Quantitative Investigation of the Role of Breast Cancer Resistance Protein (Bcrp/Abcg2) in Limiting Brain and Testis Penetration of Xenobiotic Compounds

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ABSTRACT:

The role of breast cancer resistance protein (BCRP/ABCG2) in limiting the brain and testis penetration of xenobiotic compounds in the blood-brain and -testis barriers was investigated using Bcrp−/− mice. Tissue/plasma concentration ratios in the brain (Kp,brain) and testis (Kp,testis) obtained under steady-state conditions were significantly increased in Bcrp−/− mice for PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), N-hydroxyl PhIP, MelQx (2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline), dantroline, and prazosin. In addition, the Kp,brain of triamterene and the Kp,testis of 4′-hydroxyl PhIP were also significantly increased in Bcrp−/− mice. The effect of functional impairment of Bcrp on the brain uptake of PhIP, dantroline, and daidzein in Bcrp−/− mice determined using in situ brain perfusion was weaker than that observed on the Kp values. In vitro transcellular transport experiments using cell lines expressing mouse Bcrp or P-glycoprotein (Mdr1a/Abcb1a) showed that, among the tested Bcrp substrates, PhIP, MelQx, prazosin, and triamterene are common substrates of Bcrp and P-glycoprotein. The Kp values of common substrates exhibited a smaller increase both in the brain and testis of Bcrp−/− mice than expected from the in vivo Bcrp activities. The Bcrp-specific substrates were weak acids, whereas basic or neutral BCRP substrates were also P-glycoprotein substrates. These results suggest that BCRP limits the tissue penetration of xenobiotic compounds in the blood-brain and -testis barriers, but its in vivo importance is also modulated by P-glycoprotein activity.

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ABBREVIATIONS: BBB, blood-brain barrier; BTB, blood-testis barrier; ABC, ATP-binding cassette; P-gp, P-glycoprotein; MDR (Mdr in mice), multidrug resistance-associated protein; P-gp, multidrug transporters; BCRP (Bcrp/Abcg2), breast cancer resistance protein; DHEAS, dehydroepiandrosterone sulfate; CTR, corrected flux ratio; MelQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; 4′-OH PhIP, 4′-hydroxyl PhIP; PBS, phosphate-buffered saline; LC/MS, liquid chromatography/mass spectrometry; N-OH PhIP, N-hydroxyl PhIP; Kp,brain, brain/plasma concentration ratio; Kp,testis, testis/plasma concentration ratio; GFP, green fluorescent protein; LC/MS/(MS), liquid chromatography (tandem) mass spectrometry.
brain penetration of imatinib rather than Bcrp (Bihorel et al., 2007). Recently, we reported for the first time that BCRP limits the penetration of phytoestrogens, such as daidzein, genistein, and pterostilbene, into the brain and testis (Enokizono et al., 2007b). On the other hand, there was no difference in the brain distribution of other BCRP substrates, such as dehydroepiandrosterone sulfate (DHEAS), mitoxantrone, pitavastatin, and fluoroquinolones, between wild-type and Bcrp−/− mice (Hirano et al., 2005; Lee et al., 2005; Ando et al., 2007). These results emphasize the difficulties in predicting in vivo relevance from in vitro transport assays.

The purpose of the present study is to determine the quantitative relationship between the transport activities of the compounds by mouse Bcrp and the increase in their tissue/plasma concentration ratios. Adachi et al. (2001) and Yamazaki et al. (2001) previously demonstrated that the corrected flux ratio (CFR), obtained by the ratio of the basal-to-apical and apical-to-basal transport across the monolayers of epithelial cells expressing P-gp divided by the corresponding ratio in mock cells, is a good predictor of in vivo P-gp activity at the BBB (Adachi et al., 2001; Yamazaki et al., 2001). In addition to phytoestrogens (Enokizono et al., 2007b), we investigated the effect of BCRP on the brain and testicular distribution of five compounds, including dietary carcinogens MeIQx and PhIP and drugs (dantrolene, prazosin, and triamterene), using Bcrp−/− mice. Transcellular transport of the compounds across the monolayers of MDCK cells expressing mouse Bcrp was also determined. The CFR for Bcrp was not a good predictor of the increase in Kp values in the brain and testis in Bcrp−/− mice. It was also found that some Bcrp substrates, particularly those exhibiting a marginal increase in Kp values, are also P-gp substrates. The present study proposes that Bcrp plays a significant role in limiting the tissue penetration of xenobiotic compounds, but the impact of functional impairment of Bcrp is also modulated by P-glycoprotein activity on their shared substrates.

Materials and Methods

Materials and Animals. MeIQx and PhIP were purchased from Wako Pure Chemicals (Osaka, Japan), and dantrolene, prazosin, and triamterene were purchased from Sigma-Aldrich (St. Louis, MO). 4′-Hydroxy PhIP (4′-OH PhIP) was a gift from Dr. Hitoshi Nakagama (Biochemistry Division, National Cancer Center Research Institute, Tokyo, Japan). All other chemicals were commercially available and of reagent grade. Male wild-type FVB mice and Bcrp−/− mice (Jonker et al., 2002) used in the present study were 13 to 19 weeks old and weighed 23 to 35 g. All animals were maintained at a controlled temperature of 20°C and a 12-h light/dark cycle. Food and water were available ad libitum.

In Vivo Study with Continuous Infusion. Under urethane anesthesia (1.25 g/kg i.p.), the right jugular vein of the mice was cannulated with a polyethylene tube (PE-10). The common carotid artery was catheterized with a polyethylene tube (PE-10) mounted on a 30-gauge needle. Before insertion of the catheter, the common carotid artery was ligated caudally. During surgery, the body temperature was maintained on a heated plate. The syringe containing the perfusion fluid was placed in an infusion pump (PerkinElmer Life and Analytical Sciences, M SND, CT) and connected to the catheter. Before perfusion, the thorax of the animal was opened, the heart was cut, and perfusion was started immediately at a flow rate of 1 ml/min. The perfusion fluid consisted of Krebs-Henseleit bicarbonate buffer: 25 mM NaHCO3, 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM NaH2PO4, 1.2 mM CaCl2, and 10 mM α-glucose. The perfusion fluid was gassed with 95% O2 and 5% CO2 for pH control (7.4) and warmed to 37°C in a water bath. The concentration of the test compounds was 5 μM. The 1-min perfusion was terminated by decapitation. The brain was then removed, and the cortex of the right cerebral hemisphere was collected and weighed.

Calculation of Initial Uptake Clearance. The average vascular volume (V vasc) following brain perfusion is reported to be 10 μl/g brain (Dagenais et al., 2000). This value was used to correct for vascular contamination in brain tissue. The initial uptake clearances of test compounds (Cl up, microliters per minute per gram of brain) were calculated using the equation Cl up = X brain/T V perf, where X brain is the compound in the right cortex (nanomoles per gram of brain) corrected for vascular contamination (X total − X vasc × C perf), C perf is the concentration of compound in the perfusate (nanomoles per microliter), and T is the perfusion time (minutes).

In Vitro Transport Study. In vitro transport experiments of mouse Bcrp were performed as previously reported with minor modifications (Matsumi- shita et al., 2005). Briefly, MDCK II cells were seeded into a 24-well Transwell (Corning, Cambridge, MA) at a density of 1.4 × 105 cells/well and grown for 3 days in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Sigma-Aldrich). The cells were infected with the recombinant adenovirus harboring green fluorescent protein (GFP) or mouse Bcrp expression vector at a 200 multiplicity of infection. The details of the construction of these recombinant adenoviruses are described in a previous report (Kondo et al., 2004). After a 2-day culture, both GFP- and Bcrp-expressing cells (MDCK II/GFP and MDCK II/Bcrp, respectively) were used for transport studies. The cells were preincubated in Krebs-Henseleit bicarbonate (pH 7.4) buffer for 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 (20, 40, and 60 min), 100-μl aliquots were taken from the opposite side of the cell monolayer and replaced with 100 μl of buffer. In vitro transport experiments of mouse Mdr1a were carried out using Mdr1a-expressing LLC-PK1 cells (L-Mdr1a). L-Mdr1a and parent LLC-PK1 cells were seeded in a 24-well Transwell at a density of 4.8 × 104 cells/well and grown in medium 199 (Invitrogen) with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Medium was changed on the 2nd day, and cells were subjected to the transport study on the 4th day. The procedures for the transport study were the same as those used for mouse Bcrp.

Efflux rates were calculated from the slopes of the time profiles of the apical-to-basal and basolateral transport. Flux ratios were obtained by dividing efflux ratios in the basolateral 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 CFR. CFR was used as the transport activity index.

Preparation of N-OH PhIP. After the administration of PhIP to rats, N-OH PhIP is excreted into the bile mainly in its glucuronide form (Dietrich et al., 2001). Therefore, N-OH PhIP was purified from β-glucuronidase-treated bile obtained from rats that had been given PhIP. Under ether anesthesia, the bile duct was cannulated with a polyethylene tube (PE-10), then PhIP was i.p. administered at a dose of 100 mg/kg, and bile was collected for 2.5 h. Bile was loaded onto a solid-phase extraction cartridge (OASIS HLB; Waters, Milford, MA), which was then washed with water and eluted with acetonitrile. The eluted solution was evaporated, reconstituted in PBS containing 4 μM ascorbic acid, and treated with β-glucuronidase (Roche Diagnostics, Basel, Switzerland) for 3 h at 37°C. Ascorbic acid was added to prevent the autoxidation of N-OH PhIP. The reaction was stopped by adding a 30% volume of acetonitrile, and N-OH PhIP was purified by HPLC, using a D-7000 series instrument.
(Hitachi, Tokyo, Japan) equipped with an Inertsil ODS-2, 5 μm, 4.6-mm i.d. × 250-mm column (GL Science, Tokyo, Japan). The mobile phase A was 10 mM ammonium acetate, and the mobile phase B was acetonitrile. The mobile phase B concentration was 7% at 0 min and was then linearly increased to 20% over 60 min followed by a 5-min re-equilibration with the initial concentration. The flow rate was 1.2 ml/min, the column temperature was 40°C, and the detector wavelength was 313 nm. Under these conditions, PhIP and N-OH PhIP were eluted at 38.2 and 31.1 min, respectively. The purified N-OH PhIP was used to construct standard curves for the quantification of N-OH PhIP concentrations in plasma, brain, and testis.

Quantification. Plasma samples were diluted with PBS to obtain 20% diluted plasma. This diluted plasma and 20% tissue homogenate were precipitated with 3 volumes of acetonitrile and centrifuged at 4°C, 15,000g, for 10 min. The supernatants were evaporated, reconstituted in mobile phase, and subjected to LC/MS/MS analysis. Aliquots obtained from in vitro transport studies were mixed with an equal volume of acetonitrile and centrifuged at 4°C and 15,000g for 10 min. The supernatants were diluted with an appropriate volume of mobile phase and centrifuged at 4°C, 15,000g for 10 min. The supernatants were diluted with an appropriate volume of mobile phase and subjected to LC/MS/MS analysis. PhIP, 4'-OH PhIP, and N-OH PhIP were analyzed in multireaction monitoring mode using an API2000 instrument (Applied Biosystems, Foster City, CA) equipped with a ZQ Micromass instrument equipped with an Alliance 2695 system (Waters) or an Agilent 1100 series LC system (Agilent Technologies, Palo Alto, CA), and the other compounds were analyzed in selected ion monitoring mode using an Inertsil ODS-2, 5 μm/5 μm. The analytical columns used were a CAPCELL PAK C18 MG 3 μm (Shiseido, Kanagawa, Tokyo) for the analysis of PhIP and its metabolites and CAPCELL PAK C18 MG 3 μm, 2-mm i.d.) × 50 mm for the other compounds. The column temperature was 40°C. Detailed LC conditions and mass-to-charge ratios are shown in Table 1.

Statistical Analysis. Statistical analysis of significant differences was performed using the two-tailed Student’s t test. A probability of <0.05 was considered to be statistically significant.

Results

Effects of Bcrp on the Brain and Testis Distribution at Steady State. Compounds were administered by continuous infusion to obtain tissue distribution data under steady-state conditions. In the case of PhIP, the plasma and tissue concentrations of its two metabolites, 4'-OH PhIP and N-OH PhIP, were also determined. The time courses of the plasma concentrations are shown in Fig. 1. The plasma concentrations of PhIP, 4'-OH PhIP, and prazosin were significantly lower in Bcrp+/− mice at several time points. The Kp values are shown in Fig. 2. Both the Kp,brain and Kp,testis values were significantly increased in Bcrp−/− mice in comparison with those in wild-type mice for PhIP, N-OH PhIP, MelQx, dantrolene, and prazosin. For 4'-OH PhIP, the brain concentrations of 4'-OH PhIP were below the limit of quantification (15 nM) in both wild-type and Bcrp−/− mice, whereas Kp,brain exhibited a significant increase in Bcrp−/− mice. For triamterene, both the Kp,brain and Kp,testis values were increased in Bcrp−/− mice; however, only the increase in the Kp,brain was statistically significant.

Effect of Bcrp on Uptake Transport in the BBB. The effect of functional impairment of Bcrp on the brain uptake was investigated by the in situ brain perfusion technique for PhIP, dantrolene, and daidzein. In all cases, a relatively large increase in the Kp,brain values was observed in Bcrp−/− mice. Significant increases in CLup were observed in Bcrp−/− mice for daidzein and dantrolene (Fig. 3). The increase was 2.1-fold for dantrolene and 1.4-fold for daidzein (Fig. 3). The increase was 2.1-fold for dantrolene and 1.4-fold for daidzein (Fig. 3). However, no significant change was observed for dantrolene (Table 2). Therefore, MelQx, PhIP, prazosin, and triamterene exhibited large CFR values, the effect of functional impairment of Bcrp on the tissue concentration was marginal.

The time profiles in the transcellular transport in Mdr1a-expressing cells are shown in Fig. 6 and Table 2. In Mdr1a-expressing cells, the effect of the test compounds exhibited directional transport with statistically significant changes in the permeability (Fig. 4; Table 2), indicating that all the compounds are Bcrp substrates. The comparison between the calculated values of Bcrp−/− mice and the calculated values of wild-type mice was performed. The calculated values of Bcrp−/− mice were significantly increased in comparison with those in wild-type mice for PhIP, N-OH PhIP, MelQx, dantrolene, and prazosin. For 4'-OH PhIP, the brain concentrations of 4'-OH PhIP were below the limit of quantification (15 nM) in both wild-type and Bcrp−/− mice, whereas Kp,brain exhibited a significant increase in Bcrp−/− mice. For triamterene, both the Kp,brain and Kp,testis values were increased in Bcrp−/− mice; however, only the increase in the Kp,brain was statistically significant.

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The time profiles in the transcellular transport in Mdr1a-expressing cells are shown in Fig. 6 and Table 2. In Mdr1a-expressing cells, directional transport across the cell monolayers was clearly enhanced for MelQx, PhIP, and prazosin, and apical-to-basal transport was significantly reduced for triamterene (Table 2). No significant change was observed for dantrolene (Table 2). Therefore, MelQx, PhIP, prazosin, and triamterene are Mdr1a substrates, whereas dantrolene is not. PhIP, MelQx, prazosin, and triamterene, which exhibited a marginal increase in the Kp values, were all common substrates of Bcrp.
and Mdr1a, whereas dantrolene and the phytoestrogens with a larger increase in the \( K_p \) ratios were Bcrp-specific substrates. The most acidic and basic \( pK_a \) values are shown in Table 3. The four BCRP-selective compounds were all weak acids, whereas the other compounds were basic or neutral at physiological pH.

**Discussion**

In the present study, the effects of BCRP on the brain and testicular distribution of two dietary carcinogens and three drugs were investigated using \( Bcrp^{-/-} \) mice. The increase in the \( K_p \) values in the brain and testis in \( Bcrp^{-/-} \) mice compared with wild-type mice indicates that Bcrp limits both the brain and testicular distribution of MeIQx, PhIP, N-OH PhIP, dantrolene, and prazosin. For triamterene, the \( K_{p,brain} \) was significantly increased in \( Bcrp^{-/-} \) mice, whereas the \( K_{p,testis} \) was increased, but this was not statistically significant. For 4'-OH PhIP, \( K_{p,testis} \) was significantly increased in \( Bcrp^{-/-} \) mice, whereas its brain concentration was below the limit of quantification; thus, the impact of functional impairment of Bcrp on the \( K_{p,brain} \) of 4-OH PhIP remains to be evaluated. In contrast, the impact of functional impairment of Bcrp on the uptake transport in the BBB was
smaller than that observed on the $K_p$ values (Fig. 3). Because the $K_p$ values determined under steady-state conditions represent the ratio of the clearance of uptake transport to that of efflux transport, it is likely that Bcrp mainly influences the efflux transport of its substrates in the BBB. The increase in the $K_p,\text{brain}$ of prazosin in Bcrp$^{−/−}$/H11002 mice is consistent with a previous study in which the brain concentration of prazosin was increased by coadministration of GF120918 in Mdr1a$^{−/−}$/H11002 mice (Cisternino et al., 2004). The present results on PhIP contradicted a previous study in which neither $K_p,\text{brain}$ nor $K_p,\text{testis}$ was changed (van Herwaarden et al., 2003). This discrepancy can be explained by the difference in the method of determining the plasma and tissue concentrations. van Herwaarden et al. (2003) determined the total radioactivities in the plasma and tissues without separation, whereas in the present study, the parent drug and metabolites were separately determined by LC/MS/MS. Indeed, the total radioactivity associated with plasma specimens was mainly associated with unknown metabolites, other than the metabolites 4$'$-OH and N$'$-OH PhIP, when $^{14}$C-PhIP was given to mice (data not shown). It is possible that the metabolites in the plasma made it difficult to detect an increase in the brain and testicular distribution of PhIP in Bcrp$^{−/−}$/H11002 mice.

The in vivo Bcrp activities (expressed as $K_p$ ratios) were compared with the in vitro transport activities by Bcrp (expressed as CFR) estimated using a cell line expressing mouse Bcrp. Previously, Adachi et al. (2001) and Yamazaki et al. (2001) demonstrated clear positive relationships between the $K_p$ ratios and CFR for Mdr1a. Unlike the case of Mdr1a, the in vivo and in vitro Bcrp activities did not show a positive relationship. The Bcrp substrates with a greater CFR exhibited a somewhat smaller increase in $K_p$ values (Fig. 5). We assumed
that this discrepancy is accounted for by the difference in the contribution of Bcrp to the net efflux at the BBB and BTB. Indeed, it was found that some Bcrp substrates are also P-gp substrates. In particular, the compounds exhibiting large increases in \( K_p \) ratios were Bcrp-specific substrates. Therefore, it is likely that, for common substrates of Bcrp and P-gp, the impact of functional impairment of Bcrp on the \( K_p \) values becomes smaller than predicted from the CFR because of active efflux mediated by P-gp. This will also account for the discrepancy reported previously that the brain distribution of some Bcrp substrates, such as fluoroquinolones, mitoxantrone, and pitavastatin, was unchanged in \( Bcrp^{-/-} \) mice (Hirano et al., 2005; Lee et al., 2005; Ando et al., 2007). All these compounds are also substrates of P-gp (de Lange et al., 2000; Yagi et al., 2003; Sasabe et al., 2004; Hirano et al., 2005), and the brain distributions of mitoxantrone, grepafloxacin, and ulifloxacin were significantly increased in \( Mdr1a^{-/-}/Bcrp^{-/-} \) mice (Yagi et al., 2003; Sasabe et al., 2004). As far as DHEAS is concerned, the \( CL_{up} \) of DHEAS in the brain was not increased in \( Mdr1a^{-/-}/Bcrp^{-/-} \) mice (Lee et al., 2005). The reason for the absence of a change in the \( K_{p,brain} \) in \( Bcrp^{-/-} \) remains unknown. It is possible that brain perfusion study was not suitable for the detection of the involvement of Bcrp in the transport of DHEAS. It is also possible that other transporters besides Bcrp and P-gp are involved in the transport of DHEAS in the BBB. A candidate transporter is MRP4, which is expressed on the luminal side of BMECs (Leggas et al., 2004) since DHEAS is a substrate of MRP4 (Bai et al., 2004).

The four Bcrp-specific substrates are weak acids, whereas the other compounds are basic or neutral at physiological pH (Table 3). P-gp is well characterized by its preferential substrate recognition of lipophilic and basic compounds. BCRP also recognizes basic compounds, such as cimetidine and imatinib (Burger et al., 2004; Breed-veld et al., 2005), but it also accepts acidic compounds, such as methotrexate and sulfate conjugates (Volk et al., 2002; Suzuki et al., 2003). Based on the substrate specificity, it would be speculated that BCRP plays a major role in the efflux of acidic compounds in the BBB and BTB, but only a limited role in the efflux of basic compounds, which are mainly extruded by P-gp. It should be noted that some MRPs have been identified in the BBB, which accepts anionic compounds as substrates (Kusuha et al., 1998; Zhang et al., 2000; Leggas et al., 2004). Further studies are necessary to elucidate the role of Bcrp in the BBB.

Dietary carcinogens, such as PhIP, 2-amino-3-methylimidazo[4,5-f]quinoline, and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole, have been identified as BCRP substrates (van Herwaarden et al., 2006), and MelQx was identified as a BCRP substrate for the first time in the present study. Schinkel and his collaborators (van Herwaarden et al., 2003, 2006; Jonker et al., 2005) demonstrated the involvement of BCRP in the oral availability, milk secretion, biliary, and intestinal excretion of dietary carcinogens. In addition, the present study revealed that BCRP limits penetration of dietary carcinogens into the brain and testis. N-Hydroxylation is a bioactivation pathway for dietary carcinogens (Kim and Guengerich, 2005). Our results suggest that Bcrp accepts N-OH PhIP; moreover, BCRP may limit DNA adduct formation in the brain and testis.

The plasma concentrations of PhIP, 4-OH PhIP, and prazosin were slightly, but significantly, lower in \( Bcrp^{-/-} \) mice, suggesting induction of some clearance mechanisms for these substrates in \( Bcrp^{-/-} \) mice. Previously, we reported the induction of compensatory mechanisms for the elimination of troglitazone sulfate from the systemic circulation in \( Bcrp^{-/-} \) mice (Enokizono et al., 2007a). The underlying mechanisms remain unknown. Further studies are required to investigate such compensatory mechanisms in \( Bcrp^{-/-} \) mice.

In conclusion, the present study revealed that BCRP limits the brain and testicular distribution of MelQx, PhIP, prazosin, triamterene, and dantrolene. Because of the overlapping substrate specificities of Bcrp...
and P-gp. P-gp is also involved in the efflux of some Bcrp substrates at the BBB, thereby modulating the impact of functional impairment of BCRP.

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