The Effect of Breast Cancer Resistance Protein and P-Glycoprotein on the Brain Penetration of Flavopiridol, Imatinib Mesylate (Gleevec), Prazosin, and 2-Methoxy-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl)propanoic Acid (PF-407288) in Mice

Lin Zhou, Kari Schmidt, Frederick R. Nelson, Veronica Zelesky, Matthew D. Troutman, and Bo Feng

Department of Pharmaceutics, University of Washington, Seattle, Washington (L.Z.); and Department of Pharmacokinetics, Dynamics, and Drug Metabolism, Pfizer Global Research and Development, Groton, Connecticut (K.S., F.R.N., V.Z., M.D.T., B.F.)

Received September 9, 2008; accepted February 12, 2009

ABSTRACT:
The role of breast cancer resistance protein (Bcrp) and the combined activities of Bcrp and P-glycoprotein (P-gp, Mdr1a/1b) in limiting the brain penetration of drugs at the blood-brain barrier (BBB) were investigated using wild-type FVB, Mdr1a/1b(-/-), (-/-), Bcrp(-/-), and Mdr1a/1b(-/-), (-/-)Bcrp(-/-) mice. Four drugs, flavopiridol, imatinib mesylate (Gleevec), PF-407288, and prazosin, with different transport specificity for BCRP/Bcrp and MDR1/MDR1a were selected, and the drug levels in plasma, cerebrospinal fluid, and brain of mice were determined. Flavopiridol and prazosin were identified as substrates for both mouse Bcrp and Mdr1a with greater transport associated with Bcrp. The brain/plasma (B/P) ratios at 0.5 and 2 h in Mdr1a/1b(-/-), (-/-) and Bcrp(-/-) mice were 1- to 2-fold for both compounds, whereas the ratios in Mdr1a/1b(-/-), (-/-)Bcrp(-/-) mice were more than 5-fold of those observed in FVB mice. For imatinib, a better substrate of P-gp than Bcrp, the B/P ratios in Bcrp(-/-) were comparable to those in FVB mice, whereas the B/P ratios in Mdr1a/1b(-/-), (-/-) and Mdr1a/1b(-/-), (-/-)Bcrp(-/-) mice were more than 4- and 28-fold of those in FVB mice at both time points, respectively. Finally, the Bcrp-specific substrate PF-407288 exhibited comparable B/P ratios in Mdr1a/1b(-/-), (-/-) and Bcrp(-/-) mice and slightly but significantly increased B/P ratios in Mdr1a/1b(-/-), (-/-)Bcrp(-/-) mice compared with those in FVB mice. The B/P ratios of compounds in Mdr1a/1b(-/-), (-/-), (-/-)Bcrp(-/-) mice compared with those in Mdr1a/1b(-/-), (-/-) mice clearly demonstrate that Bcrp impairs the brain penetration of its substrates. Moreover, P-gp and Bcrp at BBB function synergistically to limit the brain penetration of shared substrates.

It is widely recognized that the tight junctions between adjacent brain endothelial cells forming the blood-brain barrier (BBB) restrict the entry of compounds by paracellular diffusion from the blood to the brain. Moreover, the transcellular diffusion of compounds through the brain endothelial cells can also be impeded by transmembrane efflux transporters, such as P-glycoprotein (P-gp, MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2). These efflux transporters can eliminate xenobiotics from the brain against a concentration gradient, thereby limiting central nervous system (CNS) exposure to these compounds. Indeed, the prominent effect of P-gp at the BBB is well established, and P-gp is functionally important in limiting the brain penetration of its substrates (Schnikel et al., 1994; Chen et al., 2003; Scherrmann, 2005). Like P-gp, BCRP is another major member of the ATP-binding cassette family of drug transporters and is highly expressed in the BBB as well. BCRP has been found at the luminal side of human brain capillary endothelial cells (Cooray et al., 2002), and the mRNA level of mouse analog Bcrp was ~700 times greater in brain microvessels than in the cortex of the mice (Cisternino et al., 2004), implying that mouse Bcrp may play a key role at the BBB. BCRP has been identified as accepting sulfon conjugated organic anions as well as hydrophobic and amphiphilic compounds (Lee et al., 2004), and the substrate specificity has considerable, but varying, overlaps with that of P-gp (Doyle and Ross, 2003). To date, the importance of BCRP in the disposition of drugs has been

ABBREVIATIONS: BBB, blood-brain barrier; P-gp, P-glycoprotein; Mdr1/Mdr1, ABCB1, multidrug resistance protein; Bcrp/BCRP, ABCG2, breast cancer resistance protein; CNS, central nervous system; WT, wild-type; KO, knockout; MDCK, Madin-Darby canine kidney; PF-407288, 2-methoxy-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl)propanoic acid; HPLC, high-performance liquid chromatography; MEM, minimal essential medium; PBS, phosphate-buffered saline; A, apical; B, basolateral; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ER, efflux ratio; RR, ratio of ratios; CSF, cerebrospinal fluid; B/P, brain/plasma ratio; CP-018857, benzyl 3-(dimethylamino)-3-oxo-2-phenylpropanoate; CP-660551, 4-chloro-N-(4-methyl-3-(4-(pyridin-3-y1)pyrimidin-2-ylaminophenyl)benzamide; CE-267266, 7-hydroxy-3-(2-methoxyphenyl)-8-(piperidin-1-ylmethyl)-4H-chromen-4-one; PF-548939, 2-(4-(2-(4-methoxyphenyl)-5-methyloxazol-4-yl)ethoxy)benzyl)-tetrahydrofuran-2-carboxylic acid.
strated in various studies (Allen et al., 1999; Jonker et al., 2002; Kruijtizer et al., 2002; Lee et al., 2005; Zaher et al., 2006). However, the relevance of this transporter to the CNS distribution of drugs is not well understood. It has been reported that brain penetration of imatinib increased 4.2-fold in mice pretreated with elacridar, an inhibitor of both Bcrp and P-gp (Breedveld et al., 2005). Imatinib brain penetration has been consistently observed to increase 2.5-fold in Bcrp(−/−) mice (Breedveld et al., 2005), demonstrating that Bcrp restricts its substrates across mouse brain in vivo. The Cistermino group has also demonstrated that brain uptake of two mouse Bcrp substrates, mitoxantrone and prazosin, increased ∼3- and ∼2-fold, respectively, in Mdr1a/1b(−/−), (−/−) mice in the presence of elacridar, confirming that Bcrp plays a role in limiting brain penetration of drugs (Cistermino et al., 2004). In contrast, Lee et al. (2005) reported that mitoxantrone and dehydroepiandrosterone sulfate brain uptake did not change in Bcrp(−/−) mice, although mitoxantrone brain uptake increased ∼1.6-fold in Mdr1a/1b(−/−), (−/−) mice in the presence of elacridar (Lee et al., 2005), thus leading these authors to conclude that Bcrp may not be important in limiting brain penetration of its substrates. Additionally, another group has reported that the $K_v$ value ($C_{br}/C_{plasma}$) did not change for Bcrp substrates, 2-amino-1-methyl-6-phenylimidazo[4,5-f]quinoline (van Herwaarden et al., 2003) and 2-amino-3-methylimidazo[4,5-b]quinoline (van Herwaarden et al., 2003, 2006), in Bcrp(−/−) versus wild-type (WT) mice. Despite efforts devoted to investigate the role that BCRP/Bcrp plays in vivo in relation to brain penetration, the significance is still unclear in mice and human.

In the present research, we conducted studies in Mdr1a/1b(−/−), (−/−), Bcrp(−/−), and Mdr1a/1b(−/−), (−/−)Bcrp(−/−) knockout (KO) mice to gain understanding of the in vivo role of Bcrp and the combined role of Bcrp and P-gp in brain penetration. Previous studies have mainly used inhibitors of the Bcrp transporter to elucidate the in vivo functions of Bcrp expressed in BBB. However, the inhibitors may not be Bcrp-specific, and the inhibition may not be complete at the plasma concentrations achieved, thus potentially complicating the results and conclusions. It is noteworthy that, in this work, we highlight the great value of Mdr1a/1b(−/−), (−/−),Bcrp(−/−) so that there is no potential compensatory up-regulation of P-gp or Bcrp in this triple KO mice, which could confound attempts to assess the functions of two transporters at the BBB. In addition, in vitro-in vivo relationships regarding BCRP efflux liability are typically assumed to be analogous to those derived for P-gp but have yet to be explicitly studied. This work includes studies to provide further information on 1) the in vivo role of Bcrp efflux and the combined role of Bcrp and P-gp in brain penetration. In the course of the experiment). For both A→B and B→A transport, the donor is the A compartment and the receiver is the B compartment, because in plates used for B→A transport, cells were seeded on the other side of the insert membrane. This experimental approach allows for a self-correction of non-specific binding when efflux ratios are calculated. On day 5, medium was removed from the insert and 75 μl of fresh buffer B containing test substrates at 2 μM was added using a 96-well pipettor (Apirac Designs, Covina, CA). Mdr1a/1b(−/−), Bcrp(−/−), and Mdr1a/1b(−/−), (−/−)Bcrp(−/−) KO mice. Finally, the results of these studies were compared with the effect of Bcrp at the BBB, in attempts to establish a correlation between the in vitro transporter assay and the in vivo effects.

Materials and Methods

Materials. Flavopiridol, imatinib mesylate (Gleevec; Novartis, Basel, Switzerland), PF-407288, and prazosin were obtained from Pfizer Inc. (Groton, CT). High-performance liquid chromatography (HPLC)-grade dimethyl sulfoxide was obtained from Thermo Fisher Scientific (Waltham, MA), and used as the solvent for making stock solutions for all compounds. MEM Alpha, penicillin-streptomycin, fetal bovine serum, nonessential amino acids, t-glutamine, trypsin-EDTA, phosphate-buffered saline (PBS), buffer B (Hanks’ balanced salt solution with 25 mM t-glucose, 20 mm HEPES, 1.25 mM CaCl₂, and 0.5 mM MgCl₂), and genetin were purchased from Invitrogen (Carlsbad, CA). Feeder trays and 96-well membrane inserts were purchased from BD Biosciences (San Jose, CA), and 1.2-ml 96-well deep well plates were purchased from Marsch (Rochester, NY). Mouse plasma and brain were purchased from Pel-Freeze Biologicals (Rogers, AR).

Cell Culture. MDCK cells were obtained from American Type Culture Collection (Manassas, VA). The MDR1-transfected MDCK cell line (MDR1-MDCK) was acquired externally (Dr. Piet Borst, Netherlands Cancer Institute, Amsterdam, The Netherlands), and mouse Mdr1a-transfected MDCK (Mdr1a-MDCK) and human BCRP-transfected MDCK (BCRP-MDCK) cell lines were generated by Pfizer Inc. (Xiao et al., 2006). The mouse TReX-Bcrp-MDCK cell line, which uses the tet-inducible promoter to express the mouse Bcrp, was acquired through a collaboration with XenoPort (Santa Clara, CA). All cell lines, except the TReX-Bcrp-MDCK cell line, were grown and maintained in MEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.5 mg/ml G418 at 37°C, 95% humidity, and 5% CO₂. As an inducible cell line, the TReX-Bcrp-MDCK cells were grown and maintained as other cells except that G418 was omitted in the culture medium, and 1 μg/ml doxycycline was added for induction. Cells were grown to 80 to 90% confluence and treated with trypsin-EDTA before harvesting for subculturing or for further use in Transwell assays.

Transporter 96-Well Transwell Assays. A high-throughput 96-well Transwell assay method similar to that described previously (Feng et al., 2008) was used. On day 1, MDCK, human MDR1-MDCK, mouse Mdr1a-MDCK, human BCRP-MDCK, and mouse TReX-Bcrp-MDCK cells were seeded into a cell density of 2.5 × 10⁵ cells/ml in complete MEM Alpha on either side of the PET membrane of 96-well inserts. For apical (A) to basolateral (B) (A→B) transport studies, cells were seeded onto the A side of the insert, whereas for B→A transport studies, cells were seeded onto the underside of the membrane when the insert was placed upside-down. The inserts for B→A transport were incubated for 60 min at 37°C, 95% humidity, and 5% CO₂, to allow the attachment of cells on the membrane. Then, all of the inserts were placed into feeder trays containing 37 ml of complete MEM Alpha growth medium and cultured (37°C, 95% humidity, and 5% CO₂) for 5 days. All in vitro transport experiments were performed under sink conditions (at least 90% or greater of mass applied to the donor compartment remained over the course of the experiment). For both A→B and B→A transport, the donor is the A compartment and the receiver is the B compartment, because in plates used for B→A transport, cells were seeded on the other side of the insert membrane. This experimental approach allows for a self-correction of non-specific binding when efflux ratios are calculated. On day 5, medium was removed from the insert and 75 μl of fresh buffer B containing test substrates at 2 μM was added using a 96-well pipettor (Apirac Designs, Covina, CA). Mdr1a/1b(−/−), Bcrp(−/−), and Mdr1a/1b(−/−), (−/−)Bcrp(−/−) KO cells were grown and maintained as other cells except that G418 was omitted in the culture medium, and 1 μg/ml doxycycline was added for induction. Cells were grown to 80 to 90% confluence and treated with trypsin-EDTA before harvesting for subculturing or for further use in Transwell assays.

Transport Study Sample Analysis by LC-MS/MS. The samples were centrifuged for 10 min at 3500 rpm in a Sorvall RC 3C Plus centrifuge (Sorvall, Newton, CT) before LC-MS/MS analysis. The samples were analyzed using a Sciex API-4000 triple quadrupole mass spectrometer (PerkinElmerSciex, Ontario, ON, Canada) equipped with a turbo ion source interface. A Gilson 215 autosampler (Gilson Inc., Middleton, WI) was used to make 25-μl injections with further sample concentration, and on-line cleanup was achieved through the use of a single column/valve-switching system equipped with a two-position 10-port Valco valve (Valco Instruments, Houston, TX) and a cartridge-style 1.5 × 5 mm column (Optimize Technologies Inc., Oregon City, OR) custom packed with 1.3-μm particle size Showa Denko (Tokyo, Japan) ODP polymeric packing material. Two Shimadzu SCL-10AD VP HPLC pumps (Shimadzu Inc., Torrance, CA) were used to deliver the mobile phase at a flow rate of 1.2 ml/min. The aqueous mobile phase (sample
loading) consisted of 95% 2 mM ammonium acetate-5% 50:50 acetonitrile/methanol, and the organic mobile phase (sample elution) consisted of 10% 2 mM ammonium acetate-90% 50:50 acetonitrile/methanol. Data acquisition was carried out in the positive ionization mode (ion spray voltage of 5.5 kV) for prazosin, imatinib, and flavopiridol and in the negative mode (ion spray voltage of 4.5 kV) for PF-407288. Selected reaction monitoring was used to monitor for analyte and internal standard simultaneously. The following transitions were used for analyte detection: 384.5 m/z → 247 m/z for prazosin, 495 m/z → 395 m/z for imatinib, 402.8 m/z → 340.6 m/z for flavopiridol, and 381 m/z → 119 m/z for PF-407288. For internal standard PFP-628374, a transition of 687.3 m/z → 319.7 m/z was used in the positive mode and 685 m/z → 362 m/z in the negative mode. A Q2 offset voltage of 5 V was used with collision energies of 45 eV for prazosin and PF-407288 and 30 eV for imatinib and flavopiridol.

Transwell Data Analysis. The following procedures were used to determine compound apparent permeability ($P_{app}$) values for studies conducted with MDCK, MDR1-MDCK, mouse Mdr1a-MDCK, BCRP-MDCK, and mouse TREx-Bcrp-MDCK cell lines. The $P_{app}$ was calculated using eq. 1: \[ P_{app} = \frac{I}{\text{Area} \times C_{0}(t)} \times \frac{dM_{i}}{dt} \] where Area is the surface area of the cell monolayer (0.0625 cm²), $C_{0}(t)$ is the initial concentration of compound applied to the donor chamber, $t$ is time, $M_{i}$ is the mass of compound appearing in the receiver compartment as a function of time, and $dM_{i}/dt$ is the flux of the compound across the cell monolayer. The efflux ratio (ER) was calculated using eq. 2: \[ \text{ER} = \frac{P_{app,i}}{P_{app}} \] where A>B and B>A denote the transport direction in which $P_{app}$ was determined. The ratio of ratios (RR) was calculated using eq. 3: \[ \text{RR} = \frac{ER_{\text{transfected}}}{ER_{\text{WT}}} \] where ERtransfected is the ER determined in the transfected MDCK cells (MDR1-MDCK, Mdr1a-MDCK, BCRP-MDCK, and TREx-Bcrp-MDCK) and ERWT is the ER determined in WT MDCK cells. RRMDR1, RRMdr1a, RRBCRP, and RRbcrp denote RR determined for ER in MDR1-MDCK, Mdr1a-MDCK, BCRP-MDCK, and TREx-Bcrp-MDCK transfected cells, respectively, compared with ER in WT MDCK.

Animal Studies. Male WT FVB, Mdr1a/lb(−/−), (−/−), Bcrp(−/−), and Mdr1a/lb(−/−), (−/−), Bcrp(−/−) mice of approximately 8 weeks of age, weighing 25 to 30 g, were obtained from Taconic Farms (Germantown, NY). Upon arrival, the mice were maintained for at least 5 days on a 12-h light/dark cycle in a temperature- and humidity-controlled environment with free access to food and water. The mice were housed in clear polycarbonate boxes (n = 50) containing sawdust. The study was conducted in accordance with approved Pfizer Animal Care and Use Procedures.

Dose Administration and Sample Collection. Mice were administered a single 5 mg/kg s.c. dose (n = 4–5 per genotype per time point per drug). Dosing solutions of each drug were prepared in PBS, with or without 5% dimethyl sulfoxide or 10% glycerol formal, when necessary, or 20% hydroxypropyl β-cyclodextrin. The resulting doses were administered in a volume of 10 ml/kg. Mice were euthanized in a CO2 chamber at 0.5 and 2 h postdose. Whole blood was collected by cardiac puncture into Microtainer tubes containing lithium heparin and stored on ice until centrifuged for the preparation of plasma. Cerebrospinal fluid (CSF) was collected via puncture of the cisterna magna using a 25-gauge needle attached to polyethylene tubing (internal diameter of 2.0 mm) and a syringe. Whole brains were collected by decapitation, rinsed with PBS, and weighed. CSF and whole brains were immediately frozen on dry ice upon collection until analysis.

Animal Sample Preparation. In preparation for analysis by LC-MS/MS, brain tissue was diluted 1:4 (w/v) with PBS and homogenized with a Mini Beadbeater (BioSpec Products, Inc., Bartlesville, OK) with 2-mm zirconia beads for 2 min. For all of the drugs, the brain and plasma samples were treated in a similar manner in terms of analysis. The samples were processed through proteolysis using acetonitrile containing an internal standard that was structurally similar to each individual analyte. The CSF samples were directly injected onto the LC-MS/MS system.

Animal Study Sample Analysis Using LC-MS/MS. The samples were analyzed using a Micromass Quattro Ultima LC-MS/MS system (Waters, Milford, MA). A Leap autosampler (Gilion) was used to make 10-μl injections. Separation of the compound and corresponding internal standard was achieved using a Phenomenex Luna C18(2) column (2 × 30 mm; 5-μ particle size). Two Shimadzu HPLC pumps (Shimadzu Inc.) were used to deliver the mobile phase at a flow rate of 0.6 ml/min. The aqueous mobile phase (phase A) consisted of 20 mM ammonium acetate with the addition of 0.1% formic acid and 0.1% isopropanol. The organic mobile phase (phase B) was 100% acetonitrile. During the first ½-min of the chromatographic run, the mobile phase solvents were held at a constant ratio of 95.5 (A:B) that was followed by a 1.3-min linear gradient to a mobile phase solvent ratio of 10:90 (A:B). The intermediate condition was held for 0.2 min followed by an immediate return to the starting conditions. The starting conditions were then maintained for a 1-min period of equilibration. Data acquisition was carried out in the positive ionization mode, and the instrument was fitted with an electrospray ionization Z-spray interface for the analytes. Selected reaction monitoring was used to monitor for analyte and internal standard simultaneously. The following transitions were used for analyte detection: 384.0 m/z → 247.0 m/z for prazosin, 494.2 m/z → 394.2 m/z for imatinib, 402.0 m/z → 341.2 m/z for flavopiridol, and 382.0 m/z → 186.0 m/z for PF-407288. For internal standard detection, the following transitions were used: 414.1 m/z → 249 m/z for CP-018857, 416.1 m/z → 139.1 m/z for CP-660551, 366.1 m/z → 281 m/z for CE-267266, and 438.2 m/z → 216.1 m/z for PF-549839. The collision energy values for prazosin, imatinib, flavopiridol, and PF-407288 were 25, 30, 22, and 25 eV, respectively.

Animal Study Data and Statistical Analysis. The experiments were set up as a randomized complete block design with genotype as the main effect and time as a secondary effect. The sampling times were 0.5 and 2 h postdose. The genotypes included WT and Mdr1a/lb(−/−), (−/−), Bcrp(−/−), and Mdr1a/lb(−/−), (−/−), Bcrp/−(−)/KO. The sample size was determined assuming a small percentage of dropouts for a significance level of 0.05 and a power of 0.8 at each time point. The comparisons of concentrations and ratios were done using a Student’s t test with unequal variances.

Brain Tissue and Plasma Protein Binding Studies. Plasma and brain free fraction values were determined for all compounds through the use of a 96-well equilibration dialysis apparatus (HTDialysis, Gales Ferry, CT). Mouse plasma and brains were obtained from a commercial source. The mouse brains were diluted at a ratio of 1:2 (w/v) with PBS followed by homogenization via the Mini Beadbeater (BioSpec Products, Inc.) with 2-mm zirconia beads for 2 min. Spectra-Por number 2 membranes with molecular weight cutoff of 12 to 14 kDa were used for equilibrium dialysis (Spectrum Laboratories, Rancho Dominguez, CA). Before the experiments, membranes were sequentially soaked in HPLC grade water for 15 min and 30% ethanol for 15 min, rinsed with HPLC grade water, and then stored in PBS until use.

The pH of the plasma, brain homogenate, and buffer was adjusted to 7.4 with either 20% phosphoric acid or 5 N NaOH. Compounds at the specific concentrations were spiked into plasma, brain homogenate, and buffer, and 150 μl was then loaded into the dialysis apparatus and dialyzed against an equal amount of PBS. The plate was sealed with a CO2-permeable adhesive membrane, and equilibration was achieved over a 6-h incubation period in a Stericult cell incubator at 37°C, 95% humidity, and 5% CO2. After the conclusion of the incubation, 100 μl of the donor, receiver, and equilibrium controls was transferred to marhs tubes containing an equal amount of the alternate matrix to yield an identical matrix between the donor and receiver samples from each incubation. The mixed matrix samples were immediately frozen and stored at −20°C until analysis. Samples were analyzed using the LC-MS/MS/MS conditions described above for in vivo studies.

The unbound fraction $f_u$ was calculated using the following equation:

$$ f_u = \frac{1}{D} \left[ \frac{1}{f_{u2}} - 1 \right] + \frac{1}{D} $$

where $D$ is the dilution factor for biological matrix and $f_{u2}$ is the unbound fraction measured from diluted samples.
FUNCTION OF P-gp AND Bcrp ON THE CNS PENETRATION OF DRUGS

Fig. 1. Chemical structure of PF-407288.

Results

Compound Selection Using in Vitro 96-Well Transporter Transwell Assay. Transport studies performed in a 96-well Transwell format were used to identify human MDR1, mouse Mdr1a, human BCRP, and mouse Bcrp substrates using MDCK, human MDR1-MDCK, mouse Mdr1a-MDCK, human BCRP-MDCK, and mouse TREX-BCRP-MDCK cell lines. Four compounds of the more than 30 compounds tested, including PF-407288 (Fig. 1), flavopiridol, prazosin, and imatinib, were chosen for further evaluation in the transporter KO mouse models. The $P_{app}$ and ER values determined using MDR1-MDCK, Mdr1a-MDCK, BCRP-MDCK, and TREX-BCRP-MDCK Transwell assays are illustrated in Table 1. The magnitude of RR values was used to definitively assign whether a compound was a human MDR1 substrate, and the same approach was used to identify human BCRP, mouse Mdr1a, and mouse Bcrp substrates (Feng et al., 2008). Previously established cutoffs of 1.7 for RR$_{MDR1}$ and 1.5 for RR$_{Mdr1a}$ and RR$_{Bcrp}$ were shown to yield a positive in vitro and in vivo correlation for human and mouse MDR1/Mdr1a and BCRP/Bcrp substrates (Xiao et al., 2006; Feng et al., 2008). The RR$_{MDR1}$, RR$_{Mdr1a}$, RR$_{BCRP}$, and RR$_{Bcrp}$ values for the four compounds can be found in Table 2, and our qualitative assessment of substrate specificity and preference is based on the respective magnitude of RR values. Based on the cutoffs, PF-407288 was identified as a human and mouse BCRP/BCRP-specific substrate. Flavopiridol and prazosin were categorized as MDR1/Mdr1a and BCRP/Bcrp dual substrates, although they seem to be more strongly associated with BCRP/BCRP transport per their higher RR$_{BCRP}$ and RR$_{Bcrp}$ versus RR$_{MDR1}$ and RR$_{Mdr1a}$, than RR$_{BCRP}$ and RR$_{Bcrp}$ values.

In Vivo Pharmacokinetics of Flavopiridol, Imatinib, PF-407288, and Prazosin in Transporter KO Mice Models. To determine the individual and combined roles of Bcrp and Mdr1a/1b in the CNS penetration of drugs in mice, the systemic and central exposures of PF-407288, flavopiridol, prazosin, and imatinib were determined in Mdr1a/1b(-/-), (-/-), Bcrp(-/-), and Mdr1a/1b(-/-) Bcrp(-/-) KO mice as well as in the WT FVB strain. The compounds were administrated at a 5 mg/kg s.c. dose, and concentrations were determined in plasma, brain, and CSF samples obtained at 0.5 and 2 h postdose (Fig. 2). Concentrations in plasma and brain samples were all at quantifiable levels, whereas the concentrations in CSF were not detectable in all samples [e.g., CSF concentrations of PF-407288 at 0.5 and 2 h postdose were only detectable in Mdr1a/1b(-/-), (-/-), Bcrp(-/-) mice].

In addition, the B/P ratios for all four compounds in Mdr1a/1b(-/-), (-/-), Bcrp(-/-), and Mdr1a/1b(-/-) Bcrp(-/-) mice as well as in FVB were calculated (Table 3). To measure the effects of functional impairment of Bcrp and/or Mdr1a/1b on the brain penetration of drugs, the concentrations of the four compounds in brain, plasma, and CSF as well as B/P ratios from Mdr1a/1b(-/-), (-/-), Bcrp(-/-), and Mdr1a/1b(-/-) Bcrp(-/-) mice versus FVB mice at both 0.5 and 2 h time points were summarized in Table 4. There were 2- to 50-fold increases in the brain concentrations of the four compounds in Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice compared with those in FVB mice at both time points. The CSF concentrations consistently increased 2- to 15-fold in Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice versus those in FVB mice for all four compounds at both time points as well (Table 4). It is interesting to note that the brain and CSF increases of the four compounds in Mdr1a/1b(-/-), (-/-) and Bcrp(-/-) were not comparable with the increases in Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice. For imatinib, there was a 3- to 4-fold increase in brain concentrations in Mdr1a/1b(-/-), (-/-) mice and no increase in brain concentrations in Bcrp(-/-) mice, but a 19- to 50-fold increases of brain concentrations in Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice compared with those determined in FVB mice at both 0.5 and 2 h. In addition, for flavopiridol and prazosin, the brain concentrations increased considerably in both Mdr1a/1b(-/-), (-/-) and Bcrp(-/-) mice but to a much lower extent than the increases observed in Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice in comparison with those in the FVB mice. Slightly different from the other three compounds, PF-407288 showed no appreciable increase in brain concentrations in Mdr1a/1b(-/-), (-/-) and Bcrp(-/-) mice compared with those in the FVB mice, but there were more than 2-fold increases in Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice versus FVB mice. The CSF concentration increases for the four compounds in Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice were consistently greater than the CSF concentration increases in Mdr1a/1b(-/-), (-/-) and Bcrp(-/-) mice compared with those in the FVB mice (Table 4). On the other hand, the plasma concentrations of four compounds were comparable in Mdr1a/1b(-/-), (-/-), Bcrp(-/-), and Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice to those in FVB mice, except for prazosin, which had higher plasma concentrations in the three types of KO mice versus FVB mice ($P < 0.01$) at the 2-h time point, suggesting the potential attenuation of some transporter-mediated clearance mechanisms for prazosin in KO mice. Although the mechanism is not clear and will require further investigation, we noted that the changes in plasma concentrations were not comparable with the changes in brain and CSF concentrations.

Furthermore, the B/P ratios for all four compounds in the four types of mice (Table 3) and B/P ratios in Mdr1a/1b(-/-), (-/-), Bcrp(-/-), and Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice compared with those in FVB mice were calculated (Table 4). Consistent with the increases in brain, the increases in B/P ratios in Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice were tremendous, but those in the respective Mdr1a/1b(-/-), (-/-) and Bcrp(-/-) mice were increased to a much lower extent.

Unbound Tissue Exposures. To account for the differences in the affinity of the various compounds for brain tissue and plasma, the unbound fractions in plasma and brain were experimentally calculated for the four compounds (Table 5). Free brain and free plasma concentrations at 0.5 and 2 h were determined using $f_u$ brain, and $f_u$ plasma, respectively, and free B/P and CSF/P ratios at 0.5- and 2-h postdose are presented in Fig. 3. Consistent with the increases in total B/P ratios in Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice compared with those in FVB mice, the free B/P ratios showed commensurate increases in Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice versus FVB mice for all four compounds, although the free B/P ratio increases in Mdr1a/1b(-/-), (-/-) and Bcrp(-/-) were not as dramatic as those in Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice. For both flavopiridol and prazosin, the free B/P ratios of Mdr1a/1b(-/-), (-/-) and Bcrp(-/-) versus FVB were modestly higher; however, free B/P ratios of Mdr1a/1b(-/-), (-/-) Bcrp(-/-) mice increased dramatically versus those in FVB mice (Fig. 3). For imatinib, although the free B/P ratios of Bcrp(-/-) mice versus those of FVB mice were
The two major efflux transporters at the BBB would be clearly identified as the P-gp and BCRP. However, owing to the overlapping substrate specificities between MDR1/Mdr1a and BCRP/Bcrp, only one compound, PF-407288, was classified as a BCRP/BCRP-specific substrate with RRMDR1, RRMDR1a, RRBCRP, and RRBCRP values of 1.1, 1.1, 6.9, and 3.7, respectively, after screening of more than 30 commercial compounds and thousands of Pfizer compounds. PF-407288 is a Pfizer proprietary compound for the treatment in diabetes target (Fig. 1).

For the first compound, the BCRP/BCRP-specific substrate PF-407288, the brain concentrations were slightly increased in Bcrp2 mice compared with Mdr1a/Mdr1a mice, whereas the brain concentrations in Mdr1a/Mdr1a mice were only slightly increased in Bcrp2 mice. Moreover, the brain concentrations in Mdr1a/Mdr1a mice were significantly higher than those in Bcrp2 mice (Fig. 2).

Discussion

More than 30 commercial compounds have been tested using in vitro transporter studies to identify MDR1/Mdr1a and BCRP/BCRP substrates that could be used later in in vivo experiments to understand the respective roles of Bcrp and Mdr1a/b in affecting brain penetration. BCRP/BCRP-specific substrates were believed to be ideal compounds for elucidating the in vivo role of Bcrp at the BBB, because the prevalence of P-gp in determining brain penetration may cause P-gp to conceal the effect of BCRP in the case of dual substrates. However, owing to the overlapping substrate specificities between MDR1/Mdr1a and BCRP/BCRP, only one compound, PF-407288, was classified as a BCRP/BCRP-specific substrate with RRMDR1, RRMDR1a, RRBCRP, and RRBCRP values of 1.1, 1.1, 6.9, and 3.7, respectively, after screening of more than 30 commercial compounds and thousands of Pfizer compounds. PF-407288 is a Pfizer proprietary compound for the treatment in diabetes target (Fig. 1). The other three selected compounds, flavopiridol, prazosin, and imatinib, were chosen to display various transport characteristics for BCRP/BCRP and MDR1/Mdr1a, with the hope that the contribution of the two major efflux transporters at the BBB would be clearly weighed out. Regarding transport characteristics, flavopiridol exhibited RRMDR1, RRMDR1a, RRBCRP, and RRBCRP values of 3.1, 3.0, 6.0, and 8.2, respectively. Thus, flavopiridol was categorized as being transported more effectively by BCRP/BCRP than by MDR1/Mdr1a. The in vitro transport specificities for prazosin were similar to those for flavopiridol, with RRMDR1, RRMDR1a, RRBCRP, and RRBCRP values of 2.1, 2.3, 4.3, and 8.5, respectively. In contrast with flavopiridol and prazosin, imatinib was classified as a more effective MDR1/Mdr1a substrate, with RRMDR1, RRMDR1a, RRBCRP, and RRBCRP values of 4.7, 4.0, 2.4, and 2.3, respectively. It is interesting to note that the four compounds, as well as the other compounds tested, exhibited similar transport specificities between human and mouse for both P-gp and BCRP. This observation is consistent with our previous report that species differences for P-gp substrate specificity and the resulting transport activity are minimal between human and mouse (Feng et al., 2008) and seems to hold true for BCRP/BCRP as well.

With different transport specificities, the four compounds could help us establish the overall contribution of Mdr1a/b and Bcrp to the brain penetration in mice. Another important criterion for compound selection for in vivo studies was the compound permeability. The passive permeabilities of four compounds were assessed in the MDCK parent cell line (Table 1), and as we reported previously, P_app A>B is able to reflect the permeability of the compounds in vivo (Feng et al., 2008). Based on the P_app A>B values in MDCK cells and a comparison with known permeability standards, flavopiridol, prazosin, and PF-407288 had good passive permeabilities with P_app A>B values in MDCK higher than 10×10^(-6) cm/s. Although imatinib had moderate permeability with P_app A>B values in MDCK of 3.9×10^(-6) cm/s, the brain penetration of imatinib has been successfully measured previously in Mdr1a/b(−/−), −/− mice and Mdr1a(−/−), −/− mice (Breedveld et al., 2005; Bihorel et al., 2007). In addition, all four compounds displayed similar physicochemical properties for typical CNS drugs (Pajouhesh and Lenz, 2005), including molecular weight less than 400, polar surface area higher than 90 Å, reasonable plasma binding (f_b ≥ 3%) and good metabolic stability. Taking the MDCK P_app A>B and physicochemical properties into account, all four compounds were believed to be able to readily cross the BBB by passive permeability and were deemed suitable for in vivo CNS disposition studies.
Moreover, the CSF concentrations of PF-407288 were not detectable in FVB, Mdr1a/1b (H11002/H11002/H11002), and Bcrp (H11002/H11002/H11002) mice, whereas the CSF concentrations in Mdr1a/1b (H11002/H11002/H11002), (H11002/H11002/H11002) and Mdr1a/1b (H11002/H11002/H11002), (H11002/H11002/H11002) mice were detectable at both time points (Fig. 2). The B/P ratios of PF-407288 in Mdr1a/1b (H11002/H11002/H11002), (H11002/H11002/H11002), Bcrp (H11002/H11002/H11002), and Mdr1a/1b (H11002/H11002/H11002), (H11002/H11002/H11002) mice were slightly higher than those in FVB mice (Table 3). We were surprised to find that the brain concentrations were not considerably increased in Bcrp (H11002/H11002/H11002) mice. 

Table 4). Moreover, the CSF concentrations of PF-407288 were not detectable in FVB, Mdr1a/1b (H11002/H11002/H11002), (H11002/H11002/H11002), and Bcrp (H11002/H11002/H11002) mice, whereas the CSF concentrations in Mdr1a/1b (H11002/H11002/H11002), (H11002/H11002/H11002) and Bcrp (H11002/H11002/H11002) mice were detectable at both time points (Fig. 2). The B/P ratios of PF-407288 in Mdr1a/1b (H11002/H11002/H11002), (H11002/H11002/H11002), Bcrp (H11002/H11002/H11002), and Mdr1a/1b (H11002/H11002/H11002), (H11002/H11002/H11002) mice were slightly higher than those in FVB mice (Table 3). We were surprised to find that the brain concentrations were not considerably increased in Bcrp (H11002/H11002/H11002) mice ver-
sus those in FVB mice although PF-407288 was identified as a specific Bcrp substrate in vitro. It is very likely that Bcrp affected brain penetration of PF-407288 in mice to a lesser extent than what we expected from the in vitro transport activities. It seems that efflux transporters at the BBB must counteract the brain penetration of PF-407288, but Bcrp may not be the only transporter involved. In particular, B/P ratios generated in Bcrp(−/−) mice, as well as in other KO mice, were much less than 1, indicating some additional barriers to brain penetration of PF-407288. Taken together, although in vitro transporter assay results classified PF-407288 as a Bcrp substrate, Bcrp does not seem to be a major factor, leading to its poor brain penetration in mice. No dramatic brain concentration and B/P ratio increases in Bcrp(−/−) and Mdr1a/1b(−/−), (−/−)Bcrp(−/−) mice compared with those in FVB mice were observed. It is reasonable to conclude that Bcrp impeded CNS disposition of PF-407288, but to a small extent.

Compounds in the second group are common substrates for both MDR1/Mdr1a and BCRP/Bcrp, including flavopiridol and prazosin. The brain concentrations for both compounds exhibited increases in Bcrp(−/−) and Mdr1a/1b(−/−), (−/−) mice compared with those in FVB mice, with more enhancement in Mdr1a/1b(−/−), (−/−) than in Bcrp(−/−) mice (Fig. 2; Table 4). Likewise the B/P ratios showed a larger increase in Mdr1a/1b(−/−), (−/−) mice compared with those determined in Bcrp(−/−) mice (Table 3). This finding different from what we may have predicted based on the in vitro transport activities, given that the two compounds were demonstrated to be weaker Mdr1a substrates than Bcrp in vitro. The reason for this disconnect could be the higher expression of Mdr1a/1b in mouse BBB compared with that of Bcrp and/or the different affinities of the compounds to Mdr1a/1b versus Bcrp. Although based on mRNA level, Mdr1a expression is slightly higher than that of Bcrp in mouse brain homogenate (Lee et al., 2005), Mdr1a expression could be much higher than that of Bcrp at the mouse BBB because Mdr1a is more enriched than Bcrp in BBB compared with brain homogenate (12- versus 5.6-fold) (Lee et al., 2005). Pfizer internal data have also confirmed that Mdr1a expression is higher than Bcrp expression at the mouse BBB (Warren et al., 2009). Consequently, the impact of functional impairment on the brain penetration of Bcrp and P-gp shared substrates probably becomes smaller than that predicted from the in vitro Bcrp transport activities. In addition, the Sugiyama group recently observed that the brain penetration of common substrates of Bcrp and P-gp exhibited a smaller increase in Bcrp(−/−) mice than expected from the in vitro Bcrp activities and proposed that the effect of functional impairment of Bcrp could be modulated by P-gp activity on their shared substrates (Enokizono et al., 2008).

We were surprised to find that for flavopiridol and prazosin, the B/P ratio in Mdr1a/1b(−/−), (−/−)Bcrp(−/−) mice increased dramatically compared with that observed in FVB mice (more than 5-fold) (Table 4). It is obvious that the increases in B/P ratios in Mdr1a/1b(−/−), (−/−)Bcrp(−/−) mice are much higher than the simple addition of increases of B/P ratios in Bcrp(−/−) and Mdr1a/1b(−/−), (−/−) mice compared with those in FVB mice. This finding was confirmed by pharmacodynamic observations regarding the hypnotic side affects of prazosin. The triple KO mice became inactive shortly after dose administration and did not return to the initial active status at 2 h postdose, whereas Bcrp(−/−) or Mdr1a/1b(−/−), (−/−) mice became inactive ~10 min after dosing and came back to normal at approximately 1 h postdose. The FVB strain did not show any apparent change in activity. These results all support the hypothesis that the two transporters act in concert (synergistically and/or in a compensatory fashion), not additively, to limit the brain penetration of dual substrates. It is interesting to note that Kusuhara and Sugiyama (2009) proposed an explanation for the observed synergism of efflux transporters at the BBB in Mdr1a/1b(−/−), (−/−)Bcrp(−/−) mice recently by providing a conceptual basis describing the synergism and its ramifications for kinetics. When both P-gp- and Bcrp-mediated transports are significantly greater than passive diffusion, the remaining efflux in Bcrp(−/−) or Mdr1a/1b(−/−), (−/−) mice will prevent a marked reduction in the luminal efflux clearance, whereas in Mdr1a/1b(−/−), (−/−)Bcrp(−/−) mice, no efflux remains, and the synergism is therefore observed. The kinetic concept of this apparent synergism was illustrated in the article by Kusuhara and Sugiyama (2009). Alternatively, it is important to note that the transporter expression differences in the KO mice may produce this apparent compensation in brain impairment for Mdr1a/1b(−/−), (−/−)Bcrp(−/−) and Bcrp(−/−) versus that in WT mice. Of interest, it has been claimed that there was ~3 times more Bcrp mRNA in the microvessels from P-gp-deficient mutant mouse brains than in the microvessels of WT mouse brains (Cisternino et al., 2004). Taken together, the compensatory higher expression of Bcrp in Mdr1a/1b(−/−), (−/−)Bcrp(−/−) mice could mitigate B/P ratio increases in flavopiridol and prazosin in Mdr1a/1b(−/−), (−/−) mice to be not as dramatic as expected. Likewise, it would be reasonable to postulate that Mdr1a/1b is up-regulated in Bcrp(−/−) mice, because P-gp and Bcrp are two major efflux transporters in the mouse BBB to protect the brain from xenobiotics. If the up-regulation occurs in both cases, the increases in the B/P ratio in Bcrp(−/−) mice compared with that in FVB mice could greatly underestimate the contribution of Bcrp in brain penetration of dual substrates by virtue of compensation by Mdr1a/1b. With no confounding possibility of up-regulation, the B/P ratio in-

<table>
<thead>
<tr>
<th></th>
<th>FVB</th>
<th>Mdr1a/1b(−/−), (−/−)</th>
<th>Bcrp(−/−)</th>
<th>Mdr1a/1b(−/−), (−/−)Bcrp(−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>0.42 ± 0.06</td>
<td>0.71 ± 0.07***</td>
<td>0.55 ± 0.05*</td>
<td>3.11 ± 0.59***</td>
</tr>
<tr>
<td>Imatinib</td>
<td>0.02 ± 0.00</td>
<td>0.09 ± 0.02***</td>
<td>0.02 ± 0.00</td>
<td>0.58 ± 0.18**</td>
</tr>
<tr>
<td>PF-407288</td>
<td>0.01 ± 0.001</td>
<td>0.02 ± 0.005*</td>
<td>0.02 ± 0.002**</td>
<td>0.03 ± 0.004**</td>
</tr>
<tr>
<td>Prazosin</td>
<td>0.17 ± 0.01</td>
<td>0.31 ± 0.07*</td>
<td>0.23 ± 0.06</td>
<td>1.04 ± 0.20**</td>
</tr>
<tr>
<td>2 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>0.92 ± 0.07</td>
<td>1.45 ± 0.39</td>
<td>1.22 ± 0.09**</td>
<td>6.34 ± 0.88***</td>
</tr>
<tr>
<td>Imatinib</td>
<td>0.02 ± 0.00</td>
<td>0.11 ± 0.03**</td>
<td>0.02 ± 0.01</td>
<td>1.55 ± 0.77*</td>
</tr>
<tr>
<td>PF-407288</td>
<td>0.02 ± 0.003</td>
<td>0.02 ± 0.004</td>
<td>0.02 ± 0.003</td>
<td>0.03 ± 0.004*</td>
</tr>
<tr>
<td>Prazosin</td>
<td>0.33 ± 0.03</td>
<td>0.58 ± 0.08**</td>
<td>0.37 ± 0.01</td>
<td>1.73 ± 0.26***</td>
</tr>
</tbody>
</table>

* vs. FVB, P < 0.05.
** vs. FVB, P < 0.01.
*** vs. FVB, P < 0.001.
creases in Mdr1a/lb(−/−), (−/−)Bcrp(−/−) mice could serve to reflect the real overall contribution of both transporters in the brain penetration of shared substrates in vivo. Moreover, that B/P ratio increases in Mdr1a/lb(−/−), (−/−)Bcrp(−/−) mice were significantly higher than those in Mdr1a/lb(−/−), (−/−) mice (P < 0.01) compared with those in FVB mice at both time points (Tables 3 and 4) clearly demonstrates the effect of Bcrp on the brain penetration of flavopiridol and prazosin. If we assume that Bcrp does not contribute to the brain penetration of its substrate, the increases in B/P ratios in Mdr1a/lb(−/−), (−/−)Bcrp(−/−) mice should be comparable with that in Mdr1a/lb(−/−), (−/−) mice, even with the up-regulation of Bcrp in Mdr1a/lb(−/−), (−/−) mice. It is clear that Bcrp must contribute to the brain penetration of flavopiridol and prazosin.

Regarding the last compound, imatinib, in vitro transport studies indicated that imatinib was preferentially transported by Mdr1a rather than by Bcrp. The B/P ratios in Bcrp(−/−) and Mdr1a/lb(−/−), (−/−) mice compared with those in FVB mice were ∼1- and ∼4-fold, respectively (Table 4), which implied that Mdr1a/lb accounts for limitation of the brain penetration of imatinib. It is likely that Mdr1a/lb could offset the deletion of Bcrp and maintain the B/P ratios in Bcrp(−/−) and WT at a similar low level. This is consistent with the previous reports suggesting that P-gp plays a more important role than Bcrp in limiting the distribution of imatinib to the brain (Hamada et al., 2003; Breedveld et al., 2005; Bihorel et al., 2007). However, the remarkable increase in B/P ratios in Mdr1a/lb(−/−), (−/−) mice versus B/P ratios in Mdr1a/lb(−/−), (−/−) mice with ∼7-fold at 0.5 h and ∼14-fold at 2 h (Table 3) suggests that Bcrp must contribute to the brain penetration of imatinib as well but to a much lower extent than P-gp does. Up-regulation of the other efflux transporters in the Bcrp(−/−) or Mdr1a/lb(−/−), (−/−) mice could also explain the lower than expected B/P ratio increases in Bcrp(−/−) and Mdr1a/lb(−/−), (−/−) mice based on the extrapolation of the in vitro transport activities.

In CNS drug discovery, $K_p$ (B/P ratio) is the most commonly used parameter to evaluate brain penetration (Pardridge, 2004), but its relevance has been occasionally questioned. In a recent study at Pfizer, $K_p$ was determined for a set of the 32 most prescribed CNS drugs and ranged from 0.1 to 24 in mice (Doran et al., 2005). However, a compound having a $K_p$ value as low as 0.1, such as sulpiride, can still be a successful CNS drug, suggesting that it is difficult to assess brain penetration based upon $K_p$ alone (Doran et al., 2005). $K_p$, defined as the ratio of free brain and free plasma concentration at equilibrium, has been identified as a better parameter to assess brain penetration (Maurer et al., 2005; Syvänen et al., 2006). Thus, it is essential to understand the impact of the binding in plasma and brain on brain penetration. Unbound fractions in mouse brain and plasma were determined for all four compounds (Table 5), and free B/P and CSF/free plasma ratios at 0.5 and 2 h are presented in Fig. 3. For the four compounds, the $f_{u, \text{brain}}$ and $f_{u, \text{plasma}}$ for each compound are within a few fold. Thus, the free B/P ratio concurs with the total B/P ratio for these four compounds as shown in Table 3. Consequently, B/P ratios are valuable parameters to evaluate brain penetra-

### Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mouse Brain</th>
<th>Mouse Plasma</th>
<th>Data are presented as mean ± S.D. from three independent determinations.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavopiridol</td>
<td>0.26 ± 0.03</td>
<td>0.04 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Imatinib</td>
<td>0.09 ± 0.00</td>
<td>0.03 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>PF-407288</td>
<td>0.03 ± 0.00</td>
<td>0.07 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Prazosin</td>
<td>0.35 ± 0.09</td>
<td>0.03 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>
tion for the four compounds, as discussed earlier. In addition, CSF/free plasma ratios correlated well with the free B/P ratio (Fig. 3), suggesting that CSF is a good indicator of free brain exposure for the four compounds. As mentioned before, the free B/P ratio is used to assess a potential brain impairment of compounds in CNS drug discovery and a free B/P ratio of approximately 1 (within 3-fold error)
will suggest that the compounds have no potential brain impairment issues (Maurer et al., 2005). Because the free B/P ratios for flavopiridol, prazosin, and imatinib in Mdr1a/1b/(−/−), (−/−)Bcrp/(−/−) were close to 1 at 2 h with lower free B/P ratios at 0.5 h (Fig. 3), the data suggest that distribution of the three compounds between brain and plasma was delayed at 0.5 h, and it is expected that there will be no brain impairment for the three compounds at 2 h and longer time points. However, it should be noted that the free B/P ratios for PF-407288 were not close to unity, even in the triple KO mice, which further implies that other efflux transporters besides P-gp and Bcrp could contribute to the brain impairment of PF-407288 in mice.

These studies unambiguously indicate that Bcrp is an important component at the mouse BBB that can impede brain penetration, and Bcrp and Mdr1a/1b probably work in synergy or in a compensatory fashion to limit CNS distribution of their shared substrates across the BBB in mice. Recent work from the van Tellingen group has also demonstrated this mechanism of Bcrp and P-gp at the mouse BBB (de Vries et al., 2007). Compared with the prominent role of P-gp at the mouse BBB, our results suggest that Bcrp seems to contribute less than P-gp to the brain penetration of dual substrates. Certainly, the effect of Bcrp may differ among substrates, and thus the effects may be greater or lesser for any particular drug. However, our study provides definitive evidence that Bcrp is functional at the mouse BBB. It is also important to remember that in addition to P-gp and Bcrp, other efflux transporters, such as multidrug resistance protein 4, are highly expressed at the mouse BBB (Leggas et al., 2004) and could contribute to the brain penetration of drugs across the BBB in vivo.

Regarding in vitro-in vivo correlation, we have established a good correlation between in vitro efflux ratios in human MDR1-MDCK and mouse Mdr1a-MDCK cells with in vivo brain penetration based on Kp values (Doran et al., 2005; Feng et al., 2008), whereas a positive relationship between the in vitro and in vivo Bcrp activities has not yet been established. These studies suggest a positive relationship between the in vitro Bcrp transport activities and the in vivo CNS penetration of Bcrp substrates. Thus, in vitro efflux ratios in Bcrp transport assays could help predict in vivo relevance of Bcrp at the BBB.

Overall, knowledge of how Bcrp mediates drug transport at the BBB in vivo could provide important information on the mechanisms underlying drug transport across the BBB and CNS toxicity, and our findings show that P-gp alone is sometimes insufficient to understand the role of efflux transport in brain penetration, particularly for dual substrates. Our study underlines the fact that BCRP is also a crucial component at the BBB, which may cause brain impairment of CNS drugs. We conclude that it is more effective to use the triple KO mice rather than P-gp or Bcrp KO mice alone to study the brain penetration of their substrates drugs and to understand the overall contributions of these two efflux transporters in prediction of clinical relevance.

Acknowledgments. We thank Ralph Davidson for help with the Transwell assays and Julie Cianfrogna for help with the animal studies.

References


