrich Z (2006) Expression and transport activity of breast cancer resistance protein (Bcrp/Abcg2) in dually perfused rat placenta and HRP-1 cell line. *J Pharmacol Exp Ther* **319**:53–62.
- Summerfield SG, Read K, Begley DJ, Obradovic T, Hidalgo JJ, Coggon S, Lewis AV, Porter RA, and Jeffrey P (2007) Central nervous system drug disposition: the relationship between in situ brain permeability and brain free fraction. *J Pharmacol Exp Ther* **322**:205–213.
- Takasato Y, Rapoport SI, and Smith QR (1984) An in situ brain perfusion technique to study cerebrovascular transport in the rat. *Am J Physiol* **247**:H484–H493.
- Tanaka Y, Slitt AL, Leazer TM, Maher JM, and Klaassen CD (2005) Tissue distribution and hormonal regulation of the breast cancer resistance protein (Bcrp/Abcg2) in rats and mice. *Biochem Biophys Res Commun* **326**:181–187.
- Xia CQ, Liu N, Miwa GT, and Gan LS (2007) Interactions of cyclosporin A with breast cancer resistance protein. *Drug Metab Dispos* **35**:576–582.
- Yasuno F, Brown AK, Zoghbi SS, Krushinski JH, Chernet E, Tauscher J, Schaus JM, Phebus LA, Chesterfield AK, Felder CC, et al. (2008) The PET radioligand [11 C]MePPEP binds reversibly and with high specific signal to cannabinoid CB1 receptors in nonhuman primate brain. *Neuropsychopharmacology* **33**:259–269.
- Youdim KA, Qaiser MZ, Begley DJ, Rice-Evans CA, and Abbott NJ (2004) Flavonoid permeability across an in situ model of the blood-brain barrier. *Free Radic Biol Med* **36**:592–604.
- Yousif S, Marie-Claire C, Roux F, Scherrmann JM, and Declèves X (2007) Expression of drug transporters at the blood-brain barrier using an optimized isolated rat brain microvessel strategy. *Brain Res* **1134**:1–11.
- Zamek-Gliszczynski MJ, Nezasa K, Tian X, Kalvass JC, Patel NJ, Raub TJ, and Brouwer KL (2006) The important role of Bcrp (Abcg2) in the biliary excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in mice. *Mol Pharmacol* **70**:2127–2133.
- 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.
- Zhang Y, Gupta A, Wang H, Zhou L, Vethanayagam RR, Unadkat JD, and Mao Q (2005b) BCRP transports dipyridamole and is inhibited by calcium channel blockers. *Pharm Res* **22**:2023–2034.

Address correspondence to: Dr. Gary M. Pollack, Division of Pharmacotherapy and Experimental Therapeutics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7360. E-mail: gary_pollack@unc.edu