Kinetic Analysis of the Cooperation of P-Glycoprotein (P-gp/Abcb1) and Breast Cancer Resistance Protein (Bcrp/Abcg2) in Limiting the Brain and Testis Penetration of Erlotinib, Flavopiridol, and Mitoxantrone

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ABSTRACT

A synergistic effect of P-glycoprotein (P-gp/Abcb1a and breast cancer resistance protein (Bcrp)/Abcg2) was reported to limit the brain penetration of their common substrates. This study investigated this based on pharmacokinetics using Mdr1a/1b(−/−), Bcrp(−/−), and Mdr1a/1b(−/−)/Bcrp(−/−) mice. Comparison of the brain-to-plasma and testis-to-plasma ratios (Cbrain/Cplasma and Ctestis/Cplasma, respectively) of the reference compounds quinidine and dantrolene for P-gp and Bcrp, respectively, indicates that impairment of either P-gp and Bcrp did not cause any change in the efflux activities of Bcrp or P-gp, respectively, at both the blood–brain barrier (BBB) and blood-testis barrier (BTB). The Cbrain/Cplasma and Ctestis/Cplasma of the common substrates erlotinib, flavopiridol, and mitoxantrone were markedly increased in Mdr1a/1b(−/−)/Bcrp(−/−) mice even compared with Mdr1a/1b(−/−) and Bcrp(−/−) mice. Efflux activities by P-gp and Bcrp relative to passive diffusion at the BBB and BTB were separately evaluated based on the Cbrain/Cplasma and Ctestis/Cplasma in the knockout strains to the wild-type strain. P-gp made a larger contribution than Bcrp to the net efflux of the common substrates, but Bcrp activities were also significantly larger than passive diffusion. These parameters could reasonably account for the marked increase in Cbrain/Cplasma and Ctestis/Cplasma in the Mdr1a/1b(−/−)/Bcrp(−/−) mice. In conclusion, the synergistic effect of P-gp and Bcrp on Cbrain/Cplasma and Ctestis/Cplasma can be explained by their contribution to the net efflux at the BBB and BTB without any interaction between P-gp and Bcrp.

It is well accepted that the penetration of xenobiotic compounds into the brain and testis is restricted by the blood–brain barrier (BBB) and blood-testis barrier (BTB), respectively. The BBB is formed by brain capillary endothelial cells, whereas, in addition to endothelial cells, myoid and Sertoli cells form the BTB (Bart et al., 2002; Kusuhara and Sugiyama, 2005). Tight junctions between adjacent cells in the BBB and BTB are highly developed and limit the penetration of substances via the paracellular route. Moreover, drug transporters act as active barriers to limit the tissue penetration of substrates from the blood by extruding them back into the blood in the BBB and BTB, thereby, modulating pharmacological or adverse reactions. It has been shown that ATP binding cassette (ABC) transporters, which are known to mediate resistance to anticancer drugs and antiviral drugs, are expressed in the BBB and BTB. These include P-glycoprotein (P-gp/Mdr1/Abcb1), breast cancer resistance protein (BCRP/Abcg2), multidrug resistance-associated protein (MRP)-1/ABCC1, MRP2/ABCC2, MRP4/ABCC4, and MRP5/ABCC5 (Leggas et al., 2004; Zhang et al., 2004; Lee et al., 2005). In particular, P-gp is a well known transporter that plays a pivotal role in barrier function, and disruption of the Mdr1a gene, a predominant isoform expressed in the barriers, causes accumulation of a number of its substrates (Schinkel, 1999; Scherrmann, 2005).

ABBREVIATIONS: P-gp, P-glycoprotein; Mdr, multidrug resistance protein; Bcrp, breast cancer resistance protein; BBB, blood-brain barrier; BTB, blood-testis barrier; ABC, ATP binding cassette; MDCK, Madin-Darby canine kidney; GFP, green fluorescent protein; L-Mdr1a, LLC-PK1 cells expressing mouse Mdr1a; LC, liquid chromatography; MS/MS, tandem mass spectrometry; CFR, corrected flux ratio; PS, permeability surface area; Cbrain/Cplasma, brain-to-plasma ratio; Ctestis/Cplasma, testis-to-plasma ratio; MRP, multidrug resistance-associated protein; GF120918, N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; HMR-1275, (--)cis-5,7-dihydroxy-2-[2-chlorophenyl]-8-(4R-3S-hydroxy-1-methyl)piperidin-4-yl-1-benzopyran-4-one.
Recently, we demonstrated that Bcrp also acts as an active barrier in both the BBB and BTB. Disruption of the Bcrp gene causes a significant increase in the accumulation of isoflavonoids, drugs (dantrolene, prazosin, and triamterene), and food-derived carcinogens in the brain and testis without affecting systemic exposure (Enokizono et al., 2007, 2008). According to Adachi et al. (2001), the ratio of the brain-to-plasma ratio in Bcrp (−/−) mice to wild-type mice represents Bcrp activity in the BBB when only Bcrp accounts for the active efflux. However, the ratio showed rather negative correlation to in vitro Bcrp activities (Enokizono et al., 2008). We hypothesized that Bcrp activity at the BBB is underestimated because of P-gp-mediated efflux based on the in vivo finding that some Bcrp substrates were also found to be P-gp substrates (Enokizono et al., 2008). Later, Oostendorp et al. (2009) demonstrated that imatinib, a common substrate of P-gp and Bcrp, exhibits a considerable increase in the brain-to-plasma ratio in mice lacking both P-gp and Bcrp, Mdr1a1b (−/−)/Bcrp (−/−) mice, and in FVB mice treated with GF120918 [N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isouquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide], an inhibitor of both Bcrp and P-gp. The increase observed in Mdr1a1b (−/−)/Bcrp (−/−) mice appears to be a synergistic effect considering the increase observed in Mdr1a1b (−/−)/Bcrp (−/−) mice. In addition to imatinib, the same synergistic effect was observed for dasatinib, flavopiridol, lapatinib, and prazosin (Chen et al., 2009; Lagas et al., 2009; Polli et al., 2009; Zhou et al., 2009). Because Cisternino et al. (2004) reported an induction of Bcrp mRNA in the brain capillaries from Mdr1a1b (−/−) mice, induction of Bcrp or P-gp in Mdr1a1b (−/−) or Bcrp (−/−) mice could be the underlying mechanism. However, this remains controversial because de Vries et al. (2007) reported similar expression of Bcrp in the brain of wild-type and Mdr1a1b (−/−) mice. The equations for the $K_{p, brain}$ that we derived by taking the active efflux mediated by both P-gp and Bcrp into consideration could reasonably explain such a synergistic effect in the increase in Mdr1a1b (−/−)/Bcrp (−/−) mice without consideration of any interplay between P-gp and Bcrp (Kusuhara and Sugiyama, 2009). The present study was undertaken to support this kinetic consideration based on in vivo experiments using wild-type, Mdr1a1b (−/−), Bcrp (−/−), and Mdr1a1b (−/−)/Bcrp (−/−) mice. In addition to the BBB, we demonstrated that Bcrp limits the penetration of xenobiotic compounds into the testis (Enokizono et al., 2008). Because P-gp and Bcrp are colocalized on the luminal side of the endothelial cells in the testis and the apical side of the myoid cells in the testis (Melaine et al., 2002; Bart et al., 2004; Lee et al., 2005; Enokizono et al., 2007), it is possible that the synergistic increase in the accumulation of xenobiotic compounds is also observed in the testis. Therefore, the present study also determined the concentrations of drugs in the testis of Mdr1a1b (−/−)/Bcrp (−/−) mice.

Materials and Methods

Materials. Erlotinib (Tarceva) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada), and flavopiridol [Alvocidib, HMR-1275, (−)-cis-5,7-dihydroxy-2-(2-chlorophenyl)-8-(4R-[3S-hydroxy-1-methyl] piperidinyl)-4H-1-benzopyran-4-one] was kindly supplied by sanofi-aventis (Bridgewater, NJ). Dantrolene (Dantrium) and mitoxantrone (Novantrone) were purchased from LKT Labs (St. Paul, MN). Quinidine was purchased from Tokyo Kasei (Tokyo, Japan). All other chemicals were commercially available and of reagent grade.

Animals. Male wild-type FVB, Mdr1a1b (−/−), Bcrp (−/−), and Mdr1a1b (−/−)/Bcrp (−/−) mice, 9 weeks of age, were obtained from Taconic Farms (Germantown, NY). The mice used in the present study were 10 to 18 weeks old and weighed 23 to 36 g. All animals were maintained at a controlled temperature under a 12-h light/dark cycle. Food and water were available ad libitum.

Determination of the Transcellular Transport across Monolayers of Cell Lines Expressing P-gp or Bcrp. In vitro mouse Bcrp transport experiments were performed as reported previously (Enokizono et al., 2007, 2008). In brief, MDCK II cells were seeded into 24-well Transwell plates (Corning Life Sciences, Lowell, MA) at a density of $1.4 \times 10^5$ cells/well and grown for 2 days in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% antibiotic–antimycotic solution (Sigma-Aldrich). The cells were infected with recombinant adenovirus harboring green fluorescent protein (GFP) or mouse Bcrp expression vector at a $\times 200$ multiplicity of infection. Details of the construction of these recombinant adenoviruses have been described previously (Kondo et al., 2004). After 2 days in culture, both GFP- and Bcrp-expressing cells (GFP-MDCK and Bcrp-MDCK, respectively) were used for transport studies.

In vitro transport experiments to determine the transport activity by mouse Mdr1a were conducted with Mdr1a-expressing LLC-PK1 cells (L-Mdr1a) that were established previously (Schinkel et al., 1995, 1996). L-Mdr1a and parent LLC-PK1 cells were seeded in 24-well Transwell plates at a density of $3.2 \times 10^5$ and $2.1 \times 10^5$ cells/well, respectively, and grown in Medium 199 (Invitrogen) with 10% fetal bovine serum and 1% antibiotic–antimycotic solution. Medium was changed on the 2nd day of culture, and cells were subjected to transportation study on the 4th day.

The cells were preincubated in Krebs–Henseleit buffer (118 mM NaCl, 23.8 mM NaHCO₃, 4.83 mM KCl, 0.96 mM KH₂PO₄, 1.20 mM MgSO₄, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM CaCl₂, pH 7.4) at 37°C for more than 30 min, and transport experiments were initiated by replacing the medium on one side of the cell monolayer with Krebs–Henseleit buffer containing 3 μM test compounds. At the appropriate times (60, 120, and 180 min), 100-μl aliquots were taken from the opposite side of the cell monolayer and replaced with 100 μl of drug-free buffer. The medium (100 μl) obtained from in vitro transport studies was mixed with 50 μl of acetonitrile for all compounds except mitoxantrone, which was mixed with 50 μl of 20% (w/v) acetic acid–saline.

Transport rates were calculated from the slopes of the time profiles of the apical-to-basal and basal-to-apical transport. Flux ratios (Table 1) were obtained by dividing the efflux rates in the basal-to-apical direction by those in the apical-to-basal direction. Flux ratios in transporter-expressing cells were divided by those in control cells to give a corrected flux ratio (CFR), an in vitro index of the P-gp- and Bcrp-mediated efflux.

Determination of the Tissue-to-Plasma Ratio in Mice. Under urethane anesthesia (1.25 g/kg i.p.), the right jugular vein of the mice was cannulated with a polyethylene tube (PE-10; BD Biosciences, San Jose, CA). Compounds were administered via the cannula by continuous infusion for 120 min. The infusion rates of dantrolene, erlotinib, flavopiridol, mitoxantrone, and quinidine were 2, 4, 32, 8, and 8 μmol/h/kg after priming doses of 1, 2, 8, 2, and 6 μmol/kg, respectively. Blood samples were collected from the left jugular vein at the appropriate time points and centrifuged at 4°C and 9000g for 5 min to obtain plasma. Immediately after the final blood sampling, mice were sacrificed by exsanguination. For mitoxantrone, the plasma samples were transferred to microtubes con-
Kinetic parameters for the penetration across the monolayers of polarized cell lines expressing mouse Bcrp or Mdr1a and their corresponding control cells

Data were taken from Figs. 1 and 2. Permeabilities across GFP- and Bcrp-MDCK or parent LLC-PK1 and L-Mdr1a were determined by the slopes from 1 to 3 h in the transcellular transport. Flux ratios were calculated by the permeabilities in the basal-to-apical direction divided by those in the apical-to-basal direction and the value of flux ratio in the cell lines expressing mouse Bcrp or Mdr1a divided by that in their corresponding control cells defined as CFR. The results are shown as the mean value and S.E.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell Line</th>
<th>Permeability</th>
<th>Flux Ratio</th>
<th>CFR</th>
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\[
K_p[Mdr1a/1b]/Mdr1a/1b = \frac{PS_{b,inf} \times PS_{eff} + PS_{b,eff} \times PS_{Bcrp} + PS_{eff} \times PS_{PP}}{PS_{b,eff} \times PS_{eff} + PS_{b,eff} \times PS_{Bcrp} + PS_{eff} \times PS_{PP}}
\]  (4)

\[
K_p[Bcrp]/Bcrp = \frac{PS_{b,inf} \times PS_{eff} + PS_{b,eff} \times PS_{eff} + PS_{eff} \times PS_{PP}}{PS_{b,eff} \times PS_{eff} + PS_{b,eff} \times PS_{b,eff} + PS_{eff} \times PS_{PP}}
\]  (5)

\[
K_p[Mdr1a/1b]/Bcrp = \frac{PS_{b,inf} \times PS_{eff} + PS_{b,eff} \times PS_{Bcrp} + PS_{eff} \times PS_{PP}}{PS_{b,eff} \times PS_{eff} + PS_{b,eff} \times PS_{Bcrp} + PS_{eff} \times PS_{PP}}
\]  (6)

The ratios of \( R \) in Mdr1a/1b\(-/-\) and Mdr1a/1b\(-/-\)/Bcrp\(-/-\) mice to that in FVB mice are given by eqs. 7, 8, and 9, respectively.

\[
R_{Mdr1a/1b}/R_{Bcrp} = 1 + \frac{PS_{PP}}{PS_{eff} + PS_{PP}}
\]  (7)

\[
R_{Bcrp}/R_{Bcrp} = 1 + \frac{PS_{Bcrp}}{PS_{b,eff} + PS_{Bcrp}}
\]  (8)

\[
R_{Mdr1a/1b}/R_{Bcrp} = 1 + \frac{PS_{PP}}{PS_{eff} + PS_{PP}}
\]  (9)

The \( PS_{eff} \) and \( PS_{Bcrp} \), intrinsic efflux transport activities at the BBB and BTB relative to passive diffusion, were obtained by using a nonlinear least-squares method by fitting eqs. 7 to 9 to the actual \( R_{Mdr1a/1b}/R_{Bcrp} \) and \( R_{Mdr1a/1b}/R_{Bcrp} \) by using the MULTI program (Yamaoka et al., 1981). The algorithm used for the fitting was the Damping Gauss Newton Method (Yamaoka et al., 1981).

### Quantification of Drugs in the Biological Samples

Plasma samples were diluted with two volumes of phosphate-buffered saline for all compounds except mitoxantrone. Mitoxantrone was mixed with 0.9% saline containing 20% (w/v) ascorbic acid to obtain 33% diluted plasma. The proteins in these diluted plasma samples were diluted with two volumes of phosphate-buffered saline (pH 7.4), whereas 0.9% saline containing 20% (w/v) ascorbic acid was used to prepare the homogenates containing mitoxantrone, to obtain a 25% homogenate for all compounds. Plasma samples and tissue homogenates were stored at \(-80°C\) until use. Tissue-to-plasma ratios (\( C_{brain}/C_{plasma} \) and \( C_{testis}/C_{plasma} \)) were obtained by dividing the drug concentrations in the brain and testes by the plasma concentrations at the last sampling point.

### Determination of \( PS_{eff} \) and \( PS_{Bcrp} \)

Adachi et al. (2001) demonstrated previously that the \( K_p \) value is given by the ratio of permeability surface area (PS) products for the uptake (\( PS_{brain-to-blood} \) and efflux (\( PS_{blood-to-brain} \)). The \( PS_{brain-to-blood} \) and \( PS_{blood-to-brain} \) in wild-type mice are given by eqs. 1 and 2, respectively.

\[
PS_{blood-to-tissue} = PS_{b,inf} \times PS_{eff}
\]  (1)

\[
PS_{tissue-to-blood} = PS_{b,inf} \times PS_{eff}
\]  (2)

where \( PS_{b,inf} \) and \( PS_{b,eff} \) represent the PS product for the influx and efflux across the blood-side membrane of the endothelial cells, \( PS_{b,inf} \) and \( PS_{b,eff} \) represent the PS product for the influx and efflux across the tissue-side membrane of the endothelial cells, and \( PS_{eff} \) and \( PS_{eff} \) represent the PS product for the ef flux mediated by Bcrp and P-gp on the blood-side membrane of the endothelial cells, respectively. Because the \( K_p \) value is given by \( PS_{b,inf} \times PS_{eff}/PS_{tissue-to-blood} \) \( K_p \) in wild-type mice \( E_{k,WT} \) is given by eq. 3.

\[
K_p(\text{WT}) = \frac{PS_{b,inf} \times PS_{eff}}{PS_{tissue-to-blood} \times (PS_{b,eff} + PS_{eff} + PS_{eff} + PS_{eff})}
\]  (3)

The \( K_p \) in Mdr1a/1b\(-/-\), Bcrp\(-/-\), and Mdr1a/1b\(-/-\)/Bcrp\(-/-\) mice are given by eqs. 4, 5, and 6, respectively.

Adachi et al. (2001) demonstrated previously that the \( K_p \) value is given by the ratio of permeability surface area (PS) products for the uptake (\( PS_{brain-to-blood} \) and efflux (\( PS_{blood-to-brain} \)). The \( PS_{brain-to-blood} \) and \( PS_{blood-to-brain} \) in wild-type mice are given by eqs. 1 and 2, respectively.
jected to LC-MS/MS or LC-UV analysis. The media (100 μl) mixed with 50 μl of acetonitrile or 20% (w/v) ascorbic acid–saline in in vitro transport studies were centrifuged at 4°C and 5000g for 5 min. The supernatants were also subjected to analysis by LC-MZ/MS or LC-UV. All compounds except mitoxantrone were analyzed in a multiple reaction monitoring mode with an API2000 instrument (Applied Biosystems, Foster City, CA) equipped with an Agilent 1100 series LC system (Agilent Technologies, Santa Clara, CA). The transitions (precursor/product) used for quantification of erlotinib, flavopiridol, and quinidine were 394/278, 402/70, and 325/160, respectively, under positive atmospheric pressure chemical ionization mode, and that of dantrolene was 313/214 under negative atmospheric pressure chemical ionization mode. Mitoxantrone was detected by its absorbance at 655 nm by using an Agilent 1100 series diode array detector. All the compounds were separated by using a Capcell Pak C18 MGII column (3 μm, 3-mm i.d. × 35 mm; Shiseido, Kanagawa, Japan) at room temperature at the flow rate of 0.8 ml/min. Mobile phase A was 0.05% formic acid for all compounds except dantrolene, and 10 mM ammonium acetate was used as mobile phase A for dantrolene. Mobile phase B was acetonitrile for all compounds. For the analysis of erlotinib and quinidine, the fraction of mobile phase B was initially 5%, kept at 5% for 0.5 min, linearly increased to 90% over 2.9 min, kept at 90% for an additional 0.1 min, and finally re-equilibrated at 5% for 1.5 min. For the analysis of flavopiridol, the fraction of mobile phase B was initially 5%, kept at 5% for 1 min, linearly increased to 90% over 2.4 min, kept at 90% for an additional 0.1 min, and finally re-equilibrated at 5% for 1.5 min. For the analysis of mitoxantrone, the fraction of mobile phase B was initially 5%, kept at 5% for 0.5 min, linearly increased to 75% over 3 min, kept at 75% for an additional 0.5 min, and finally re-equilibrated at 5% for 1.5 min. For the analysis of dantrolene, the fraction of mobile phase B was initially 5%, kept at 5% for 0.5 min, linearly increased to 90% over 2.95 min, kept at 90% for an additional 0.05 min, and finally re-equilibrated at 5% for 1.5 min.

**Statistical Analysis.** The presented values are all mean ± S.E. For comparison between genotype groups, log-transformed data were processed by using one-way analysis of variance, followed by a Tukey post hoc test. For comparison of the in vitro transcellular transport between transporter-expressing cells and the corresponding control cells, Student’s two-tailed t test was used. Differences were considered significant at P < 0.05. All statistical calculations were performed with SAS software (version 9; SAS Institute, Cary, NC).

**Results**

**Transcellular Transport of Erlotinib, Flavopiridol, Mitoxantrone, Dantrolene, and Quinidine in Cell Lines Expressing Mouse Bcrp or Mdr1a.** Transcellular transport of erlotinib, flavopiridol, and mitoxantrone was determined in the basal-to-apical and apical-to-basal directions across cells expressing Bcrp or Mdr1a (Bcrp-MDCK or L-Mdr1a). In the present study, based on previous knowledge, dantrolene and quinidine were selected as specific substrates of Bcrp and P-gp, respectively. Although dantrolene exhibited a directional transport in the basal-to-apical direction in parent LLC-PK1, the directional transport was unchanged by the expression of P-gp (Fig. 1). The transcellular transport of dantrolene was nondirectional in mock MDCK II cells, but expression of Bcrp induced directional transport in the basal-to-apical direction (Fig. 2). There was no directional transport of quinidine in parent LLC-PK1, but the expression of P-gp induced directional transport in the basal-to-apical direction (Fig. 1). Expression of Bcrp did not affect the transcellular transport of quinidine (Fig. 2).

The time profiles for the transcellular transport of erlotinib, flavopiridol, and mitoxantrone are shown in Figs. 1

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![Graphs showing transcellular transport profiles](image-url)

**Fig. 1.** Transcellular transport across the monolayers of parent LLC-PK1 cells and L-Mdr1a. Transcellular transport of test compounds (3 μM) was determined in the apical-to-basal direction (○, ●) and the basal-to-apical direction (○, □) across the monolayers of L-Mdr1a (○, □) and parent LLC-PK1 (●, ○) at 37°C. Each symbol with a bar represents the mean and S.E. (n = 3).
and 2. In both Mdr1a- and Bcrp-expressing cells, the permeability of erlotinib and flavopiridol in the basal-to-apical direction was significantly greater than those in the opposite direction. The transcellular transport of mitoxantrone showed directional transport in the basal-to-apical direction in both parent LLC-PK1 and MDCK II cells; however, the expression of both P-gp and Bcrp clearly increased the basal-to-apical transport. The ratios of the permeability in the basal-to-apical direction to those in the opposite direction were higher for L-Mdr1a and Bcrp-MDCK than those for parent LLC-PK1 and GFP-MDCK (Table 1), suggesting that erlotinib, flavopiridol, and mitoxantrone are common substrates of P-gp and Bcrp.

**Tissue Distribution in the Brain and Testis of Wild-Type, Mdr1a/1b(+/–), Bcrp(–/–), and Mdr1a/1b(+/–)/Bcrp(–/–) Mice.** The test drugs were administered by continuous infusion and the concentrations in plasma samples during infusion and in brain and testis samples obtained at 2 h after administration were determined. The time profiles of the plasma concentrations are shown in Fig. 3. The plasma concentrations of the test drugs in Mdr1a/1b(+/–), Bcrp(–/–), and Mdr1a/1b(+/–)/Bcrp(–/–) mice were comparable with those in FVB mice except for flavopiridol and dantrolene. Flavopiridol exhibited significantly lower plasma concentrations in Bcrp(–/–) mice and Mdr1a/1b(+/–)/Bcrp(–/–) mice compared with FVB mice only at 1 h, and dantrolene exhibited significantly higher plasma concentrations in Mdr1a/1b(+/–)/Bcrp(–/–) mice compared with FVB mice at 2 h.

Both the C_{brain}/C_{plasma} and C_{tissue}/C_{plasma} of dantrolene were significantly increased in Bcrp(–/–) and Mdr1a/1b(+/–)/Bcrp(–/–) mice compared with those in Mdr1a/1b(+/–) and FVB mice (Fig. 4). The C_{brain}/C_{plasma} and C_{tissue}/C_{plasma} of dantrolene were slightly higher in Mdr1a/1b(+/–)/Bcrp(–/–) mice than in Bcrp(–/–) mice, and the C_{tissue}/C_{plasma} was higher in Mdr1a/1b(+/–) mice than in wild-type mice. The C_{brain}/C_{plasma} and C_{tissue}/C_{plasma} of quinidine exhibited a significant increase only in Mdr1a/1b(+/–) and Mdr1a/1b(+/–)/Bcrp(–/–) mice, and there were no significant differences in these parameters between Bcrp(–/–) mice and FVB mice or Mdr1a/1b(+/–) and Mdr1a/1b(+/–)/Bcrp(–/–) mice (Fig. 4).

The C_{brain}/C_{plasma} and C_{tissue}/C_{plasma} of erlotinib, flavopiridol, and mitoxantrone were markedly increased in Mdr1a/1b(+/–)/Bcrp(–/–) mice compared with FVB, Mdr1a/1b(+/–), and/or Bcrp(–/–) mice (Fig. 4). Both the C_{brain}/C_{plasma} and C_{tissue}/C_{plasma} of erlotinib were significantly higher in both Mdr1a/1b(+/–) and Bcrp(–/–) mice compared with FVB mice. The C_{brain}/C_{plasma} of flavopiridol and mitoxantrone was significantly increased in Mdr1a/1b(+/–) mice compared with FVB mice, whereas there were no significant differences in the C_{brain}/C_{plasma} between Bcrp(–/–) and FVB mice. The C_{tissue}/C_{plasma} of flavopiridol and mitoxantrone was significantly increased in both Mdr1a/1b(+/–) and Bcrp(–/–) mice compared with FVB mice.

**Determination of PS_{p-gp} and PS_{Bcrp} of Erlotinib, Flavopiridol, and Mitoxantrone at the BBB and BTB.** Equations 7 to 9 were fitted to the C_{tissue}/C_{plasma} in the knockout mice to wild-type mice (Table 2) to obtain the PS_{Bcrp} and PS_{p-gp} of erlotinib, flavopiridol, and mitoxantrone (Table 2). The PS products could reproduce the observed values in both the BBB and the BTB, validating the equations (Table 2). In the BBB, both PS_{Bcrp} and PS_{p-gp} of erlotinib, flavopiridol, and mitoxantrone were higher than the passive diffusion, but PS_{p-gp} was higher.
than PS_{Bcrp}. This holds true for the BTB; however, the absolute values of both PS_{Bcrp} and PS_{P-gp} were slightly lower than the corresponding parameters at the BBB.

Discussion

The overlap in the membrane localization and substrate specificities of Bcrp and P-gp has suggested their cooperation in the active efflux in the blood–tissue barriers. A considerable increase in the accumulation of common substrates in the brain of Mdr1a/1b(−/−)/Bcrp(−/−) mice compared with that observed in either Mdr1a/1b(−/−) or Bcrp(−/−) mice has been interpreted as synergistic effect. The present study investigated this synergistic effect kinetically by using erlotinib, flavopiridol, and mitoxantrone as test compounds. Because Cistermino et al. (2004) reported an induction of Bcrp mRNA in the brain capillaries from Mdr1a/1b(−/−) mice, quinidine and dantrolene were used as probes for P-gp and Bcrp, respectively, to examine their adaptive regulation in the knockout strain.

Adaptive regulation of P-gp at the BBB and BTB of Bcrp(−/−) mice is negligible because neither the C_{brain}/C_{plasma} nor the C_{tissue}/C_{plasma} of quinidine changed in Bcrp(−/−) mice compared with the values in wild-type mice. Although the in vitro transport study could not detect it, dantrolene is likely a P-gp substrate because the C_{brain}/C_{plasma} and C_{tissue}/C_{plasma} of dantrolene were slightly higher in Mdr1a/1b(−/−)/Bcrp(−/−) mice compared with Bcrp(−/−) mice. However, the P-gp-mediated efflux (PS_{P-gp}) was 10- to 20-fold lower than the Bcrp-mediated efflux (PS_{Bcrp}) both at the BBB and BTB (PS_{P-gp} was 0.4 and 0.5 at the BBB and BTB, whereas PS_{Bcrp} was 4 and 8 at the BBB and BTB, respectively), supporting the rationality of dantrolene as a Bcrp probe. Namely, the unchanged C_{brain}/C_{plasma} of dantrolene in Mdr1a/1b(−/−) mice compared with that in wild-type mice suggests that the adaptive regulation of Bcrp is negligible even if it occurs in the BBB in Mdr1a/1b(−/−) mice. On the other hand, the C_{tissue}/C_{plasma} of dantrolene was significantly increased in Mdr1a/1b(−/−) mice for some unknown reason, and further studies of this are necessary.

A marked increase was observed in the C_{brain}/C_{plasma} of erlotinib, flavopiridol, and mitoxantrone in Mdr1a/1b(−/−)/Bcrp(−/−) mice, even compared with Mdr1a/1b(−/−) and Bcrp(−/−) mice. In addition, as expected, a marked increase was observed in the C_{tissue}/C_{plasma} of erlotinib, flavopiridol, and mitoxantrone in Mdr1a/1b(−/−)/Bcrp(−/−) mice, even compared with Mdr1a/1b(−/−) and Bcrp(−/−) mice (Fig. 4). The impact of the defect of both P-gp and Bcrp on the accumulation of their common substrates in the brain and testis could be reasonably explained by using the PS_{P-gp} and PS_{Bcrp} without introducing any interplay between P-gp and Bcrp (Table 2). This holds true for the drugs (dasatinib, flavopiridol, imatinib, lapatinib, and prazosin) for which the brain-to-plasma ratio in triple knockout mice was reported previously (Table 2) (Chen et al., 2009; Oostendorp et al., 2009; Polli et al., 2009; Zhou et al., 2009). It is reasonable that the impact of the defect of Bcrp was not as marked for the common
substrates considering that P-gp makes a larger contribution to the net efflux of the common substrates tested than Bcrp at the BBB and BTB (Table 2). However, the fact that Bcrp-mediated efflux is larger than the passive diffusion at the BBB and BTB (Table 2) indicates that Bcrp can play a more significant role when P-gp is unfunctional. Actually, without...
considering P-gp-mediated efflux, the $C_{brain}/C_{plasma}$ of erlotinib, flavopiridol, and mitoxantrone could be increased 3-, 4-, and 4-fold, respectively, by the defect of Bcrp compared with wild-type mice (the actual increases were only 1.3-, 1.3-, and 1.4-fold). This is the reason $Mdr1a/1b(-/-)/Bcrp(-/-)$ mice show further increase in the $C_{brain}/C_{plasma}$ of the common substrates compared with $Mdr1a/1b(-/-)$ mice. It is worth mentioning that the present study did not confirm that the tissue concentrations reached a plateau, although the mass balance equations were solved under steady-state conditions to obtain eqs. 7 to 9. Therefore, fitting the equations to the observed data shown in Table 2 may underestimate the PS products of P-gp and Bcrp.

Bcrp cooperates with P-gp in the active efflux at both the BBB and BTB because of their overlapped substrate specificity. Such cooperation is undoubtedly important to protect the brain and testis. Even though one transporter is unfunctional by chemicals or shows reduced transport function caused by genetic polymorphisms, the other remained intact and can still limit tissue penetration. $Bcrp(-/-)$ mice did not show any increase in the brain concentrations of Bcrp substrates, such as dehydroepiandrosterone sulfate, pitavastatin, and fluoroquinolones, compared with wild-type mice (Hirano et al., 2005; Lee et al., 2005; Matsushima et al., 2005; Ando et al., 2007). It is possible that transporters other than Bcrp are involved in the efflux of these drugs at the BBB, and, thereby, attenuate the impact of Bcrp dysfunction although Bcrp-mediated efflux is larger than passive diffusion. In fact, P-gp also accepts some anionic Bcrp substrates and fluoroquinolones as substrates (Tsuji et al., 1992; Matsushima et al., 2005; Ando et al., 2007).

### TABLE 2

Comparison of in vivo $C_{brain}/C_{plasma}$ on the brain and testis distribution and the $K_a$ ratios determined by PS products of P-gp and Bcrp.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tissue</th>
<th>$PS_{Bcrp}$</th>
<th>$PS_{P-gp}$</th>
<th>$R_{Mdr1a/1b}/Bcrp(-/-)$</th>
<th>$R_{Bcrp(-/-)}$</th>
<th>$R_{Mdr1a/1b}/Bcrp(-/-)/Bcrp(-/-)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erlotinib</td>
<td>Brain</td>
<td>1.89 ± 0.01</td>
<td>5.63 ± 0.01</td>
<td>Calculated</td>
<td>2.95</td>
<td>1.28</td>
</tr>
<tr>
<td>Testis</td>
<td>0.93 ± 0.27</td>
<td>4.32 ± 0.38</td>
<td>Calculated</td>
<td>3.23</td>
<td>1.18</td>
<td>6.25</td>
</tr>
<tr>
<td>Flavopiridol</td>
<td>Brain</td>
<td>3.07 ± 0.02</td>
<td>10.1 ± 0.0</td>
<td>Calculated</td>
<td>3.48</td>
<td>1.28</td>
</tr>
<tr>
<td>Testis</td>
<td>2.83 ± 0.71</td>
<td>3.50 ± 0.71</td>
<td>Calculated</td>
<td>1.91</td>
<td>1.63</td>
<td>7.33</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Brain</td>
<td>3.14 ± 0.55</td>
<td>3.86 ± 0.56</td>
<td>Calculated</td>
<td>1.93</td>
<td>1.65</td>
</tr>
<tr>
<td>Testis</td>
<td>1.50 ± 0.41</td>
<td>2.00 ± 0.41</td>
<td>Calculated</td>
<td>1.80</td>
<td>1.50</td>
<td>4.50</td>
</tr>
<tr>
<td>Dasatinib$^c$</td>
<td>Brain</td>
<td>1$^a$</td>
<td>7$^a$</td>
<td>Calculated</td>
<td>1.99</td>
<td>1.77</td>
</tr>
<tr>
<td>Imaatinib$^b$</td>
<td>Brain</td>
<td>5.31 ± 0.42</td>
<td>21.9 ± 0.5</td>
<td>Calculated</td>
<td>4.47</td>
<td>1.23</td>
</tr>
<tr>
<td>Lapatinib$^a$</td>
<td>Brain</td>
<td>12.2 ± 0.5</td>
<td>26.8 ± 0.5</td>
<td>Calculated</td>
<td>3.02</td>
<td>1.44</td>
</tr>
<tr>
<td>Prazosin$^a$</td>
<td>Brain</td>
<td>9.55 ± 0.76</td>
<td>32.0 ± 0.8</td>
<td>Calculated</td>
<td>4.03</td>
<td>1.29</td>
</tr>
</tbody>
</table>

$^a$ Chen et al. (2009). $^b$ Polli et al. (2009). $^c$ Zhou et al. (2009). $^d$ Oostendorp et al. (2009). $^e$ Determined 0.5 h after subcutaneous administration. $^f$ Determined 1 h after subcutaneous administration. $^g$ Determined 2 h after oral administration. $^h$ Determined 4 h after oral administration. $^i$ Determined 24 h after constant infusion at a rate of 0.3 mg/h/kg.
uncover such cooperation of xenobiotic transporters at the BBB.

In conclusion, the present study elucidated that the synergistic effect of P-gp and Bcrp on the accumulation of their common substrates in the brain and testis can be explained by their contribution to the net efflux at the BBB and BTB without any direct interaction between P-gp and Bcrp.

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References


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