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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Abbreviations:
Mdr, multidrug resistance protein; P-gp, P-glycoprotein; Bcrp, breast cancer resistance protein; BBB, blood-brain barrier; BTB, blood-testis barrier; MDCK, Madin–Darby canine kidney; GFP, green fluorescent protein; L-Mdr1a, LLC-PK1 cells expressing mouse Mdr1a; LC-MS/MS, liquid chromatography-tandem mass spectrometry; CFR, corrected flux ratio
Abstract

A synergistic effect of P-glycoprotein/Abcb1a and Bcrp/Abcg2 was reported to limit the brain penetration of their common substrates. This study investigated this based on pharmacokinetics using Mdr1a/1b<sup>−/−</sup>, Bcrp<sup>−/−</sup> and Mdr1a/1b<sup>−/−</sup>/Bcrp<sup>−/−</sup> mice. Comparison of the brain- and testis-to-plasma ratios (C<sub>brain</sub>/C<sub>plasma</sub> and C<sub>testis</sub>/C<sub>plasma</sub>) 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, both at the blood-brain barrier (BBB) and blood-testis barrier (BTB). C<sub>brain</sub>/C<sub>plasma</sub> and C<sub>testis</sub>/C<sub>plasma</sub> of the common substrates, erlotinib, flavopiridol, and mitoxantrone, were markedly increased in Mdr1a/1b<sup>−/−</sup>/Bcrp<sup>−/−</sup> mice even compared with Mdr1a/1b<sup>−/−</sup> and Bcrp<sup>−/−</sup> mice. Efflux activities by P-gp and Bcrp relative to passive diffusion at the BBB and BTB were separately evaluated based on the ratio of C<sub>brain</sub>/C<sub>plasma</sub> and C<sub>testis</sub>/C<sub>plasma</sub> in the knockout strains to the wild-type strain. P-gp made a greater contribution than Bcrp to the net efflux of the common substrates, but the Bcrp activities were also significantly greater than passive diffusion. These parameters could reasonably account for the marked increase in the C<sub>brain</sub>/C<sub>plasma</sub> and C<sub>testis</sub>/C<sub>plasma</sub> in the Mdr1a/1b<sup>−/−</sup>/Bcrp<sup>−/−</sup> mice. In conclusion, the synergistic effect of P-gp and Bcrp on the C<sub>brain</sub>/C<sub>plasma</sub> and C<sub>testis</sub>/C<sub>plasma</sub> can be explained by their contribution to the net efflux at the BBB and BTB without any interaction between P-gp and Bcrp.
Introduction

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 while, 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 and, 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 which 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).

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; Enokizono et al., 2008). According to Adachi et al (2001), the ratio of the brain-to-plasma ratio in Bcrp<sup>-/-</sup> 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 in vitro 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, Mdr1a<sup>+/+</sup>/Bcrp<sup>-/-</sup> mice and in FVB mice treated with GF120918, an inhibitor of both Bcrp and P-gp (Oostendorp et al., 2009). The increase observed in Mdr1a<sup>+/+</sup>/Bcrp<sup>-/-</sup> mice appears to
be a synergistic effect considering the increase observed in Mdr1a/1b−/− or Bcrp−/− mice. In addition to imatinib, the same synergistic effect was also 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 Mdr1a/1b−/− mice, induction of Bcrp or P-gp in Mdr1a/1b−/− or Bcrp−/− mice could be the underlying mechanism. However, this remains controversial because de Vries et al. (2007) reported similar expression of Bcrp protein in the brain of wild-type and Mdr1a/1b−/− mice. The equations for the \( K_{p,brain} \) that we derived taking the active efflux mediated by both P-gp and Bcrp into consideration could reasonably explain such a synergistic effect in the increase in Mdr1a/1b−/−/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, Mdr1a/1b−/−, Bcrp−/−, and Mdr1a/1b−/−/Bcrp−/− mice. In addition to the BBB, we demonstrated that Bcrp limits the penetration of xenobiotic compounds into the testis (Enokizono et al., 2008). Since P-gp and Bcrp are colocalized on the luminal side of the endothelial cells in the testis and on 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 Mdr1a/1b−/−/Bcrp−/− mice.
Methods

Materials

Erlotinib (Tarceva) was purchased from Toronto Research Chemicals (Ontario, Canada), and flavopiridol (Alvocidib, HMR-1275) was kindly supplied by Sanofi-Aventis (Bridgewater, NJ). Dantrolene (Dantrium) and mitoxantrone (Novantrone) were purchased from LKT Labs (Minneapolis, MN). Quinidine was purchased from Tokyo Kasei (Tokyo, Japan). All other chemicals were commercially available and of reagent grade.

Animals

Male wild-type FVB, Mdr1a/b−/−, Bcrp−/−, and Mdr1a/b−/−/Bcrp−/− mice, nine 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 previously reported (Enokizono et al., 2007; Enokizono et al., 2008). Briefly, MDCK II cells were seeded into 24-well Transwell plates (Corning, Cambridge, MA) at a density of 1.4 × 10^5 cells/well and grown for two 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 ×200 multiplicity of infection. Details of the construction of these recombinant adenoviruses are described in a previous report (Kondo et al., 2004). After two 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 using Mdr1a-expressing LLC-PK1 cells (L-Mdr1a) that were established previously (Schinkel et al., 1995; Schinkel et al., 1996). L-Mdr1a and parent LLC-PK1 cells were seeded in 24-well Transwell plates at a
density of $3.2 \times 10^5$ cells/well 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 second day of culture, and cells were subjected to the transport study on the fourth 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 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 20% (w/v) ascorbic 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 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, Franklin Lakes, NJ). 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 appropriate time-points and centrifuged at 4°C and 9,000g 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 containing ascorbic acid. For dantrolene, erlotinib, flavopiridol, and quinidine, the brain and testes specimens were homogenized with 3 volumes of phosphate buffered saline (PBS, pH 7.4), while 0.9% saline containing

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20% (w/v) ascorbic acid was used to prepare the homogenates containing mitoxantrone, to obtain a 25% homogenate for all compounds. Plasma specimens and tissue homogenates were stored at –80°C until use. Tissue-to-plasma ratios (C_{brain}/C_{plasma} and C_{testes}/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_{P-gp} and PS_{Bcrp}**

Adachi et al. (2001) previously demonstrated that the K_{p,brain} is given by the ratio of PS products for the uptake (PS_{brain-to-blood}) and efflux (PS_{blood-to-brain}) (Adachi et al., 2001). The PS_{tissue-to-blood} and PS_{blood-to-tissue} in wild-type mice are given by Equation 1 and 2, respectively:

\[
\begin{align*}
PS_{\text{blood-to-tissue}} &= PS_{b,\text{inf}} \times \frac{PS_{\text{t,eff}}}{PS_{\text{t,eff}} + PS_{b,\text{eff}} + PS_{\text{Bcrp}} + PS_{\text{P-gp}}} \\
PS_{\text{tissue-to-blood}} &= PS_{t,\text{inf}} \times \frac{PS_{b,\text{eff}} + PS_{\text{Bcrp}} + PS_{\text{P-gp}}}{PS_{\text{t,eff}} + PS_{b,\text{eff}} + PS_{\text{Bcrp}} + PS_{\text{P-gp}}}
\end{align*}
\] (1)

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_{t,inf} and PS_{t,eff} represent the PS product for the influx and efflux across the tissue-side membrane of the endothelial cells, and PS_{Bcrp} and PS_{P-gp} represent the PS product for the efflux 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_{blood-to-tissue} / PS_{tissue-to-blood}, K_{p} in wild-type mice (K_{p} (WT)) is given by Equation 3:

\[
K_{p} (\text{WT}) = \frac{PS_{b,\text{eff}} \times PS_{\text{t,eff}}}{PS_{t,\text{inf}} \times (PS_{b,\text{eff}} + PS_{\text{Bcrp}} + PS_{\text{P-gp}})}
\] (3)

The K_{p} in Mdr1a/1b^{-/-}, Bcrp^+/-, and Mdr1a/1b^{-/-}/Bcrp^+/- mice are given by Equation 4, 5 and 6, respectively:

\[
K_{p} (\text{Mdr1a/1b}^{-/-}) = \frac{PS_{b,\text{eff}} \times PS_{\text{t,eff}}}{PS_{t,\text{inf}} \times (PS_{b,\text{eff}} + PS_{\text{Bcrp}})}
\] (4)

\[
K_{p} (\text{Bcrp}^+/-) = \frac{PS_{b,\text{eff}} \times PS_{\text{t,eff}}}{PS_{t,\text{inf}} \times (PS_{b,\text{eff}} + PS_{\text{P-gp}})}
\] (5)

\[
K_{p} (\text{Mdr1a/1b}^{-/-}/\text{Bcrp}^+/-) = \frac{PS_{b,\text{eff}} \times PS_{\text{t,eff}}}{PS_{t,\text{inf}} \times PS_{b,\text{eff}}}
\] (6)

The ratios of K_{p} (R) in Mdr1a/1b^{-/-}, Bcrp^+/-, and Mdr1a/1b^{-/-}/Bcrp^+/- mice to that in FVB mice, are given by Equation 7, 8 and 9, respectively:

\[
R_{\text{Mdr1a/1b}^{-/-}} = 1 + \frac{PS_{\text{P-gp}}}{PS_{b,\text{eff}} + PS_{\text{Bcrp}}}
\] (7)
The PSP-gp and PS-Bcrp intrinsic efflux transport activities at the BBB and BTB relative to passive diffusion, were obtained using a non-linear least squares method by fitting the following equations (7)–(9) to the actual $R_{\text{Mdr1a/1b}^{-/-}, \text{Bcrp}^{-/-}, \text{Mdr1a/1b}^{-/-}/\text{Bcrp}^{-/-}}$ using MULTI program (Yamaoka et al., 1981). The algorithm used for the fitting was the Damping Gauss Newton Method (Yamaoka et al., 1981).

The PS-gp and PS-Bcrp intrinsic efflux transport activities at the BBB and BTB relative to passive diffusion, were obtained using a non-linear least squares method by fitting the following equations (7)–(9) to the actual $R_{\text{Mdr1a/1b}^{-/-}, \text{Bcrp}^{-/-}, \text{Mdr1a/1b}^{-/-}/\text{Bcrp}^{-/-}}$ using 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 PBS for all the 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 and tissue homogenates were precipitated with 2 volumes of acetonitrile and the suspensions centrifuged twice at 4°C and 5000g, for 10 min. The supernatant were evaporated, and the remaining residues reconstituted in mobile phase and subjected 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 5,000g for 5 min. The supernatants were also subjected to analysis by LC-MS/MS or LC-UV. All compounds except mitoxantrone were analyzed in a multiple reaction monitoring mode using an API2000 instrument (Applied Biosystems, Foster City, CA) equipped with an Agilent 1100 series LC system (Agilent Technologies, Palo Alto, 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 using an Agilent 1100 series Diode Array Detector. All the compounds was separated 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 the compounds except dantrolene, and 10 mM ammonium acetate was used as mobile phase A for dantrolene. Mobile phase B was acetonitrile for all
the 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 up to 90% over 2.9 min, kept at 90% for a further 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 up to 90% over 2.4 min, kept at 90% for a further 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 1 min, linearly increased up to 75% over 3 min, kept at 75% for a further 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 up to 90% over 2.95 min, kept at 90% for a further 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 using a one-way ANOVA, 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 using SAS software (version 9; SAS Institute, Cary, NC).
Results

Transcellular transport of erlotinib, flavopiridol, mitoxantrone, dantrolene, and quinidine in cell lines expressing mBcrp 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 expression of P-gp (Fig. 1). The transcellular transport of dantrolene was non-directional 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 Fig. 1 and Fig. 2. In both Mdr1a- and Bcrp-expressing cells, the permeability of erlotinib and flavopiridol, in the basal-to-apical direction were significantly greater than those in the opposite direction. The transcellular transport of mitoxantrone showed directional transport in the basal-to-apical direction both in 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, 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<sup>−/−</sup>, Bcrp<sup>−/−</sup>, and Mdr1a/1b<sup>−/−</sup>/Bcrp<sup>−/−</sup> 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 Cbrain/Cplasma and Ctestis/Cplasma of dantrolene were all significantly increased in Bcrp and Mdr1a/1b/Bcrp mice compared with those in Mdr1a/1b and FVB mice (Fig. 4). The Cbrain/Cplasma and Ctestis/Cplasma of dantrolene was slightly greater in Mdr1a/1b/Bcrp mice than in Bcrp mice, and the Ctestis/Cplasma was greater in Mdr1a/1b mice than in wild-type mice. The Cbrain/Cplasma and Ctestis/Cplasma 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 Cbrain/Cplasma and Ctestis/Cplasma 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 Cbrain/Cplasma and Ctestis/Cplasma of erlotinib were significantly greater both in Mdr1a/1b and Bcrp mice compared with FVB mice. The Cbrain/Cplasma of flavopiridol and mitoxantrone were significantly increased in Mdr1a/1b mice compared with FVB mice, while there were no significant differences in the Cbrain/Cplasma between Bcrp and FVB mice. The Ctestis/Cplasma of flavopiridol and mitoxantrone were significantly increased both in 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 ratio of tissue-to-plasma ratios (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 both in the BBB and in the BTB, validating the equations (Table 2). In the BBB, both the PS_{Bcrp} and PS_{P-gp} of erlotinib, flavopiridol, and mitoxantrone were greater than the passive diffusion, but PS_{P-gp} was greater 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 \textit{Mdr1a/1b\textsuperscript{-/-}Bcrp\textsuperscript{-/-}} mice compared with that observed in either \textit{Mdr1a/1b\textsuperscript{-/-}} or \textit{Bcrp\textsuperscript{-/-}} mice has been interpreted as synergistic effect. The present study investigated this synergistic effect kinetically using erlotinib, flavopiridol, and mitoxantrone as test compounds. Since Cisternino et al. (2004) reported an induction of Bcrp mRNA in the brain capillaries from \textit{Mdr1a/1b\textsuperscript{-/-}} mice, quinidine and dantrolene were used as probe 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 \textit{Bcrp\textsuperscript{-/-}} mice is negligible since neither the C\textsubscript{brain}/C\textsubscript{plasma} nor C\textsubscript{testis}/C\textsubscript{plasma} of quinidine changed in \textit{Bcrp\textsuperscript{-/-}} mice compared with the values in wild-type mice. Although the \textit{in vitro} transport study could not detect it, dantrolene is likely a P-gp substrate since the C\textsubscript{brain}/C\textsubscript{plasma} and C\textsubscript{testis}/C\textsubscript{plasma} of dantrolene were slightly greater in \textit{Mdr1a/1b\textsuperscript{-/-}Bcrp\textsuperscript{-/-}} mice compared with \textit{Bcrp\textsuperscript{-/-}} mice. However, the P-gp mediated efflux (PS\textsubscript{P-gp}) was 10 to 20 fold lower than the Bcrp mediated efflux (PS\textsubscript{Bcrp}) both at the BBB and BTB (PS\textsubscript{P-gp} 0.4 and 0.5 at BBB and BTB, while PS\textsubscript{Bcrp} 4 and 8 at the BBB and BTB, respectively), supporting the rationality of dantrolene as a Bcrp probe. Namely, the unchanged C\textsubscript{brain}/C\textsubscript{plasma} of dantrolene in \textit{Mdr1a/1b\textsuperscript{-/-}} 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 \textit{Mdr1a/1b\textsuperscript{-/-}} mice. On the other hand, the C\textsubscript{testis}/C\textsubscript{plasma} of dantrolene was significantly increased in \textit{Mdr1a/1b\textsuperscript{-/-}} mice for some unknown reason, and further studies of this are necessary.

A marked increase was observed in the C\textsubscript{brain}/C\textsubscript{plasma} of erlotinib, flavopiridol, and mitoxantrone in \textit{Mdr1a/1b\textsuperscript{-/-}Bcrp\textsuperscript{-/-}} mice, even compared with \textit{Mdr1a/1b\textsuperscript{-/-}} and \textit{Bcrp\textsuperscript{-/-}} mice. In addition, as expected, a marked increase was also observed in the C\textsubscript{testis}/C\textsubscript{plasma} of erlotinib, flavopiridol, and mitoxantrone in \textit{Mdr1a/1b\textsuperscript{-/-}Bcrp\textsuperscript{-/-}} mice, even compared with \textit{Mdr1a/1b\textsuperscript{-/-}} and \textit{Bcrp\textsuperscript{-/-}} 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 using the PS\textsubscript{P-gp} and PS\textsubscript{Bcrp} without introducing any interplay between P-gp and Bcrp (Table 2). This holds true for the drugs the brain-to-plasma ratio of which in the triple knockout mice was
reported previously, dasatinib, flavopiridol, imatinib, lapatinib, and prazosin (Table 2) (Chen et al., 2009; Polli et al., 2009; Oostendorp et al., 2008; 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 greater 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 greater 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 $C_{\text{brain}}/C_{\text{plasma}}$ of erlotinib, flavopiridol, and mitoxantrone could be respectively increased 3-, 4-, and 4-fold by the defect of Bcrp compared with wild-type mice (the actual increase was only 1.3-, 1.3-, and 1.4-fold). This is the reason why $\text{Mdr1a/1b}^{-/-}/\text{Bcrp}^{-/-}$ mice show further increase in the $C_{\text{brain}}/C_{\text{plasma}}$ of the common substrates compared with $\text{Mdr1a/1b}^{-/-}$ 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 the equations (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 due to genetic polymorphisms, the other remained intact can still limit the tissue penetration. $\text{Bcrp}^{-/-}$ 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 transporter(s) other than Bcrp is involved in the efflux of these drugs at the BBB, and, thereby, attenuating the impact of Bcrp dysfunction although Bcrp-mediated efflux is greater 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; Kitamura et al., 2008). In addition to P-gp, Mrp4 can be an alternative candidate. MRP4 has been demonstrated to limit the brain penetration of anionic drugs at the BBB (Leggas et al., 2004; Belinsky et al., 2007; Ose et al., 2009). Furthermore, Takenaka et al. (2007) found that enhanced toxicity of adefovir is enhanced in mice lacking both Bcrp and Mrp4. Mrp4 may act as an active barrier cooperatively with P-gp and Bcrp, and
Bcrp(-/-)/Mrp4(-/-) or Mdr1a/lb(-/-)/Bcrp(-/-)/Mrp4(-/-) mice may exhibit a marked accumulation of their common substrates. Further studies are necessary to 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.
Acknowledgements

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References


Lagas JS, van Waterschoot RA, van Tilburg VA, Hillebrand MJ, Lankheet N, Rosing H, Beijnen JH and Schinkel AH (2009) Brain accumulation of dasatinib is restricted by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) and can be enhanced by elacridar treatment. *Clin Cancer Res* **15**:2344-2351.


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. Drug Metab Dispos 37:946-955.
Footnote

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Legends of figure

Fig. 1. Transcellular transport across the monolayers of parent LLC-PK1 cells and LLC-PK1 expressing mouse Mdr1a (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 LLC-PK1 expressing Mdr1a (L-Mdr1a, □ and ○) and parent LLC-PK1 (■ and ●) at 37 °C. Each symbol with a bar represents the mean and S.E. (n = 3).

Fig. 2. Transcellular transport across the monolayers of MDCK II cells expressing GFP (GFP-MDCK) or mouse Bcrp (Bcrp-MDCK).

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 MDCK II expressing mBCRP (Bcrp-MDCK, □ and ○) and GFP (GFP-MDCK, ■ and ●) at 37 °C. Each symbol with a bar represents the mean and S.E. (n = 3).

Fig. 3. Plasma concentration–time profiles in FVB, Mdr1a/1b(-/-), Bcrp(-/-), and Mdr1a/1b(-/-)/Bcrp(-/-) mice during continuous infusion.

FVB (■), Mdr1a/1b(-/-) (□), Bcrp(-/-) (Δ), and Mdr1a/1b(-/-)/Bcrp(-/-) (○) mice were administered compounds by continuous infusion at rates of 2 μmol/h/kg for dantrolene, 4 μmol/h/kg for erlotinib, 32 μmol/h/kg for flavopiridol, 8 μmol/h/kg for mitoxantrone, and 8 μmol/h/kg for quinidine. Blood was collected by sampling at the designated times. Drug concentrations in the plasma specimens were determined by LC-MS/MS or LC-UV. Each symbol with a bar represents the mean and S.E. obtained from three male mice.

Statistically significant differences; *P < 0.05 FVB versus Bcrp(-/-) or Mdr1a/1b(-/-)/Bcrp(-/-) mice; *P < 0.05 Mdr1a/1b(-/-) versus Mdr1a/1b(-/-)/Bcrp(-/-) mice

Fig. 4. Comparison of the brain and testicular distributions among FVB, Mdr1a/1b(-/-), Bcrp(-/-), and Mdr1a/1b(-/-)/Bcrp(-/-) mice.
Tissue/plasma concentration ratios ($C_{\text{brain}}/C_{\text{plasma}}$, $C_{\text{testis}}/C_{\text{plasma}}$) were obtained in the plasma, brain, and testis at the end of the infusions. The ratios in FVB, $Mdr1a/1b(-/-)$, $Bcrp(-/-)$, and $Mdr1a/1b(-/-)/Bcrp(-/-)$ mice are represented by closed, open, dotted, and striped bars, respectively. Each bar represents the mean ± S.E. obtained from three mice. Statistically significant differences; *$P < 0.05$ and **$P < 0.01$, FVB versus $Mdr1a/1b(-/-)$, $Bcrp(-/-)$, or $Mdr1a/1b(-/-)/Bcrp(-/-)$ mice; ‡‡$P < 0.01$ $Mdr1a/1b(-/-)$ versus $Bcrp(-/-)$ mice; #$P < 0.01$ $Mdr1a/1b(-/-)$ versus $Mdr1a/1b(-/-)/Bcrp(-/-)$ mice; ††$P < 0.01$ $Bcrp(-/-)$ versus $Mdr1a/1b(-/-)/Bcrp(-/-)$ mice
TABLE 1 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 Fig. 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 hr 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 corrected flux ratio (CFR). The results are shown as the mean value and S.E. Asterisks represent statistically significant differences in the permeability between transporter-expressing cells and the corresponding control cells; *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

<table>
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<tr>
<th>Compound</th>
<th>Cell Line</th>
<th>Permeability (µL/h/well) A to B</th>
<th>Permeability (µL/h/well) B to A</th>
<th>Flux ratio A to B</th>
<th>Flux ratio B to A</th>
<th>CFR</th>
</tr>
</thead>
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<td>Erlotinib</td>
<td>LLC-PK1</td>
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<td>38.3 ± 1.8</td>
<td>1.09</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>L-Mdr1a</td>
<td>12.2 ± 0.9***</td>
<td>60.7 ± 3.9**</td>
<td>4.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GFP-MDCK</td>
<td>28.0 ± 0.4</td>
<td>31.5 ± 0.4</td>
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<td></td>
<td>Bcrp-MDCK</td>
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<td>33.5 ± 3.1</td>
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<td>39.6 ± 1.7</td>
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<td>L-Mdr1a</td>
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<td>67.3 ± 9.8**</td>
<td>7.03</td>
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<td>Flavopiridol</td>
<td>GFP-MDCK</td>
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<td>34.3 ± 0.5</td>
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<td>Bcrp-MDCK</td>
<td>22.1 ± 0.9***</td>
<td>41.2 ± 2.3*</td>
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<td></td>
<td>LLC-PK1</td>
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<td>9.08 ± 0.63</td>
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<td>L-Mdr1a</td>
<td>4.79 ± 0.41</td>
<td>14.3 ± 0.7**</td>
<td>2.97</td>
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<td>Mitoxantrone</td>
<td>GFP-MDCK</td>
<td>1.11 ± 0.33</td>
<td>4.26 ± 0.43</td>
<td>3.84</td>
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<td>Bcrp-MDCK</td>
<td>0.801 ± 0.06</td>
<td>8.06 ± 0.24**</td>
<td>10.1</td>
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<td>LLC-PK1</td>
<td>22.5 ± 0.8</td>
<td>37.0 ± 1.9</td>
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<tr>
<td>Dantrolene</td>
<td>L-Mdr1a</td>
<td>24.3 ± 0.4</td>
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<td>GFP-MDCK</td>
<td>29.9 ± 0.9</td>
<td>30.8 ± 0.7</td>
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<td>Quinidine</td>
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<td>GFP-MDCK</td>
<td>Bcrp-MDCK</td>
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</tr>
<tr>
<td>Bcrp-MDCK</td>
<td>22.1 ± 0.8**</td>
<td>42.2 ± 1.3**</td>
<td>1.91</td>
<td></td>
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<tr>
<td>LLC-PK1</td>
<td>22.3 ± 0.8</td>
<td>22.9 ± 1.1</td>
<td>1.03</td>
<td>6.21</td>
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<tr>
<td>L-Mdr1a</td>
<td>6.62 ± 0.58***</td>
<td>42.2 ± 2.2**</td>
<td>6.38</td>
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<tr>
<td>GFP-MDCK</td>
<td>42.4 ± 3.9</td>
<td>37.8 ± 1.0</td>
<td>0.892</td>
<td>1.04</td>
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<td>Bcrp-MDCK</td>
<td>38.4 ± 3.6</td>
<td>35.6 ± 3.6</td>
<td>0.928</td>
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TABLE 2 Comparison of in vivo the ratios of $C_{tissue}/C_{plasma}$ on the brain and testis distribution and the $K_p$ ratios determined by PS products of P-gp and Bcrp

PS products for the efflux of Bcrp and P-gp (PS$_{Bcrp}$ and PS$_{P-gp}$) can be obtained by the method of least squares for the sum of squares of the residual between in vivo the ratios of $C_{tissue}/C_{plasma}$ in the knockout mice to wild-type mice on the brain and testis distribution and $K_p$ ratio calculated by equation (7)-(9), if the PS product for the efflux across the blood-side membrane is unity. In vivo the ratios of $C_{tissue}/C_{plasma}$ were calculated from the data in Fig. 4. PS$_{Bcrp}$ and PS$_{P-gp}$ are shown as the mean value and S.D. except for dasatinib.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PS$_{Bcrp}$</th>
<th>PS$_{P-gp}$</th>
<th>$R_{Mdr1a/1b(-/-)}$</th>
<th>$R_{Bcrp(-/-)}$</th>
<th>$R_{Mdr1a/1b(-/-)/Bcrp(-/-)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>erlotinib</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<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></td>
<td></td>
<td></td>
<td>In vivo</td>
<td>2.95</td>
<td>1.29</td>
</tr>
<tr>
<td>testis</td>
<td>0.933 ± 0.279</td>
<td>4.32 ± 0.38</td>
<td>Calculated</td>
<td>3.23</td>
<td>1.18</td>
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<td>In vivo</td>
<td>3.29</td>
<td>1.59</td>
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<tr>
<td>flavopiridol</td>
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<td>3.48</td>
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<tr>
<td>brain</td>
<td>3.07 ± 0.02</td>
<td>10.1 ± 0.0</td>
<td>In vivo</td>
<td>3.49</td>
<td>1.27</td>
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</table>

Chen et al (2009), b Polli et al (2009), c Zhou et al (2009), d Oostendorp et al (2008), e determined at 0.5h after s.c. administration, f determined at 2h after s.c. administration, g determined at 2h following oral administration, h determined at 1h following oral administration, i determined at 4h following oral administration, j determined at 24h after constant infusion at a rate of 0.3 mg/h/kg, k determined 24h after constant infusion at a rate of 3 mg/h/kg.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Brain</th>
<th>Testis</th>
<th>Imatinib</th>
<th>Dasatinib</th>
<th>Mitoxantrone</th>
<th>Imatinib</th>
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<tr>
<td>Brain</td>
<td>2.83±0.71</td>
<td>3.50±0.71</td>
<td>1.91</td>
<td>1.63</td>
<td>7.33</td>
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<tr>
<td>Testis</td>
<td>2.73±0.69</td>
<td>3.15±0.71</td>
<td>1.84</td>
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<td>6.87</td>
<td>1.84</td>
<td>1.66</td>
<td>6.87</td>
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<tr>
<td>In vivo</td>
<td>4.49±1.3</td>
<td>4.91±1.3</td>
<td>4.47</td>
<td>4.47</td>
<td>4.97</td>
<td>4.47</td>
<td>4.47</td>
<td>4.97</td>
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<tr>
<td>Calculated</td>
<td>1.75±1.1</td>
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<tr>
<td>Brain</td>
<td>5.31±0.72</td>
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<td>3.86±0.56</td>
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<tr>
<td>In vivo</td>
<td>1.80±1.3</td>
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<td>1.65±1.3</td>
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<tr>
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<tr>
<td>In vivo</td>
<td>1.26±1.2</td>
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Fig 1

- **Erlotinib**
- **Flavopiridol**
- **Mitoxantrone**

- **Dantrolene**
- **Quinidine**

- A-to-B direction in L-Mdr1a
- A-to-B direction in parent LLC-PK1
- B-to-A direction in parent LLC-PK1
- B-to-A direction in L-Mdr1a
Fig 2
Fig 3

Erlotinib

Flavopiridol

Mitoxantrone

Dantrolene

Quinidine

Plasma concentration (μM)

Time (h)

Plasma concentration (μM)

Time (h)

Plasma concentration (μM)

Time (h)

Plasma concentration (μM)

Time (h)

- FVB mice
- Mdr1a/1b−/− mice
- Bcrp−/− mice
- Mdr1a/1b−/−/Bcrp−/− mice

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**Fig 4**

### Erlotinib

- **K\(_{p,\text{brain}}\)**
  - **FVB**
  - Mdr1a/1b\(^{-/}\)
  - Bcrp\(^{-/-}\)
  - Mdr1a/1b\(^{-/-}\)/Bcrp\(^{-/-}\)

- **K\(_{p,\text{testis}}\)**
  - **FVB**
  - Mdr1a/1b\(^{-/}\)
  - Bcrp\(^{-/-}\)
  - Mdr1a/1b\(^{-/-}\)/Bcrp\(^{-/-}\)

### Flavopiridol

- **K\(_{p,\text{brain}}\)**
  - **FVB**
  - Mdr1a/1b\(^{-/}\)
  - Bcrp\(^{-/-}\)
  - Mdr1a/1b\(^{-/-}\)/Bcrp\(^{-/-}\)

- **K\(_{p,\text{testis}}\)**
  - **FVB**
  - Mdr1a/1b\(^{-/}\)
  - Bcrp\(^{-/-}\)
  - Mdr1a/1b\(^{-/-}\)/Bcrp\(^{-/-}\)
Fig 4 (continued)

Mitoxantrone

Dantrolene

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Fig 4 (continued)

Quinidine

Kp,brain

FVB  Mdr1a/1b<sup>-/-</sup>  Bcrp<sup>-/-</sup>  Mdr1a/1b<sup>-/-</sup>/Bcrp<sup>-/-</sup>

Kp,testis

FVB  Mdr1a/1b<sup>-/-</sup>  Bcrp<sup>-/-</sup>  Mdr1a/1b<sup>-/-</sup>/Bcrp<sup>-/-</sup>