

**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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Abbreviations: BCRP (Bcrp in mice), breast cancer resistance protein; BBB, blood–brain barrier; BTB, blood–testis barrier;  $K_{p,brain}$ , brain-to-plasma concentration ratio;  $K_{p,testis}$ , testis-to-plasma concentration ratio; P-gp, P-glycoprotein; MDR (Mdr in mice), multidrug resistance protein; MRP (Mrp in mice), multidrug resistance-associated protein; ABC, ATP-binding cassette; PBS, phosphate-buffered saline; GFP, green fluorescent protein; CFR, corrected flux ratio; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; 4'-OH PhIP, 4'-hydroxyl PhIP; *N*-OH PhIP, *N*-hydroxyl PhIP; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline.; DHEAS, dehydroepiandrosterone sulfate.

## 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*<sup>−/−</sup> mice. Tissue-to-plasma concentration ratios in the brain ( $K_{p, \text{brain}}$ ) and testis ( $K_{p, \text{testis}}$ ) obtained under steady-state conditions were significantly increased in *Bcrp*<sup>−/−</sup> mice for PhIP, *N*-hydroxyl PhIP, MeIQx, dantrolene and prazosin. In addition, the  $K_{p, \text{brain}}$  of triamterene, and the  $K_{p, \text{testis}}$  of 4'-hydroxyl PhIP were also significantly increased in *Bcrp*<sup>−/−</sup> mice. The effect of functional impairment of Bcrp on the brain uptake of PhIP, dantrolene and daidzein in *Bcrp*<sup>−/−</sup> mice determined using *in situ* brain perfusion was weaker than that observed on the  $K_p$  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, MeIQx, prazosin and triamterene are common substrates of Bcrp and P-glycoprotein. The  $K_p$  values of common substrates exhibited a smaller increase both in the brain and testis of *Bcrp*<sup>−/−</sup> mice than expected from the *in vitro* Bcrp activities. The Bcrp-specific substrates were weak acids, while 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.

## Introduction

The brain and testis are highly protected from the invasion of xenobiotic compounds by the blood–brain barrier (BBB) and blood–testis barrier (BTB). The BBB is formed by brain microvascular endothelial cells, while myoid and Sertoli cells are involved in the BTB in addition to the endothelial cells (Bart et al., 2002; Kusuvara and Sugiyama, 2005; Scherrmann, 2005). In addition to the highly developed tight junctions between adjacent cells, the BBB and BTB express multiple xenobiotic transporters characterized by broad substrate specificities. They actively extrude xenobiotic compounds into the circulating blood and, thereby, limit tissue penetration from the circulating blood. On the luminal side of the BBB, ABC transporters, such as P-glycoprotein (P-gp/MDR1/*ABCB1*), multidrug resistance-associated protein 1 (MRP1/*ABCC1*) and -4 (MRP4/*ABCC4*), and breast cancer resistance protein (BCRP/*ABCG2*), are expressed (Schinkel, 1999; Leggas et al., 2004; Lee et al., 2005; Leslie et al., 2005; Scherrmann, 2005). Among them, P-gp is the best characterized transporter providing a barrier function for the BBB. Cumulative studies have shown that it plays a key role in limiting the penetration of a variety of drugs and toxins (Schinkel, 1999; Scherrmann, 2005). Immunohistochemical analysis has demonstrated the expression of P-gp, MRP1, and BCRP in the BTB (Bart et al., 2004; Augustine et al., 2005). In vivo studies using *Mdr1a*<sup>-/-</sup> or *Mrp1*<sup>-/-</sup> mice have shown their roles in protecting the testis from invasion by their substrates (Wijnholds

et al., 1998; Uhr et al., 2000).

The present study is focused on BCRP in the BBB and BTB. BCRP is a member of the ABC G-transporter family (Kusuhara and Sugiyama, 2007). Cumulative in vivo studies, particularly using *Bcrp*<sup>-/-</sup> mice, have shown the importance of BCRP in limiting oral absorption, and mediating the biliary and urinary excretion of xenobiotic compounds (Jonker et al., 2002; van Herwaarden et al., 2003; Mizuno et al., 2004; Adachi et al., 2005; Kondo et al., 2005; Ando et al., 2007). The functional role of BCRP in the BBB was first demonstrated by Breedveld et al. (Breedveld et al., 2005) who showed that the brain distribution of imatinib was significantly increased in *Bcrp*<sup>-/-</sup> mice although subsequent analysis showed that P-gp plays a significant role in limiting 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 coumestrol, 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*<sup>-/-</sup> 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-to-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 [2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and drugs (dantrolene, prazosin and triamterene), using *Bcrp*<sup>-/-</sup> 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  $K_p$  values in the brain and testis in *Bcrp*<sup>-/-</sup> mice. It was also found that some Bcrp substrates, particularly those exhibiting a marginal increase in  $K_p$  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.

## Material and Methods

### Materials and animals

2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) were purchased from Wako Pure Chemicals Industries (Osaka, Japan), and dantrolene, prazosin and triamterene were purchased from Sigma-Aldrich (St. Louis, MO). 4'-hydroxyl PhIP (4'-OH PhIP) was a gift from Dr. Hitoshi Nakagama (Biochemistry Division, National Cancer Center Research Institute). All other chemicals were commercially available and of reagent grade. Male wild-type FVB mice and *Bcrp*<sup>-/-</sup> mice (Jonker et al., 2002) used in the present study were 13-19 weeks old and weighed 23-35 g. All animals were maintained at a controlled temperature with 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; Becton Dickinson, Franklin Lakes, NJ). Compounds were administered via the cannula by continuous infusion for 120 or 150 min. Blood samples were collected from the left jugular vein at appropriate time points and centrifuged at 4 °C and 10,000g for 5 min to obtain plasma. Immediately after the last blood

sampling, mice were sacrificed by cervical dislocation and the brain and testes were collected and homogenized with 4 volumes of phosphate-buffered saline (PBS) to obtain a 20% homogenate. Plasma samples and tissue homogenates were stored at  $-80^{\circ}\text{C}$  until use. The concentrations of the parent forms were measured using LC/MS for all the compounds except PhIP. For PhIP, the concentrations of two hydroxylated metabolites (4'-OH and *N*-OH PhIP) were also measured in addition to that of the parent form.  $K_{p, \text{brain}}$  and  $K_{p, \text{testis}}$  were obtained by dividing the brain and testis concentrations by the plasma concentrations at the last sampling point (Adachi et al., 2001).

### **In situ brain perfusion**

In situ brain perfusion was carried out according to the previous report by Dagenais et al. (Dagenais et al., 2000). Briefly, mice were anesthetized by i.p. injection of pentobarbital sodium (50 mg/kg), and the right 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 (Packard Instruments, Meriden, 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 (millimoles): 25 NaHCO<sub>3</sub>, 118 NaCl, 4.7 KCl, 1.2 MgSO<sub>4</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 1.2 CaCl<sub>2</sub>, and 10 D-glucose. The perfusion fluid was gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> for pH control (7.4) and warmed to 37 °C in a water bath. The concentration of the test compounds was 5 μM. The one-minute 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<sub>vasc</sub>) 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<sub>up</sub>, μL/min/g brain) were calculated using the following equation:

$$CL_{up} = X_{brain}/T/C_{perf}$$

where X<sub>brain</sub> is the compound in the right cortex (nmol/g brain) corrected for vascular contamination (X<sub>total</sub> - V<sub>vasc</sub> · C<sub>perf</sub>), C<sub>perf</sub> is the concentration of compound in the perfusate (nmol/μL), and T is the perfusion time (min).

#### **In vitro transport study**

In vitro transport experiments of mouse Bcrp were performed as previously reported with minor modifications (Matsushima et al., 2005). Briefly, MDCK II cells were seeded into a 24-well Transwell® (Corning, Cambridge, MA) at a density of  $1.4 \times 10^5$  cells/well and grown for three 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 two-day culture, both GFP and Bcrp expressing cells (MDCK II/GFP and MDCK II/Bcrp, respectively) were used for transport studies. The cells were pre-incubated 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  $\mu$ M test compounds. At appropriate times (20, 40 and 60 min), 100  $\mu$ L aliquots were taken from the opposite side of the cell monolayer and replaced with 100  $\mu$ L 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  $4.8 \times 10^5$  cells/well and grown in medium 199 (Invitrogen) with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Medium was changed on the second day and

cells were subjected to the transport study on the fourth 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 basal-to-apical transport. Flux ratios were obtained by dividing 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)”. CFR was used as the transport activity index.

### **Preparation of *N*-hydroxyl (*N*-OH) PhIP**

After the administration of PhIP to rats, *N*-hydroxyl PhIP (*N*-OH PhIP) is excreted into the bile mainly in its glucuronide form (Dietrich et al., 2001). Therefore, *N*-OH PhIP was purified from  $\beta$ -glucuronidase-treated bile obtained from rats that had been given PhIP. Under ether anesthesia, the bile duct was cannulated with a polyethylene tube (PE10) then PhIP was intraperitoneally 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 5 mM ascorbic acid and treated with  $\beta$ -glucuronidase (Roche, Basel, Switzerland) for 3 h at 37 °C. Ascorbic acid was added to prevent the autooxidation 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) equipped with an Inertsil ODS-2, 5  $\mu$ m, 4.6 mm I.D. x 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

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 an Agilent 1100 series LC system (Agilent Technologies, Palo Alto, CA), and the other compounds were analyzed in selected ion monitoring mode using a ZQ Micromass instrument equipped with an Alliance 2695 system (Waters) or LCMS-2010 EV equipped with a Prominence LC system (Shimadzu, Kyoto, Japan). The analytical columns used were a CAPCELL PAK C18 MG 3  $\mu$ m, 3 mm I.D. x 35 mm (Shiseido, Kanagawa, Tokyo) for the analysis of PhIP and its metabolites, and CAPCELL PAK C18 MG 3  $\mu$ m, 2 mm I.D. x 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 Figure 1. The plasma concentrations of PhIP, 4'-OH PhIP, and prazosin were significantly lower in *Bcrp*<sup>-/-</sup> mice at several time points. The  $K_p$  values are shown in Figure 2. Both the  $K_{p,brain}$  and  $K_{p,testis}$  values were significantly increased in *Bcrp*<sup>-/-</sup> mice in comparison with those in wild-type mice for PhIP, *N*-OH PhIP, MeIQx, 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*<sup>-/-</sup> mice, while  $K_{p,testis}$  exhibited a significant increase in *Bcrp*<sup>-/-</sup> mice. For triamterene, both the  $K_{p,brain}$  and  $K_{p,testis}$  values were increased in *Bcrp*<sup>-/-</sup> mice, however, only the increase in the  $K_{p,brain}$  was statistically significant.

### Effect of Bcrp on the 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  $K_{p,brain}$  values was observed in *Bcrp*<sup>-/-</sup> mice. Significant increases in  $CL_{up}$

were observed in *Bcrp*<sup>-/-</sup> mice for dantrolene and daidzein (Figure 3). The increase was 2.1-fold for dantrolene and 1.4-fold for daidzein. The CL<sub>up</sub> of PhIP was not significantly changed in *Bcrp*<sup>-/-</sup> mice.

### **In vitro transport by mouse Bcrp and Mdr1a, and a comparison with in vivo data**

Transcellular transport of the compounds was examined in polarized cell lines expressing Bcrp and Mdr1a where Bcrp and Mdr1a are localized in the apical membrane (Yamazaki et al., 2001; Matsushima et al., 2005). In Bcrp-expressing cells, all the test compounds exhibited directional transport with statistically significant changes in the permeability (Figure 4 and Table 2), indicating that all the compounds are Bcrp substrates. The comparison between K<sub>p</sub> ratios and CFR values in Bcrp-expressing cells is shown in Figure 5 and Table 3. The data of three phytoestrogens were taken from our previous study (Enokizono et al., 2007b). The relationships did not show any positive correlation (Figure 5). Although PhIP, MeIQx, 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 Figure 6 and Table 2. In Mdr1a-expressing cells, directional transport across the cell monolayers was clearly enhanced for MeIQx, 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 MeIQx, PhIP, prazosin and triamterene are Mdr1a substrates, while dantrolene is not. PhIP, MeIQx, prazosin and triamterene which exhibited a marginal increase in the Kp ratios were all common substrates of Bcrp and Mdr1a, while dantrolene and the phytoestrogens with a larger increase in the Kp ratios were Bcrp-specific substrates. The most acidic and basic pKa values are shown in Table 3. The four BCRP-selective compounds were all weak acids while 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*<sup>-/-</sup> mice.

The increase in the  $K_p$  values in the brain and testis in *Bcrp*<sup>-/-</sup> 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  $K_{p, \text{brain}}$  was significantly increased in *Bcrp*<sup>-/-</sup> mice, while the  $K_{p, \text{testis}}$  was increased but this was not statistically significant. For 4'-OH PhIP,  $K_{p, \text{testis}}$  was significantly increased in *Bcrp*<sup>-/-</sup> mice, while its brain concentration was below the limit of quantification and, thus, the impact of functional impairment of Bcrp on the  $K_{p, \text{brain}}$  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 (Figure 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*<sup>-/-</sup> mice is consistent with a previous study in which the brain concentration of prazosin was increased by coadministration of GF120918 in *Mdr1a*<sup>-/-</sup> mice (Cisternino et al., 2004). The present results on PhIP contradict 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. (van Herwaarden et al., 2003) determined the total radioactivities in the plasma and tissues without separation while, 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 <sup>14</sup>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*<sup>-/-</sup> mice.

The in vivo Bcrp activities (expressed as K<sub>p</sub> 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. (Adachi et al., 2001) and Yamazaki et al. (Yamazaki et al., 2001) demonstrated clear positive relationships between the K<sub>p</sub> 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<sub>p</sub> values (Figure 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<sub>p</sub> 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*<sup>-/-</sup> 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 was significantly increased in *Mdr1a/1b*<sup>-/-</sup> 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/1b*<sup>-/-</sup> mice (Lee et al., 2005). The reason for the absence of a change in the  $K_{p,brain}$  in *Bcrp*<sup>-/-</sup> 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 while 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; Breedveld 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 (Kusuhara 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 MeIQx was identified as a BCRP substrate for the first time in the present study. Schinkel and his collaborators (van Herwaarden et al., 2003; Jonker et al., 2005; van Herwaarden et al., 2006) 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 and, 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*<sup>-/-</sup> mice, suggesting induction of some clearance mechanisms for these substrates in *Bcrp*<sup>-/-</sup> mice. Previously, we reported the induction of compensatory mechanisms for the elimination of troglitazone sulfate from the systemic circulation in *Bcrp*<sup>-/-</sup> mice (Enokizono et al., 2007a). The underlying mechanisms remain unknown. Further studies are required to investigate such compensatory mechanisms in *Bcrp*<sup>-/-</sup> mice.

In conclusion, the present study revealed that BCRP limits the brain and testicular distribution of MeIQx, 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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## References

- Adachi Y, Suzuki H, Schinkel AH and Sugiyama Y (2005) Role of breast cancer resistance protein (Bcrp1/Abcg2) in the extrusion of glucuronide and sulfate conjugates from enterocytes to intestinal lumen. *Mol Pharmacol* **67**:923-928.
- Adachi Y, Suzuki H and Sugiyama Y (2001) Comparative studies on in vitro methods for evaluating in vivo function of MDR1 P-glycoprotein. *Pharm Res* **18**:1660-1668.
- Ando T, Kusuhara H, Merino G, Alvarez AI, Schinkel AH and Sugiyama Y (2007) Involvement of Breast Cancer Resistance Protein (ABCG2) in the biliary excretion mechanism of fluoroquinolones. *Drug Metab Dispos*.
- Augustine LM, Markelewicz RJ, Jr., Boekelheide K and Cherrington NJ (2005) Xenobiotic and endobiotic transporter mRNA expression in the blood-testis barrier. *Drug Metab Dispos* **33**:182-189.
- Bai J, Lai L, Yeo HC, Goh BC and Tan TM (2004) Multidrug resistance protein 4 (MRP4/ABCC4) mediates efflux of bimepridol-glutathione. *Int J Biochem Cell Biol* **36**:247-257.
- Bart J, Groen HJ, van der Graaf WT, Hollema H, Hendrikse NH, Vaalburg W, Sleijfer DT and de Vries EG (2002) An oncological view on the blood-testis barrier. *Lancet Oncol* **3**:357-363.

Bart J, Hollema H, Groen HJ, de Vries EG, Hendrikse NH, Sleijfer DT, Wegman TD, Vaalburg

W and van der Graaf WT (2004) The distribution of drug-efflux pumps, P-gp, BCRP,

MRP1 and MRP2, in the normal blood-testis barrier and in primary testicular tumours.

*Eur J Cancer* **40**:2064-2070.

Breedveld P, Pluim D, Cipriani G, Wielinga P, van Tellingen O, Schinkel AH and Schellens JH

(2005) The effect of Bcrp1 (Abcg2) on the in vivo pharmacokinetics and brain

penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer

resistance protein and P-glycoprotein inhibitors to enable the brain penetration of

imatinib in patients. *Cancer Res* **65**:2577-2582.

Burger H, van Tol H, Boersma AW, Brok M, Wiemer EA, Stoter G and Nooter K (2004)

Imatinib mesylate (STI571) is a substrate for the breast cancer resistance protein

(BCRP)/ABCG2 drug pump. *Blood* **104**:2940-2942.

Cisternino S, Mercier C, Bourasset F, Roux F and Scherrmann JM (2004) Expression,

up-regulation, and transport activity of the multidrug-resistance protein Abcg2 at the

mouse blood-brain barrier. *Cancer Res* **64**:3296-3301.

Dagenais C, Rousselle C, Pollack GM and Scherrmann JM (2000) Development of an in situ

mouse brain perfusion model and its application to mdr1a P-glycoprotein-deficient mice.

*J Cereb Blood Flow Metab* **20**:381-386.

de Lange EC, Marchand S, van den Berg D, van der Sandt IC, de Boer AG, Delon A, Bouquet S

and Couet W (2000) In vitro and in vivo investigations on fluoroquinolones; effects of the P-glycoprotein efflux transporter on brain distribution of sparfloxacin. *Eur J Pharm Sci* **12**:85-93.

Dietrich CG, de Waart DR, Ottenhoff R, Bootsma AH, van Gennip AH and Elferink RP (2001)

Mrp2-deficiency in the rat impairs biliary and intestinal excretion and influences metabolism and disposition of the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo. *Carcinogenesis* **22**:805-811.

Enokizono J, Kusuhara H and Sugiyama Y (2007a) Involvement of breast cancer resistance

protein (BCRP/ABCG2) in the biliary excretion and intestinal efflux of troglitazone sulfate, the major metabolite of troglitazone with a cholestatic effect. *Drug Metab Dispos* **35**:209-214.

Enokizono J, Kusuhara H and Sugiyama Y (2007b) Role of Breast Cancer Resistance Protein

(Bcrp/Abcg2) in the Disposition of Phytoestrogens : the Importance of Bcrp in the Efflux Transport in the Blood-Brain and -Testis Barriers. *Mol Pharmacol*.

Hirano M, Maeda K, Matsushima S, Nozaki Y, Kusuhara H and Sugiyama Y (2005)

Involvement of BCRP (ABCG2) in the biliary excretion of pitavastatin. *Mol Pharmacol* **68**:800-807.

- Jonker JW, Buitelaar M, Wagenaar E, Van Der Valk MA, Scheffer GL, Scheper RJ, Plosch T, Kuipers F, Elferink RP, Rosing H, Beijnen JH and Schinkel AH (2002) The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. *Proc Natl Acad Sci U S A* **99**:15649-15654.
- Jonker JW, Merino G, Musters S, van Herwaarden AE, Bolscher E, Wagenaar E, Mesman E, Dale TC and Schinkel AH (2005) The breast cancer resistance protein BCRP (ABCG2) concentrates drugs and carcinogenic xenotoxins into milk. *Nat Med* **11**:127-129.
- Kim D and Guengerich FP (2005) Cytochrome P450 activation of arylamines and heterocyclic amines. *Annu Rev Pharmacol Toxicol* **45**:27-49.
- Kondo C, Onuki R, Kusuhara H, Suzuki H, Suzuki M, Okudaira N, Kojima M, Ishiwata K, Jonker JW and Sugiyama Y (2005) Lack of improvement of oral absorption of ME3277 by prodrug formation is ascribed to the intestinal efflux mediated by breast cancer resistant protein (BCRP/ABCG2). *Pharm Res* **22**:613-618.
- Kondo C, Suzuki H, Itoda M, Ozawa S, Sawada J, Kobayashi D, Ieiri I, Mine K, Ohtsubo K and Sugiyama Y (2004) Functional analysis of SNPs variants of BCRP/ABCG2. *Pharm Res* **21**:1895-1903.
- Kusuhara H and Sugiyama Y (2005) Active efflux across the blood-brain barrier: role of the solute carrier family. *NeuroRx* **2**:73-85.

- Kusuhara H, Suzuki H, Naito M, Tsuruo T and Sugiyama Y (1998) Characterization of efflux transport of organic anions in a mouse brain capillary endothelial cell line. *J Pharmacol Exp Ther* **285**:1260-1265.
- Lee YJ, Kusuhara H, Jonker JW, Schinkel AH and Sugiyama Y (2005) Investigation of efflux transport of dehydroepiandrosterone sulfate and mitoxantrone at the mouse blood-brain barrier: a minor role of breast cancer resistance protein. *J Pharmacol Exp Ther* **312**:44-52.
- Leggas M, Adachi M, Scheffer GL, Sun D, Wielinga P, Du G, Mercer KE, Zhuang Y, Panetta JC, Johnston B, Scheper RJ, Stewart CF and Schuetz JD (2004) Mrp4 confers resistance to topotecan and protects the brain from chemotherapy. *Mol Cell Biol* **24**:7612-7621.
- Leslie EM, Deeley RG and Cole SP (2005) Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol Appl Pharmacol* **204**:216-237.
- Matsushima S, Maeda K, Kondo C, Hirano M, Sasaki M, Suzuki H and Sugiyama Y (2005) Identification of the Hepatic efflux transporters of organic anions using double-transfected madin-darby canine kidney II cells expressing human organic anion-transporting polypeptide 1B1 (OATP1B1)/multidrug resistance-associated protein 2, OATP1B1/multidrug resistance 1, and OATP1B1/breast cancer resistance protein. *J*

*Pharmacol Exp Ther* **314**:1059-1067.

Mizuno N, Suzuki M, Kusuhara H, Suzuki H, Takeuchi K, Niwa T, Jonker JW and Sugiyama Y

(2004) Impaired renal excretion of

6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040)

sulfate in breast cancer resistance protein (BCRP1/ABCG2) knockout mice. *Drug*

*Metab Dispos* **32**:898-901.

Sasabe H, Kato Y, Suzuki T, Itose M, Miyamoto G and Sugiyama Y (2004) Differential

involvement of multidrug resistance-associated protein 1 and P-glycoprotein in tissue

distribution and excretion of grepafloxacin in mice. *J Pharmacol Exp Ther*

**310**:648-655.

Scherrmann JM (2005) Expression and function of multidrug resistance transporters at the

blood-brain barriers. *Expert Opin Drug Metab Toxicol* **1**:233-246.

Schinkel AH (1999) P-Glycoprotein, a gatekeeper in the blood-brain barrier. *Adv Drug Deliv*

*Rev* **36**:179-194.

Suzuki M, Suzuki H, Sugimoto Y and Sugiyama Y (2003) ABCG2 transports sulfated

conjugates of steroids and xenobiotics. *J Biol Chem* **278**:22644-22649.

Uhr M, Steckler T, Yassouridis A and Holsboer F (2000) Penetration of amitriptyline, but not of

fluoxetine, into brain is enhanced in mice with blood-brain barrier deficiency due to

mdr1a P-glycoprotein gene disruption. *Neuropsychopharmacology* **22**:380-387.

van Herwaarden AE, Jonker JW, Wagenaar E, Brinkhuis RF, Schellens JH, Beijnen JH and

Schinkel AH (2003) The breast cancer resistance protein (Bcrp1/Abcg2) restricts

exposure to the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.

*Cancer Res* **63**:6447-6452.

van Herwaarden AE, Wagenaar E, Karnekamp B, Merino G, Jonker JW and Schinkel AH (2006)

Breast cancer resistance protein (Bcrp1/Abcg2) reduces systemic exposure of the

dietary carcinogens aflatoxin B1, IQ and Trp-P-1 but also mediates their secretion into

breast milk. *Carcinogenesis* **27**:123-130.

Volk EL, Farley KM, Wu Y, Li F, Robey RW and Schneider E (2002) Overexpression of

wild-type breast cancer resistance protein mediates methotrexate resistance. *Cancer Res*

**62**:5035-5040.

Wijnholds J, Scheffer GL, van der Valk M, van der Valk P, Beijnen JH, Scheper RJ and Borst P

(1998) Multidrug resistance protein 1 protects the oropharyngeal mucosal layer and the

testicular tubules against drug-induced damage. *J Exp Med* **188**:797-808.

Yagi Y, Shibutani S, Hodoshima N, Ishiwata K, Okudaira N, Li Q, Sai Y, Kato Y and Tsuji A

(2003) Involvement of multiple transport systems in the disposition of an active

metabolite of a prodrug-type new quinolone antibiotic, prulifloxacin. *Drug Metab*

*Pharmacokinet* **18**:381-389.

Yamazaki M, Neway WE, Ohe T, Chen I, Rowe JF, Hochman JH, Chiba M and Lin JH (2001)

In vitro substrate identification studies for p-glycoprotein-mediated transport: species difference and predictability of in vivo results. *J Pharmacol Exp Ther* **296**:723-735.

Zhang Y, Han H, Elmquist WF and Miller DW (2000) Expression of various multidrug

resistance-associated protein (MRP) homologues in brain microvessel endothelial cells.

*Brain Res* **876**:148-153.

## Legends to Figures

### **Figure 1. Plasma concentration-time profiles in wild-type and *Bcrp*<sup>-/-</sup> mice during continuous infusion.**

Compounds were administered by continuous infusion at infusion rates ( $\mu\text{mol/h/kg}$ ) of 20 for PhIP, 2 for MeIQx, 1 for dantrolene, 2 for prazosin and 1 for triamterene. The plasma concentrations of the compounds in wild-type and *Bcrp*<sup>-/-</sup> mice are represented by closed and open symbols, respectively. Each symbol with a bar represents the mean value and standard error obtained from three or four mice. Asterisks represent statistically significant differences between wild-type and *Bcrp*<sup>-/-</sup> mice; \* $P < 0.05$  and \*\* $P < 0.01$ .

### **Figure 2. Comparison of the brain and testicular distributions between in wild-type and *Bcrp*<sup>-/-</sup> mice**

Tissue-to-plasma concentration ratios ( $K_p$  values) were obtained in both the brain and testis at the end of infusion. The  $K_p$  values in wild-type and *Bcrp*<sup>-/-</sup> mice are represented by closed and open bars, respectively. Each bar with an error bar represents the mean value and standard error obtained from three or four mice. Asterisks represent statistically significant differences between wild-type and *Bcrp*<sup>-/-</sup> mice; \* $P < 0.05$  and \*\* $P < 0.01$ .

**Figure 3. Comparison of the uptake clearances ( $CL_{up}$ ) in the BBB between wild-type and *Bcrp*<sup>-/-</sup> mice.**

The brain uptake for 1 min was determined by the in situ brain perfusion technique.  $CL_{up}$  was obtained by subtracting the amount associated with the capillary space from that associated with brain specimens divided by the concentration in the infusate.  $CL_{up}$  values in wild-type and *Bcrp*<sup>-/-</sup> mice are represented by closed and open bars, respectively. Each bar with an error bar represents the mean value and standard error obtained from three or four mice. Asterisks represent statistically significant differences between wild-type and *Bcrp*<sup>-/-</sup> mice; \* $P < 0.05$  and \*\* $P < 0.01$ .

**Figure 4. Transcellular transport across MDCKII cell monolayers expressing GFP (MDCKII/GFP) or mouse Bcrp (MDCKII/Bcrp).**

The transcellular transport of test compounds (3  $\mu$ M) in the apical-to-basal (circles) and basal-to-apical directions (triangles) was compared between in MDCKII/GFP (closed symbols with dotted line) and MDCKII/Bcrp cells (open symbols and solid line). Each symbol with a bar represents the mean value and standard error of triplicate experiments.

**Figure 5. Relationships between the in vitro transport activities and in vivo BCRP effects**

**on the brain and testicular distribution.**

The transport activities of test compounds were estimated from the study using MDCK II cell monolayers expressing mouse Bcrp and expressed as corrected flux ratios (CFR). In vivo BCRP activities were evaluated by the ratio of  $K_p$  values in wild-type and *Bcrp*<sup>-/-</sup> mice.  $K_p$  ratios were plotted against CFR. 1, PhIP; 2, MeIQx; 3, dantrolene; 4, prazosin; 5, triamterene; 6, daidzein; 7, genistein; 8, coumestrol.  $K_p$  values and CFR of phytoestrogens were obtained from Enokizono et al., (2007).

**Figure 6. Transcellular transport across the monolayers of parent LLC-PK1 cells and LLC-PK1 expressing mouse Mdr1a (L-Mdr1a).**

The transcellular transport of test compounds (3  $\mu$ M) in the apical-to-basal (circles) and basal-to-apical directions (triangles) was compared between parent LLC-PK1 cells (closed symbols with dotted line) and L-Mdr1a cells (open symbols and solid line). Each symbol with a bar represents the mean value and standard error of triplicate experiments.

**Table 1** Analytical conditions in LC/MS(/MS) analysis.

Compound	Ionization mode	Detection mode	<i>m/z</i>	Mobile phase		Gradient condition (B conc. %)
				A	B	
MelQx	ESI, positive	SIM	214	10 mM acetic ammonium	acetonitrile	0 min; 5%→1.5 min; 50%→3 min; 50%→3.01 min; 5%→ 6 min; 5%
PhIP	APCI, positive	MRM	225/210	10 mM acetic ammonium	acetonitrile	0 min; 10%→0.2 min; 10%→2.2 min; 60%→3 min; 70%→3.01 min; 10%→4 min; 10%
4'-OH PhIP	APCI, positive	MRM	241/226	10 mM acetic ammonium	acetonitrile	0 min; 10%→0.2 min; 10%→2.2 min; 60%→3 min; 70%→3.01 min; 10%→4 min; 10%
N-OH PhIP	APCI, positive	MRM	241/223	10 mM acetic ammonium	acetonitrile	0 min; 10%→0.2 min; 10%→2.2 min; 60%→3 min; 70%→3.01 min; 10%→4 min; 10%
dantrolene	ESI, negative	SIM	313	0.05 % formic acid	acetonitrile	0 min; 25%→1.5 min; 90%→2.5 min; 90%→2.51 min; 25%→5 min; 25%
prazosine	ESI, positive	SIM	384	10 mM acetic ammonium	acetonitrile	0 min; 20%→1.5 min; 80%→2.5 min; 80%→2.51 min; 20%→5 min; 20%
triamterene	ESI, positive	SIM	254	0.05 % formic acid	acetonitrile	0 min; 8%→1.5 min; 20%→2.5 min; 20%→2.51 min; 8%→5 min; 8%

abbreviations: APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization;

MRM, multiple reaction monitoring; SIM, selected ion monitoring

**Table 2 Transcellular transport of the test compounds across the monolayers of polarized cell lines expressing mouse Bcrp or Mdr1a expressing, and their corresponding control cells.**

Data were taken from Figure 4 and 6. Permeabilities were obtained from the slopes of the transcellular transport from 40 to 120 min, and the concentration in the donor compartment. Asterisks represent statistically significant differences in the permeability between in transporter-expressing cells and the corresponding control cells; \* $P < 0.05$  and \*\* $P < 0.01$ .

Compound	Cell line	Permeability ( $\mu\text{L}/\text{min}/\text{well}$ )	
		Apical-to-basal	Basal-to-apical
MeIQx	MDCK II/GFP	$0.371 \pm 0.038$	$0.387 \pm 0.042$
	MDCK II/Bcrp	$0.180 \pm 0.014^{**}$	$0.754 \pm 0.005^{**}$
	LLC-PK1	$0.577 \pm 0.04$	$0.733 \pm 0.007$
	L-Mdr1a	$0.389 \pm 0.02^{*}$	$0.867 \pm 0.018^{**}$
PhIP	MDCK II/GFP	$0.771 \pm 0.047$	$0.947 \pm 0.032$
	MDCK II/Bcrp	$0.410 \pm 0.030^{**}$	$1.61 \pm 0.06^{**}$
	LLC-PK1	$0.832 \pm 0.056$	$1.65 \pm 0.04$
	L-Mdr1a	$0.452 \pm 0.025^{**}$	$2.39 \pm 0.06^{**}$
Dantrolene	MDCK II/GFP	$0.643 \pm 0.071$	$0.723 \pm 0.019$
	MDCK II/Bcrp	$0.394 \pm 0.028^{*}$	$1.11 \pm 0.05^{**}$
	LLC-PK1	$0.719 \pm 0.047$	$1.32 \pm 0.01$
	L-Mdr1a	$0.732 \pm 0.026$	$1.43 \pm 0.05$
Prazosin	MDCK II/GFP	$0.546 \pm 0.019$	$0.971 \pm 0.029$
	MDCK II/Bcrp	$0.339 \pm 0.038^{**}$	$1.56 \pm 0.06^{**}$
	LLC-PK1	$0.678 \pm 0.063$	$0.996 \pm 0.049$
	L-Mdr1a	$0.270 \pm 0.026^{**}$	$2.59 \pm 0.17^{**}$
Triamterene	MDCK II/GFP	$0.397 \pm 0.016$	$0.358 \pm 0.009$
	MDCK II/Bcrp	$0.207 \pm 0.008^{**}$	$0.730 \pm 0.045^{**}$
	LLC-PK1	$0.384 \pm 0.038$	$0.781 \pm 0.004$
	L-Mdr1a	$0.196 \pm 0.007^{**}$	$0.860 \pm 0.039$

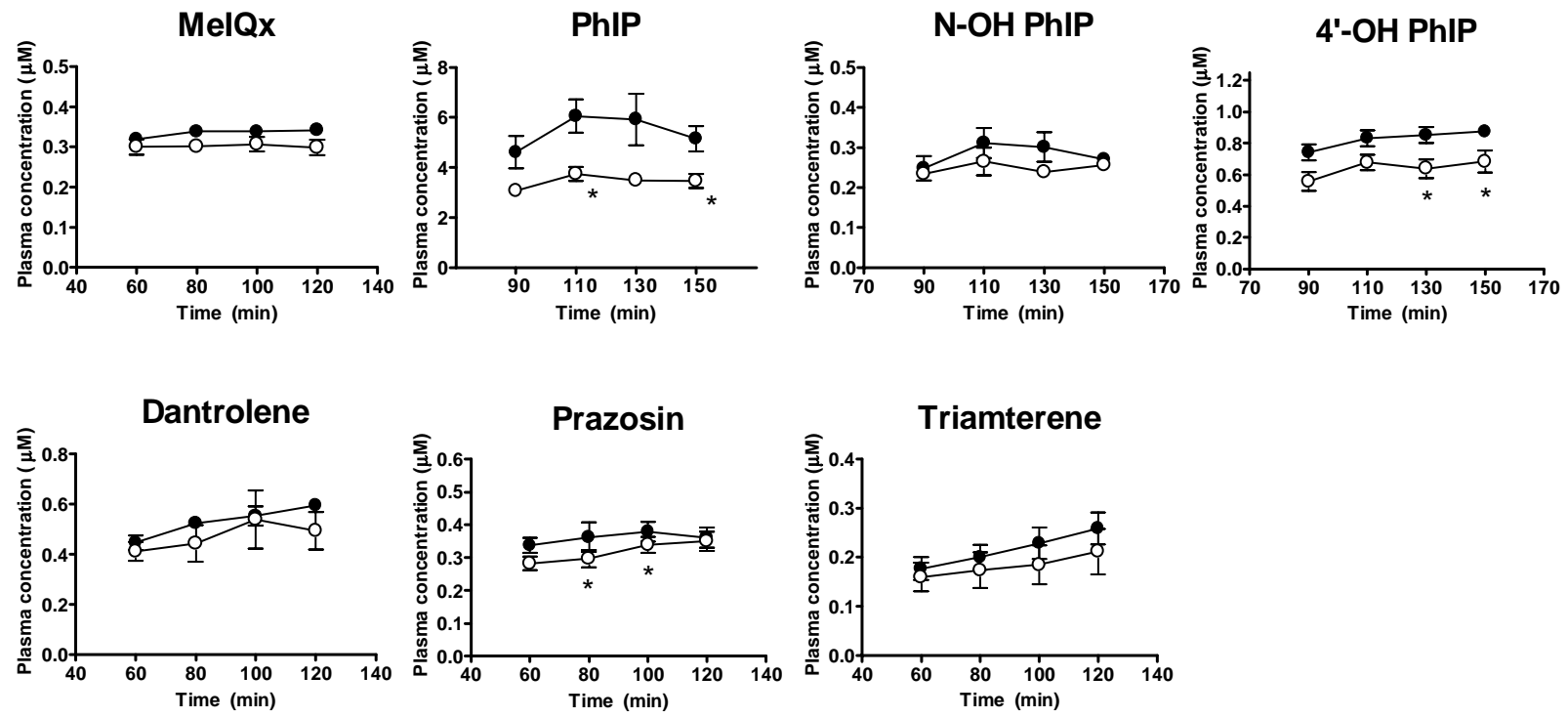
**Table 3** Comparison among  $K_p$  ratios, in vitro transport profiles and pKa values.

Compound	$K_p$ ratio		CFR		pKa <sup>b</sup>	
	Brain	Testis	Bcrp	Mdr1a	Acid	Base
MelQx	2.36	1.99	4.03	1.76	-	7.7
PhIP	2.80	3.17	3.20	2.67	-	2.5
dantrolene	6.30	16.5	2.52	1.06	7.7	-
prazosine	1.52	2.14	2.58	6.52	-	6.5
triamterene	2.10	1.71	3.91	2.16	-	6.3
daidzein <sup>a</sup>	5.63	5.75	2.21	0.714	7.5	-
genistein <sup>a</sup>	9.17	5.78	1.69	0.841	7.4	-
coumestrol <sup>a</sup>	3.94	4.13	1.90	0.801	8.1	-

a. Data were cited from Enokizono et al. (2007b).

b. The most acidic and basic pKa values were calculated using ACD/pKa software (Advanced Chemistry Development, Toronto, Canada).

Figure 1



**Figure 2**

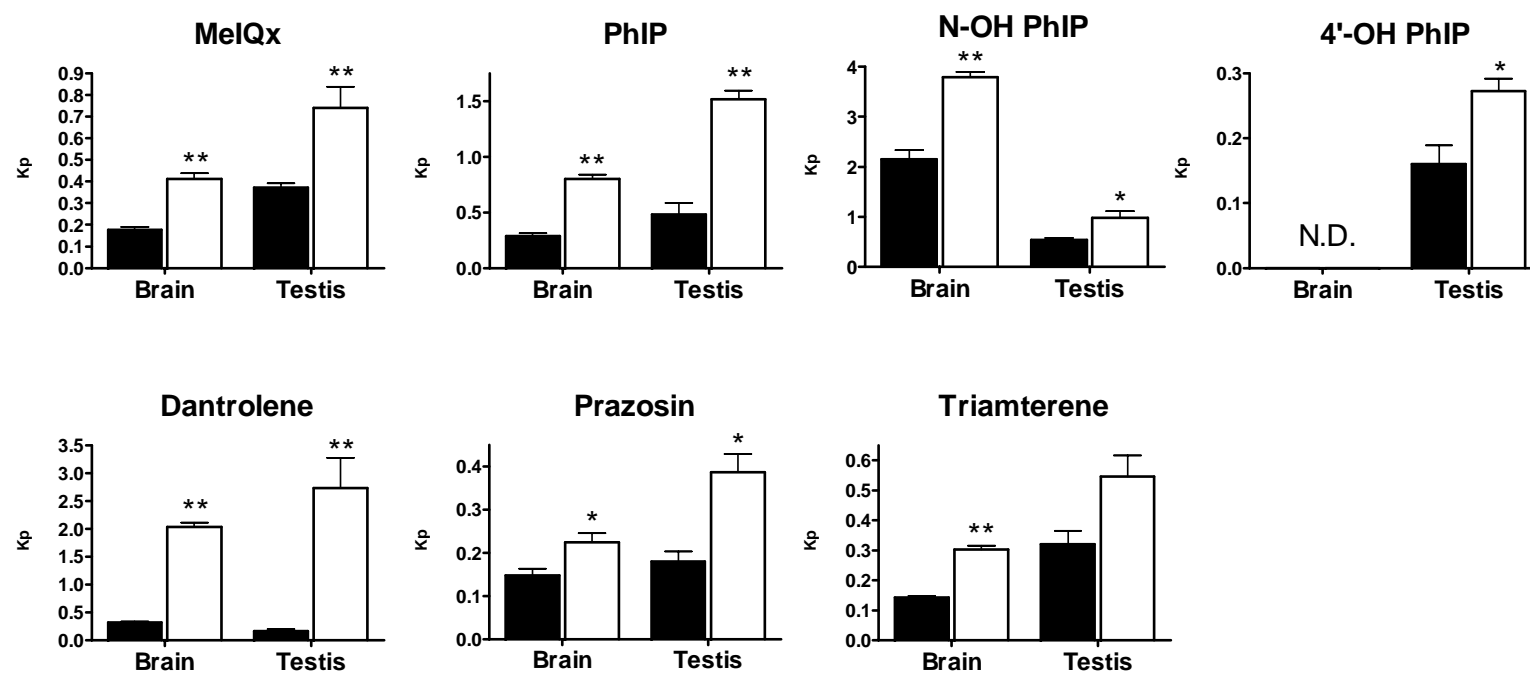
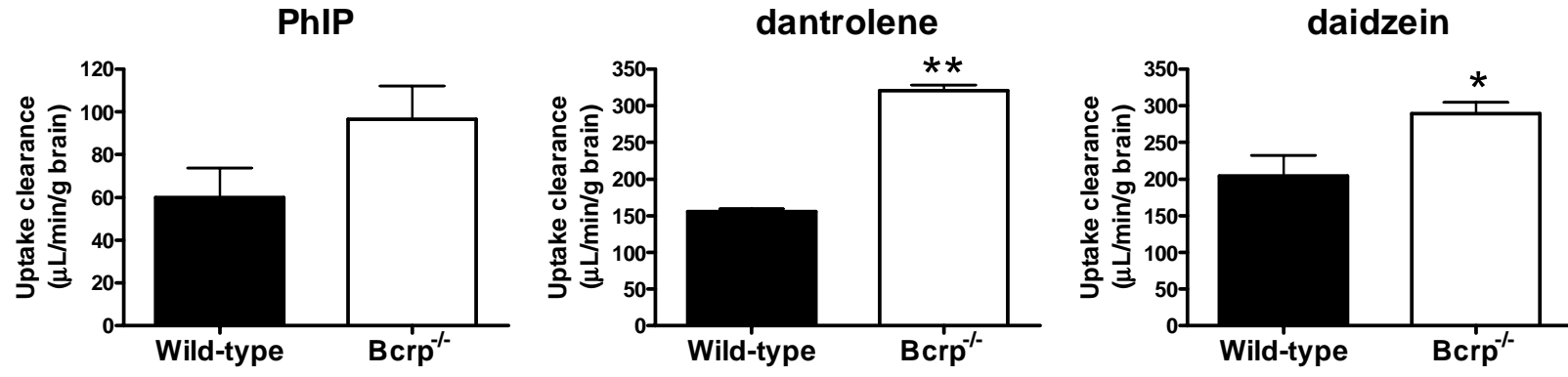


Figure 3



**Figure 4**

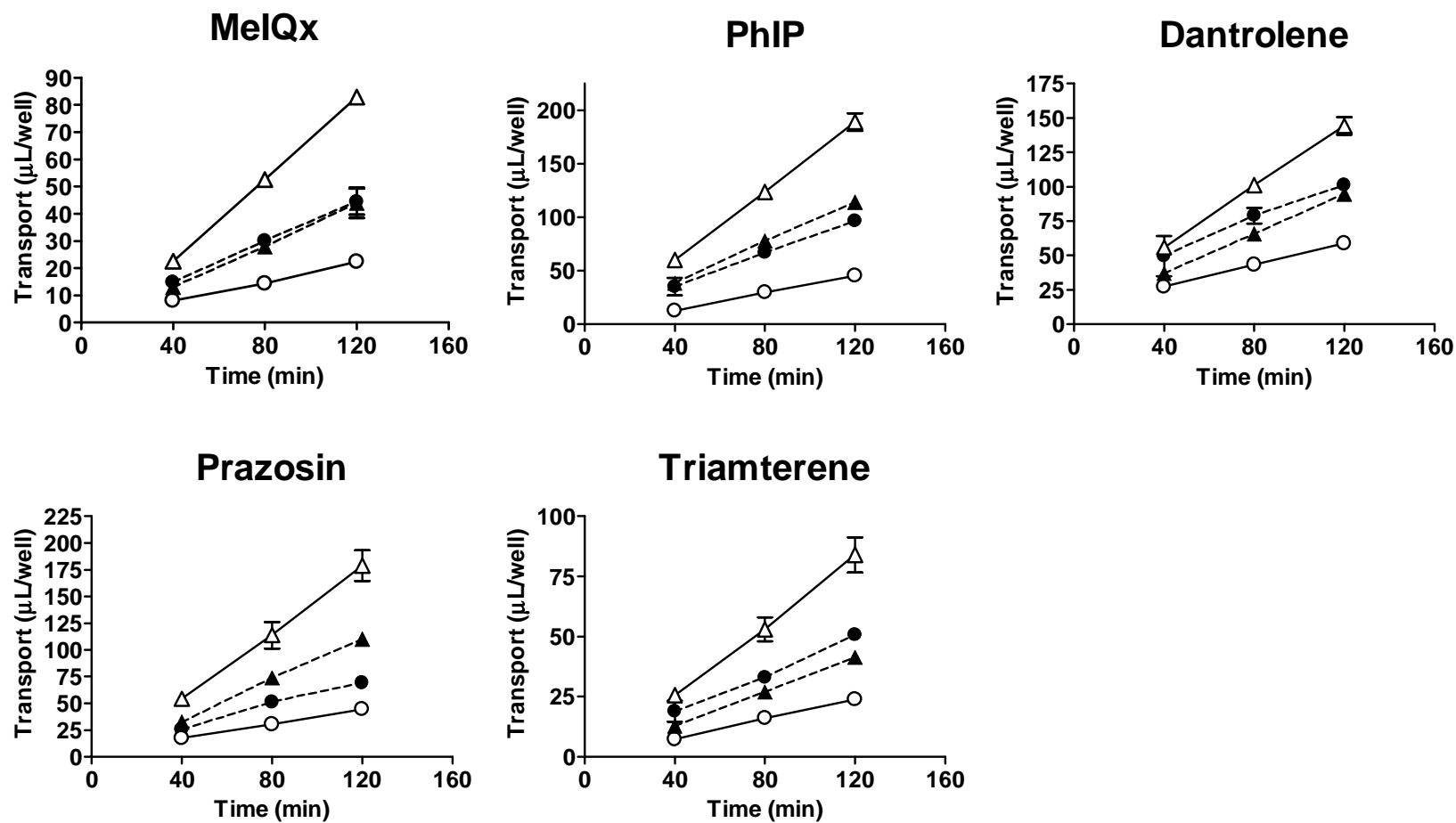
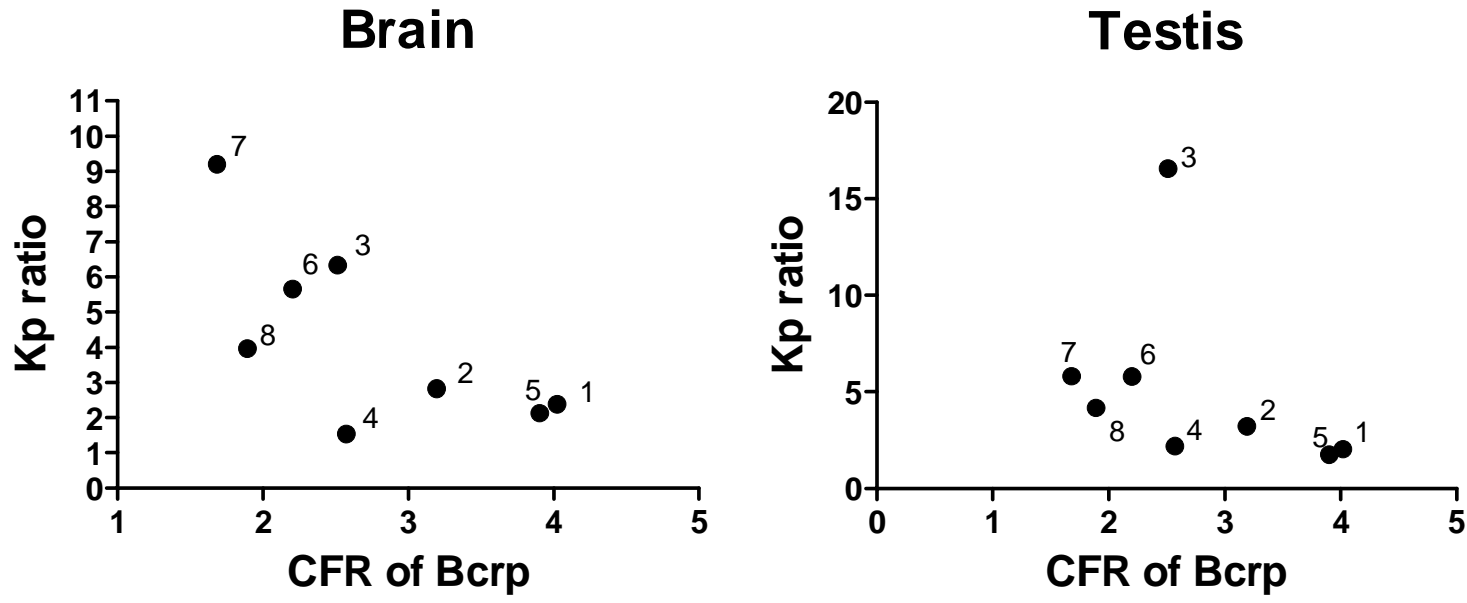


Figure 5



**Figure 6**

