Molecular Pharmacology Fast Forward. Published on July 20, 2007 as doi: 10.1124/mol.107.034751
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MOL #34751

Effect of Breast Cancer Resistance Protein (Bcrp/*Abcg2*) on The Disposition of Phytoestrogens

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Running title: role of Bcrp in the pharmacokinetics of phytoestrogens

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The number of text pages : 32

The number of tables : 1

The number of figures : 7

The number of references : 38

The number of words in Abstract : 223

The number of words in Introduction : 692

The number of words in Discussion : 1165

Abbreviations : Bcrp, breast cancer resistance protein; mBcrp, mouse Bcrp, hBCRP; human BCRP.

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GFP, green fluorescent protein; L-Mdr1a, LLC-PK1 cells expressing mouse Mdr1a; AUC, area under the curve; Kp, tissue-to-plasma concentration ratio

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Abstract

The effect of breast cancer resistance protein (Bcrp/*Abcg2*) on the disposition of the three phytoestrogens, daidzein, genistein and coumestrol, was investigated using *Bcrp*$^{-/-}$ mice. Expression of the genes for either mouse Bcrp or human BCRP in MDCK II cells induced apically directed transport of the three phytoestrogens, while their transcellular transport was identical in mock and LLC-PK1 cells expressing mouse Mdr1a. After oral administration, the plasma levels of daidzein and genistein were increased in *Bcrp*$^{-/-}$ mice, but only a minimal change was observed for coumestrol. At steady state, tissue-to-plasma concentration ratios of the three phytoestrogens in the brain and testis of wild-type mice were very small and similar to those of ^{14}C-inulin, whereas those were significantly increased in the brain and testis of *Bcrp*$^{-/-}$ mice. The largest increases were observed with genistein (9.2 and 5.8-fold in the brain and testis, respectively). The distributions of genistein in the epididymis and fetus, but not the ovary, were also increased in *Bcrp*$^{-/-}$ mice. The Bcrp protein was localized in the luminal membrane of the endothelial cells in the testis and the body of the epididymis, and in both the luminal and abluminal side of ducts in the head of the epididymis. These results suggest that Bcrp limits the oral availability and distribution into the brain and testis, epididymis and fetus of phytoestrogens.

Introduction

Phytoestrogens are plant compounds that produce estrogen-like activity in mammals. Daidzein and genistein are two major isoflavonoids included in soy-based food and are the most commonly consumed phytoestrogens. Coumestrol, an isoflavone present in high concentrations in red clover, is known as the most potent estrogen among phytoestrogens (Mueller et al., 2004). Because of their estrogenic activity, phytoestrogens have been proposed as alternative agents for the treatment of postmenopausal disease; this concept has been supported by some clinical studies (Cassidy et al., 1995; Watanabe et al., 2000). Furthermore, epidemiological studies suggest that the low incidences of prostate, breast and colon cancer, and coronary disease in Asian populations are associated with the high consumption of isoflavonoids, a group of phytoestrogens highly included in soy-based diets (Adlercreutz, 1995). Based on these findings, phytoestrogens are thought to be beneficial for human health and are widely consumed in food and food supplements. On the other hand, warnings against the excessive consumption of phytoestrogens have been issued, because some adverse effects of phytoestrogens have been reported. For example, perinatal and neonatal exposure to genistein caused abnormalities in the ovary and vagina, reduced size of the testis and prostate and suppression of sexual behavior in rodents (Delclos et al., 2001; Wisniewski et al., 2003; Kyselova et al., 2004). Suppressive effects on sexual behavior were also reported for coumestrol in rats (Whitten et al., 2002).

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The disposition of phytoestrogens has been well studied for genistein and daidzein. These exist naturally in the glycoside forms. Upon ingestion, they are hydrolyzed by bacterial β-glycosidase and are absorbed mainly as an aglycon (Setchell et al., 2002). Genistein and daidzein are predominantly metabolized to the glucuronide conjugates in the intestine and liver, followed by excretion into the bile (Chen et al., 2005). In the intestinal lumen, the glucuronide conjugates are hydrolyzed by bacterial β-glucuronidase and reabsorbed (Sfakianos et al., 1997). Thus, both genistein and daidzein undergo enterohepatic circulation. Tissue and fetal distributions of genistein have been investigated in rats (Chang et al., 2000; Doerge et al., 2001). Very low distributions of genistein were observed in the brain and testis, whereas moderate and high distributions were found in the other hormone target organs (prostate, ovary and uterus) (Chang et al., 2000). Furthermore, in pregnant rats exposed to genistein, serum genistein concentrations were about five times less in fetuses than in maternal rats (Doerge et al., 2001). These findings suggest that penetration of genistein into the brain, testis and fetus are limited by the blood–brain, –testis and –placental barriers, attenuating the toxicological effects of genistein on the development of the brain, testis and fetus.

Here we investigated the role of the breast cancer resistance protein (Bcrp/*Abcg2*) in the disposition of phytoestrogens. Bcrp is a member of the ATP-binding cassette transporter family and mediates the efflux transport of endo- and xenobiotics. Bcrp is expressed in various

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normal tissues (Maliepaard et al., 2001) and cumulative *in vivo* studies, particularly using *Bcrp*$^{-/-}$ mice, revealed important roles for this protein in the site of absorption and in clearance organs (van Herwaarden et al., 2003; Breedveld et al., 2004; Mizuno et al., 2004; Hirano et al., 2005). In addition, BCRP is also expressed in various tissue barriers, such as those in the brain, testis and placenta (Jonker et al., 2002; Zhang et al., 2003; Bart et al., 2004). In these tissue barriers, Bcrp is expressed in the plasma membranes facing circulating blood and has its protective role against xenobiotics. Indeed, it was shown using *Bcrp*$^{-/-}$ mice that Bcrp restricts the penetration of imatinib and topotecan into the brain and fetus, respectively (Jonker et al., 2002; Breedveld et al., 2005). As far as phytoestrogens are concerned, many of them can interact with human BCRP at least as inhibitors, but genistein was found to be a BCRP substrate (Imai et al., 2004). Here we compared the plasma concentration time profiles and tissue distribution of phytoestrogens (daidzein, genistein and coumestrol), in the brain, testis, epididymis and fetus using *Bcrp*$^{-/-}$ mice. We found that Bcrp limited the oral availability and penetration into the brain, testis and epididymis of genistein and the penetration of daidzein and coumestrol into the brain and testis.

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Material and Methods

Materials and animals

Daidzein, genistein and coumestrol were purchased from Sigma-Aldrich (St. Louis, MO), and ^{14}C-labeled inulin from Moravek Biochemicals (Brea, CA). All the other chemicals were commercially available and of reagent grade. Wild-type FVB mice and *Bcrp*$^{-/-}$ mice (Jonker et al., 2002) were used in the present study. Male mice (9-17 weeks old and 23-32 g body weight) were used in the studies of oral administration and tissue distribution (brain, testis and epididymis). Female mice (15-16 weeks old and 24-28 g body weight) were used in the ovary distribution study. Pregnant female mice used in the fetus distribution study were at two-weeks gestation and weighed 35-48 g. All these animals were maintained under controlled temperature with a light/dark cycle. Food and water were available ad libitum except for the oral administration study where mice were fasted for about 12 h before administration.

Transcellular transport study

The transcellular transport study was performed as previously reported with minor modifications (Matsushima et al., 2005). Briefly, MDCK II were seeded in the 24-well Transwell® (Corning, Cambridge, MA) at a density of 1.4 x 10^5 cells/well and grown for three days in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine

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serum (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Sigma-Aldrich). The cells were infected with the recombinant adenovirus harboring expression vector for green fluorescent protein (GFP), mouse Bcrp (mBcrp) or human BCRP (hBCRP) at 200 multiplicity of infection. The details of the construction of these recombinant adenoviruses were described in a previous report (Kondo et al., 2004). After two-days culture, the cells were used for transport studies. The cells were pre-incubated in Krebs-Henseleit buffer (142 mM NaCl, 23.8 mM Na_2CO_3, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM $CaCl_2$, pH 7.4) at 37 ºC 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, 100 μL aliquots were taken from the opposite side of the cell monolayer and replaced with 100 μL buffer.

In vitro transport by mMdr1a was examined using Mdr1a-expressing LLC-PK1 cells (L-Mdr1a). L-Mdr1a was established previously (Smith et al., 1998) L-Mdr1a and parent LLC-PK1 cells were seeded in the 24-well Transwell® at a density of 4.8 x 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 of the transport study were the same as those used for mBcrp and hBCRP. Efflux rates were calculated from the slopes of the time-profiles of

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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.

Animal experiments

Oral administration

Animals were fasted 12 h before administration. Compounds were suspended in 0.5% methylcellulose and orally administered at a dose of 30 μmol/kg. Blood was collected from tail vein at appropriate time points and were centrifuged at 4 ℃ and 1,000 xg for 5 min to obtain plasma.

Tissue and fetus distribution

Under urethane anesthesia (1.25 g/kg, i.p.), the right jugular vein was cannulated with a polyethylene tube (PE-50; Becton Dickinson). Compounds were solubilized at a concentration of 1.25 mM in 10% dimethylsulfoxide/90% saline containing 2 mM NaOH and continuously infused through the cannula at a dose rate of 5 μmol/h/kg. Blood was collected from the left jugular vein at 60, 80, 100 and 120 min and centrifuged at 4 ℃ and 1,000 xg for 5 min to obtain plasma. Immediately after the last blood sampling, mice were sacrificed by cervical dislocation and tissues or fetus were collected. PBS was added to tissues or fetus and homogenized to make

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20% homogenate for brain and testis, 10% homogenate for epididymis, 5% homogenate for ovary and 33% homogenate for fetus. All the samples were stored at -80 ℃ until use.

^{14}C-inulin distribution

Inulin can not penetrate cellular membrane, therefore, ^{14}C-inulin was used as a marker of space outside barriers in the brain and testis. Under urethane anesthesia (1.25 g/kg, i.p.), ^{14}C-inulin was intravenously administered from the right jugular vein (50 μCi/kg). At 10 min postdose, blood was collected from the left jugular vein and mice were sacrificed by cervical dislocation, and then the brain, testis and epididymis were collected. Blood was centrifuged at 4 ℃ and 1,000 xg for 5 min and plasma was obtained. Plasma (10 μL) was mixed with 8 mL of Hionicfluor (PerkinElmer Life and Analytical Sciences, Boston, MA) and the radioactivity was measured with a liquid scintillation counter (LS 6000SE; Beckman Coulter, Fullerton, CA). Tissues were mixed with 400 μL of hydrogen peroxide and 800 μL of 2-propanol and left for 1 h at room temperature. Subsequently, 1 mL of Soluene-350 (Packard Instrument Co., Downers Grove, IL) was added and incubated at 55 ℃ for 4 h to solubilized the tissues. 10 mL of Hionicfluor was added and then subjected to a liquid scintillation counter (LS 6000SE).

LC/MS analysis

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Samples were precipitated with two (for *in vitro* samples) or three (for *in vivo* samples) volumes of acetonitrile and centrifuged at 4 ºC and 15,000 xg for 10 min. After the evaporation of supernatants, the pellets were reconstituted with 10% acetonitrile/90% water and subjected to LC/MS analysis. LCMS-2010 EV equipped with a Prominence LC system (Shimadzu, Kyoto, Japan) was used for the analysis. Samples were separated on a CAPCELL PAK C18 MGII column (3 µm, 2 x 50 mm; Shiseido, Tokyo, Japan) in a binary gradient mode. Mobile phase A was 0.05% formic acid and mobile phase B was acetonitrile. For the analysis of daidzein and genistein, the concentration of mobile phase B was initially 18%, linearly increased up to 60% over 1.5 min, kept at 60% for a further 1 min and finally re-equilibrated at 18% for 2.5 min. The total run time was 5 min. Daidzein and genistein were eluted at 3.0 and 3.3 min, respectively. For the analysis of coumestrol, the concentration of mobile phase B was initially 25%, linearly increased up to 90% over 1.5 min, kept at 90% for a further 1 min and finally re-equilibrated at 25% for 3 min. Coumestrol was eluted at 2.7 min. Daidzein, genistein and coumestrol were detected at mass-to-charge ratios of 255, 269 and 267, respectively under negative electron spray ionization mode. The interface voltage was -3.5 kV and the nebulizer gas (N_2) flow was 1.5 L/min. The heat block and curved desolvation line temperatures were 200 and 150 ºC, respectively.

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Quantification of mRNA level of BCRP in mouse and human epididymis

Mice were anesthetized with ether and sacrificed by exsanguination from the femoral artery and vein. Immediately after sacrifice, the epididymis was collected. Total RNA was isolated from these tissues with using ISOGEN (Wako pure chemical industries). Total RNA of human epididymis was purchased from BioChain Institute (Hayward, CA). Total RNA from mouse and human epididymis was converted to cDNA using random primer and avian myeloblastosis virus reverse transcriptase. Real-time PCR was performed with a QuantiTect SYBR Green PCR Kit (QIAGEN, Valencia, CA) and a LightCycler system (Roche Diagnostics, Mannheim, Germany). PCR primers were as follows, moue Bcrp; forward AAATGGAGCACCTCAACCTG, reverse CCCATCACAACGTCATCTTG, human BCRP; forward CAGGTCTGTTGGTCAATCTCACA, reverse TCCATATCGTGGAATGCTGAAG, mouse GAPDH; forward ATTGTCAGCAATGCATCCTG, reverse ATGGACTGTGGTCATGAGCC and human GAPDH; forward AATGACCCCTTCATTGAC, reverse TCCACGACGTACTCAGCGC. External standard curves were generated by dilution of the target PCR product purified by agarose gel electrophoresis. The absolute concentration of the external standard was measured with PicoGreen dsDNA Quantification Reagent (Molecular Probes, Eugene, OR).

Pharmacokinetic analysis

Area under curve (AUC) from 0 to 240 min after oral administration was calculated by trapezoidal method. Tissue-to-plasma or fetus-to-plasma concentration ratios (Kps) were calculated by dividing tissue or fetus concentrations by plasma concentrations at 120 min postdose.

Immunohistochemical analysis

Frozen sections of the testis and epididymis were prepared from FVB wild-type and *Bcrp*$^{-/-}$ mice and fixed to glass slides in methanol (-20 ºC). The sections were incubated in 1% triton X-100 for 30 min at room temperature and subsequently washed with PBS three times. The sections were then incubated in PBS containing 5% bovine serum albumin (BSA-PBS) to block non-specific protein binding. After washing with PBS three times, the sections were incubated with 1:40 dilution of anti-Bcrp monoclonal antibody (BXP-53; Signet Laboratories, Dedham, MA) in BSA-PBS at 4 ºC over night. After washing with PBS three times, the sections were incubated with secondary antibody (Alexa 488 anti-rat IgG; Molecular Probes, Eugene, OR) and Topro3 (Topro3; Molecular Probes) in BSA-PBS for 1 h at room temperature. The sections were mounted in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA).

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Statistical analysis

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

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Results

***In vitro* transport of phytoestrogens by mBcrp, hBCRP and Mdr1a**

The transport activities of daidzein, genistein and coumestrol by mBcrp, hBCRP and mMdr1a were investigated using transporter expressing cell systems (MDCK II/mBcrp, MDCK II/hBCRP and L-Mdr1a). In those systems, mBcrp, hBCRP and Mdr1a are localized in the apical membrane, and directs the *trans*-cellular transport of their substrates in the apical direction (Smith et al., 1998; Matsushima et al., 2005). In MDCK II/mBcrp and MDCK II/hBCRP, the permeability of basal-to-apical direction was greater than that of apical-to-basal direction for all three phytoestrogens, whereas the transcellular transport was almost identical in both directions in MDCK II/GFP (Fig. 1 and Table 1). The flux ratios were higher in MDCK II/mBcrp and MDCK II/hBCRP than those in MDCK II/GFP, suggesting the three phytoestrogens are the substrate of mBcrp and hBCRP. In LLC-PK1 and L-Mdr1a, the permeability of basal-to-apical direction was slightly higher than that of apical-to-basal direction, however, the flux ratios were almost identical between the two cell systems, suggesting the three phytoestrogens are not the substrate of mMdr1a (Fig. 2 and Table 1).

Effects of Bcrp on the *in vivo* disposition of phytoestrogens

Plasma levels of daidzein, genistein and coumestrol following oral administration

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were compared between wild-type and *Bcrp*$^{-/-}$ mice (Fig. 3). Daidzein and genistein exhibited significantly higher plasma exposure in *Bcrp*$^{-/-}$ mice than in wild-type mice (Fig. 3A). The area under the curve over four hours ($AUC_{0-240\ min}$) was 3.7- and 2.0-fold greater for daidzein and genistein, respectively, than the corresponding control values (Fig. 3B). The plasma exposure of coumestrol was very low compared with the other phytoestrogens, and no significant difference was observed in the $AUC_{0-240\ min}$ of coumestrol between the wild-type and *Bcrp*$^{-/-}$ mice although significant changes were observed in plasma concentrations at several time points.

Tissue distributions of the phytoestrogens were determined at steady state achieved by a constant intravenous infusion (Fig. 4). Plasma concentrations were almost constant between 60 and 120 min, indicating that plasma concentrations reached a plateau at 60 min (Fig. 4A). The plasma concentrations of daidzein were significantly higher in *Bcrp*$^{-/-}$ mice than that in wild-type mice, but no significant changes were observed for genistein and coumestrol. The Kps of brain and testis were significantly increased in *Bcrp*$^{-/-}$ mice for all the three phytoestrogens (Fig. 4B). The fold-increases in the Kps of the brain were 5.6, 9.2 and 3.9 and those in the testis were 5.8, 5.8 and 4.1 for daidzein, genistein and coumestrol, respectively. The Kps of ^{14}C-inulin were investigated to estimate the volume of the capillary space in the brain and testis. The Kps of ^{14}C-inulin were 0.011 ± 0.004 and 0.031 ± 0.011 (mean ± S.E., n = 3) for the brain and testis, respectively (indicated by a dotted line in Figure 4B). In addition, the effects of Bcrp on the

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distributions of genistein in the epididymis and ovary were also investigated (Fig. 5). The Kp of the epididymis was increased in *Bcrp*$^{-/-}$ mice with a fold-increase of 2.5, while Kp of the ovary was unchanged.

Localization of Bcrp in mouse testis and epididymis, and mRNA expression of *BCRP* in the human epididymis

Immunohistochemical analysis was performed to identify the membrane localization of Bcrp protein in the testis and epididymis using the anti-Bcrp antibody (BXP-53) (Fig. 6). In the testis of wild-type mice, Bcrp was detected only in the endothelial cells (Fig. 6A), and specifically in the luminal membrane (Fig. 6C). In the epididymis, the localization of Bcrp differed between the head and body regions (Fig. 6D). In the head (Fig. 6D, left), Bcrp was detected in both the luminal and abluminal sides of ducts, while in the body (Fig. 6D, right), Bcrp staining was observed in the endothelial cells. Immunofluorescence by BXP-53 was diminished in *Bcrp*$^{-/-}$ mice, indicating that the signals were associated specifically with Bcrp. We also examined the mRNA expression level of *BCRP* in the human epididymis. The ratio of *BCRP* mRNA to that of *GAPDH* was 1.16×10^{-2} and was similar to that found in the mouse epididymis (1.42×10^{-2}).

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Role of Bcrp on the accumulation of genistein in the fetus and fetus brain

Role of Bcrp on the fetal distribution of genistein was investigated in pregnant mice. Genistein was given to pregnant mice by constant intravenous infusion, and the plasma concentrations were similar between wild-type and *Bcrp*$^{-/-}$ mice (Fig. 7A). The fetus-to-maternal plasma concentration ratio was 1.8-fold greater in *Bcrp*$^{-/-}$ mice than controls (Fig. 7B). Brain-to-whole body concentration ratios were compared between wild-type and *Bcrp*$^{-/-}$ fetus to evaluate the Bcrp function in fetal BBB. Brain-to-whole body concentration ratio was 1.4-fold increased in *Bcrp*$^{-/-}$ mice (Fig. 7C).

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Discussion

In the present study, the role of Bcrp in limiting oral absorption of the phytoestrogens and their penetration into the brain, testis, epididymis and fetus was investigated using *Bcrp*$^{-/-}$ mice. We tested three phytoestrogens, daidzein, genistein and coumestrol. Daidzein and genistein are two major isoflavonoids in soy-based meal, and the most frequently ingested phytoestrogens. Coumestrol is known to be the most potent phytoestrogen. We found that all three phytoestrogens are substrates of Bcrp (Fig. 1). We also investigated the transport of the phytoestrogens by mMdr1a, which exhibits overlapped tissue distribution with Bcrp, and has been shown to limit the oral availability and tissue distributions of a variety of compounds. However, it was found that all three phytoestrogens were not the substrate of Mdr1a (Fig. 2).

After oral administration, the plasma AUC of daidzein and genistein was significantly increased in *Bcrp*$^{-/-}$ mice (Fig. 3A and 3B). For coumestrol, the plasma concentrations exhibited a significant change in *Bcrp*$^{-/-}$ mice only at early time points, and thus, the AUC did not exhibit a statistically significant change. There are three potential sites to account for the increase in the plasma concentration following oral administration: an increase in intestinal absorption, and a decrease in intestinal and hepatic extraction. When given intravenously, the change in the plasma concentrations of phytoestrogens between wild-type and *Bcrp*$^{-/-}$ mice were marginal (Fig. 4). This is reasonable because the major elimination pathway of phytoestrogens from the

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systemic circulation is glucuronidation. Impaired Bcrp hardly affects the hepatic first-pass effect for phytoestrogens. Glucuronidation may be part of the mechanism limiting oral availability of the phytoestrogens as reported for quercetin (Crespy et al., 1999). We have reported that the intestinal glucuronidation activity of 4-methylumberifellone exhibited no change in *Bcrp*$^{-/-}$ mice (Enokizono et al., 2007). Therefore, the enhanced plasma exposure following oral administration in *Bcrp*$^{-/-}$ mice is likely due to an increased intestinal absorption. The effect of Bcrp on the oral availability of coumestrol was minimal whereas the *in vitro* transport activity by mBcrp was similar among the three phytoestrogens (Fig. 1). This indicates the smaller contribution of Bcrp to the intestinal absorption of coumestrol. The contribution of paracellular transport and intestinal metabolism may be greater for coumestrol than the other phytoestrogens. Indeed, transcellular transport of coumestrol in the apical-to-basal direction in control cells (MDCKII/GFP and LLC-PK1) was lowest among the three phytoestrogens, suggesting the lowest transcellular transport of coumestrol. The mechanism governing the increased plasma concentrations of daidzein during intravenous infusion in *Bcrp*$^{-/-}$ mice remains unknown. Impaired urinary excretion could be one possible mechanism since daidzein also undergoes urinary excretion : about 10% of the dose after oral administration (Bayer et al., 2001).

The Kps of brain and testis of all three phytoestrogens were significantly increased in *Bcrp*$^{-/-}$ mice (Fig. 4B). Considering that the Kp values of the phytoestrogens and ^{14}C-inulin

were similar in wild-type mice, the brain and testis distribution of the phytoestrogens are almost completely limited by Bcrp. Bcrp has been identified on the luminal membrane of both the human and mouse brain capillary endothelial cells that form the BBB (Cooray et al., 2002; Lee et al., 2005), while there is an interspecies difference in membrane localization in the testis. Bcrp is localized on both the luminal side of the endothelial cells and the apical membrane of myoid cells in the human testis (Bart et al., 2004), whereas Bcrp expression is restricted to the luminal membrane of capillary-like structures in the mouse testis (Fig. 6A, C). It is generally considered that the Sertoli and myoid cells form the blood-testis barrier, but endothelial cell–cell junctions are more leaky in rats (Dym and Fawcett, 1970). However, from the present results, testicular endothelial cells evidently have an adequate barrier function against phytoestrogens, at least in mice.

In addition to the testis, we investigated the role of Bcrp in other reproductive organs, the epididymis and ovary. The epididymis is divided into three segments (head, body and tail). Sperm formed in the testis enter the head, and finally reach the tail. During this transition, they undergo maturation and are finally stored in the tail region. It was found that the distribution of genistein was also increased in the epididymis of *Bcrp*$^{-/-}$ mice (Fig. 5). Therefore, we propose that Bcrp limits the penetration of genistein into the epididymis. The membrane localization of Bcrp was regionally dependent in the epididymis. Bcrp was mainly localized in capillary-like

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structures in the body (Fig. 6D, left), while Bcrp was found both in the luminal and abluminal membranes of the ducts in the head (Fig. 6D, right). Bcrp in the capillary-like structure and the abluminal membranes of ducts may contribute to the reduced distribution of genistein in wild-type mice. The physiological role of the Bcrp in the luminal membranes of the ducts in the head is unknown. It may mediate the luminal secretion of some endogenous compounds. Unlike male reproductive organs, the ovaries did not exhibit any change in the tissue distribution of genistein (Fig. 5) although the *Bcrp* mRNA level in the ovaries is similar to that in the testis (Tanaka et al., 2005). The reason for the discrepancy between mRNA expression and functional activity in the ovaries remains unknown.

Fetal and newborn mice are more sensitive to estrogens than adults. The distribution of genistein in the fetus was increased in pregnant *Bcrp*$^{-/-}$ mice (Fig. 7B), suggesting that Bcrp limits the penetration of genistein into the fetus in the placenta. Furthermore, the brain-to-whole body concentration ratio was also increased in fetal *Bcrp*$^{-/-}$ mice (Fig. 7C). Therefore, fetal brain capillaries may develop a barrier function to some degree even at this stage. The smaller increase (1.4-fold) than that observed in adult mice (9.2-fold) suggests that the barrier function is still immature at this stage (Nico et al., 1999). Taken together, the exposure of phytoestrogens to the fetal brain is limited by Bcrp in the fetal blood-brain barrier, and the placenta as well as the maternal small intestine.

Estrogen plays a key role in the development of reproductive systems and sexual differentiation and estrogenic chemicals are known to influence reproductive functions, such as reduced testicular weight, sperm counts and induction of the acrosome reaction in both human and mouse (Atanassova et al., 2000; Adeoya-Osiguwa et al., 2003; Kyselova et al., 2004; Fraser et al., 2006), as well as adult sexual behavior, such as reduced mounting and ejaculation in males and reduced lordosis in females (Patisaul et al., 2004). Bcrp will prevent these adverse effects of phytoestrogens by limiting the exposure to the reproductive organs and brain.

In conclusion, we have demonstrated the importance of Bcrp in limiting the oral availability of phytoestrogens and their penetration into the brain and male reproductive organs. In addition, Bcrp also limited the exposure of the mouse fetus to phytoestrogens by extruding them to the blood from the placenta. These results indicate the important roles of Bcrp in protecting the body from the adverse effects of phytoestrogens on sexual behavior and spermatogenesis.

Molecular Pharmacology Fast Forward. Published on July 20, 2007 as DOI: 10.1124/mol.107.034751
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Acknowledgment

We would like to thank Dr. Alfred H. Schinkel (The Netherlands Cancer Institute, The Netherlands) and for supplying *Bcrp*$^{-/-}$ mice and L-Mdr1a cells.

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Molecular Pharmacology Fast Forward. Published on July 20, 2007 as DOI: 10.1124/mol.107.034751
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Legends to Figures

Figure 1. The transcellular transport of daidzein (A), genistein (B) and coumestrol (C) across monolayers of MDCK II cells expressing the gene for green fluorescent protein (GFP), mBcrp and hBCRP. Transport in the apical-to-basal direction is represented by open circles and that in the basal-to-apical direction by closed circles. Data are represented by means ± S.E. of triplicate experiments.

Figure 2. The transcellular transport of daidzein (A), genistein (B) and coumestrol (C) across monolayers of control and mMdr1a expressing LLC-PK1 cells. Transport in the apical-to-basal direction is represented by open circles and that in the basal-to-apical direction by closed circles. Data are represented by means ± S.E. of triplicate experiments.

Figure 3. Comparison of the plasma concentrations of daidzein, genistein and coumestrol after oral administration (30 μmol/kg) of daidzein, genistein or coumestrol between wild-type and *Bcrp*$^{-/-}$ mice. (A) The time profiles of plasma concentrations. (B) Plasma area under the curve (AUC) from time 0 to 240 min. Data are represented by means ± S.E. of three or four mice. Asterisks represent statistically significant differences between wild-type and *Bcrp*$^{-/-}$ mice; * $P < 0.05$, ** $P < 0.01$.

Molecular Pharmacology Fast Forward. Published on July 20, 2007 as DOI: 10.1124/mol.107.034751
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Figure 4. Comparison of the brain and testis distributions of daidzein, genistein or coumestrol between wild-type and *Bcrp*$^{-/-}$ mice. Compounds were infused intravenously at a dose rate of 5 μmol/h per kilogram, and Kps in the brain and testis were measured at 120 min. (A) The time profiles of plasma concentrations. (B) Kps in the brain and testis. Kps values of ^{14}C-inulin are represented by dotted lines. Data are represented by means ± S.E. of three or four mice. Asterisks represent statistically significant differences between wild-type and *Bcrp*$^{-/-}$ mice; * $P < 0.05$, ** $P < 0.01$.

Figure 5 Comparison of the epididymal and ovarian distributions of genistein between wild-type and *Bcrp*$^{-/-}$ mice. Genistein was infused intravenously at a dose rate of 5 μmol/h per kilogram and Kp values in the epididymis and ovary were obtained at 120 min in male and female mice, respectively. Data are represented by means ± S.E. of three or four mice. Asterisks represent statistically significant differences between wild-type and *Bcrp*$^{-/-}$ mice; ** $P < 0.01$.

Figure 6. Immunohistochemical analysis of Bcrp in the testis and epididymis. Localizations of Bcrp protein and DNA are shown by green and blue staining, respectively. (A) Testis of

wild-type mice. (B) Testis of *Bcrp*$^{-/-}$ mouse. (C) Endothelial cells in the testis of wild-type mice. (D) Epididymis of wild-type mouse. (E) Epididymis of *Bcrp*$^{-/-}$ mouse. In (D) and (E), the white lines discriminate the head (left) from the body (right).

Figure 7. Comparison of the distributions of genistein into the fetuses and fetal brain between wild-type and *Bcrp*$^{-/-}$ mice. Genistein was intravenously infused to pregnant mice at a dose rate of 5 μmol/h per kilogram and the distributions into the fetuses and fetal brain were measured at 120 min. (A) The time profiles of plasma concentrations. (B) Fetus-to-maternal plasma concentration ratios. (C) Brain-to-whole body concentration ratios in fetal mice. Data are represented by means ± S.E. of three mice. Asterisks represent statistically significant differences between wild-type and *Bcrp*$^{-/-}$ mice; ** $P < 0.01$.

Molecular Pharmacology Fast Forward. Published on July 20, 2007 as DOI: 10.1124/mol.107.034751
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Table 1 Transcellular transport of phytoestrogens across MDCK II monolayers expressing mBcrp and hBCRP, and across LLC-PK1 monolayer expressing mMdr1a.

Data are taken from Figures 1 and 2. Each value represents the mean ± S.E.

A to B represents the permeability of the transcellular transport in the apical to basal direction. B to A represents the permeability of the transcellular transport in the basal to apical direction.

Compound	Cell line	Permeability (μL/h/well)		flux ratio
		A to B	B to A	
Daidzein	MDCKII/GFP	17.4 ± 0.2	23.7 ± 0.3	1.36
	MDCKII/hBCRP	12.9 ± 0.7	53.2 ± 4.1	4.12
	MDCKII/mBcrp	14.7 ± 0.3	44.1 ± 0.8	3.00
	LLC-PK1	26.7 ± 1.2	49.9 ± 1.0	1.87
	L-Mdr1a	27.9 ± 0.8	47.3 ± 1.0	1.70
Genistein	MDCKII/GFP	13.0 ± 0.3	15.7 ± 0.4	1.21
	MDCKII/hBCRP	12.2 ± 0.8	28.8 ± 1.0	2.36
	MDCKII/mBcrp	12.5 ± 0.5	25.6 ± 0.3	2.05
	LLC-PK1	54.0 ± 2.3	77.5 ± 0.8	1.44
	L-Mdr1a	54.2 ± 1.1	76.0 ± 1.8	1.40
Coumestrol	MDCKII/GFP	2.80 ± 0.04	4.08 ± 0.18	1.40
	MDCKII/hBCRP	3.13 ± 0.15	10.2 ± 0.6	3.26
	MDCKII/mBcrp	3.29 ± 0.23	8.75 ± 0.41	2.66
	LLC-PK1	11.3 ± 0.4	18.7 ± 0.4	1.65
	L-Mdr1a	18.9 ± 0.5	30.9 ± 0.6	1.63

Figure 1

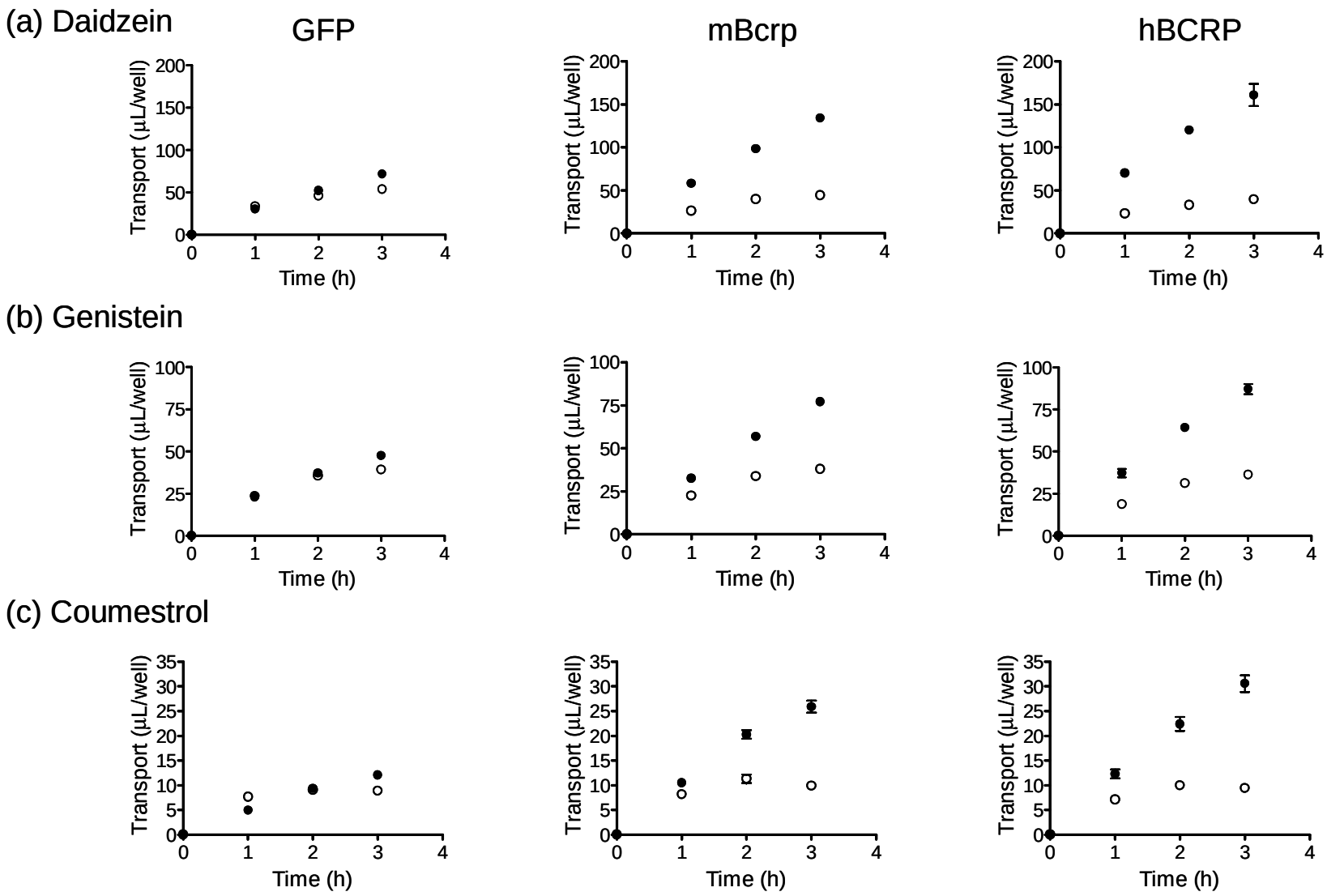

Figure 2

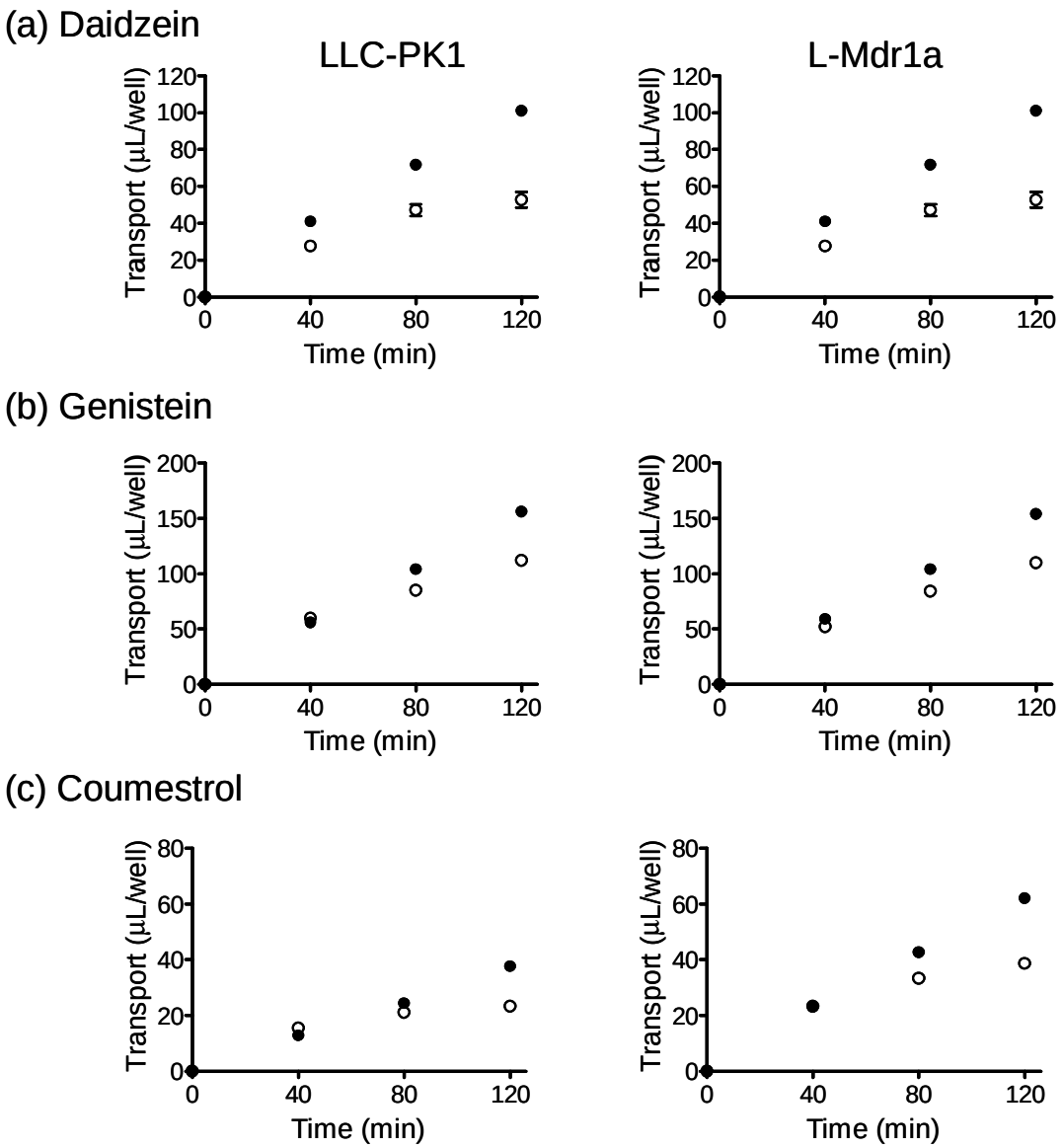

Figure3

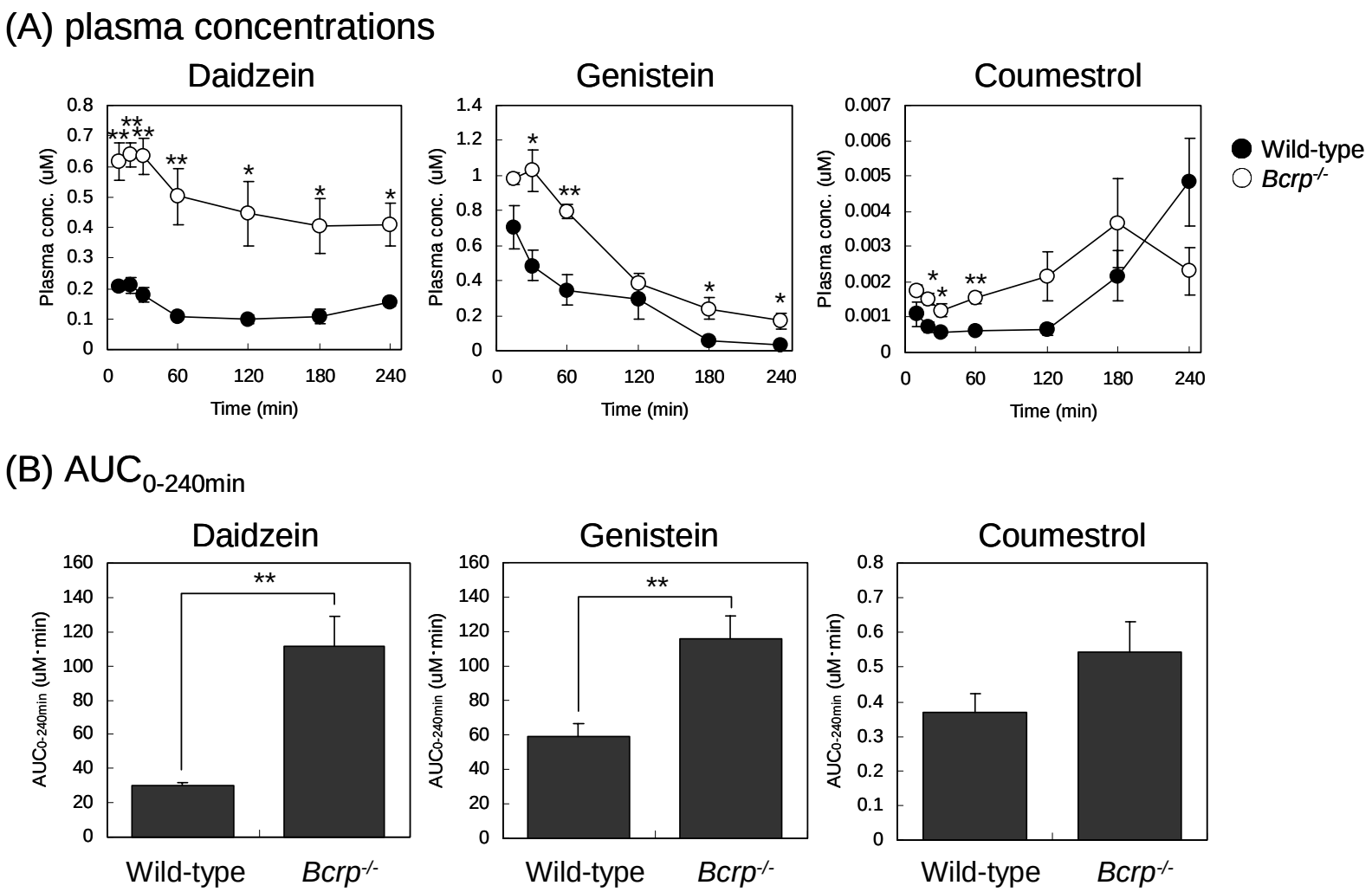

Figure4

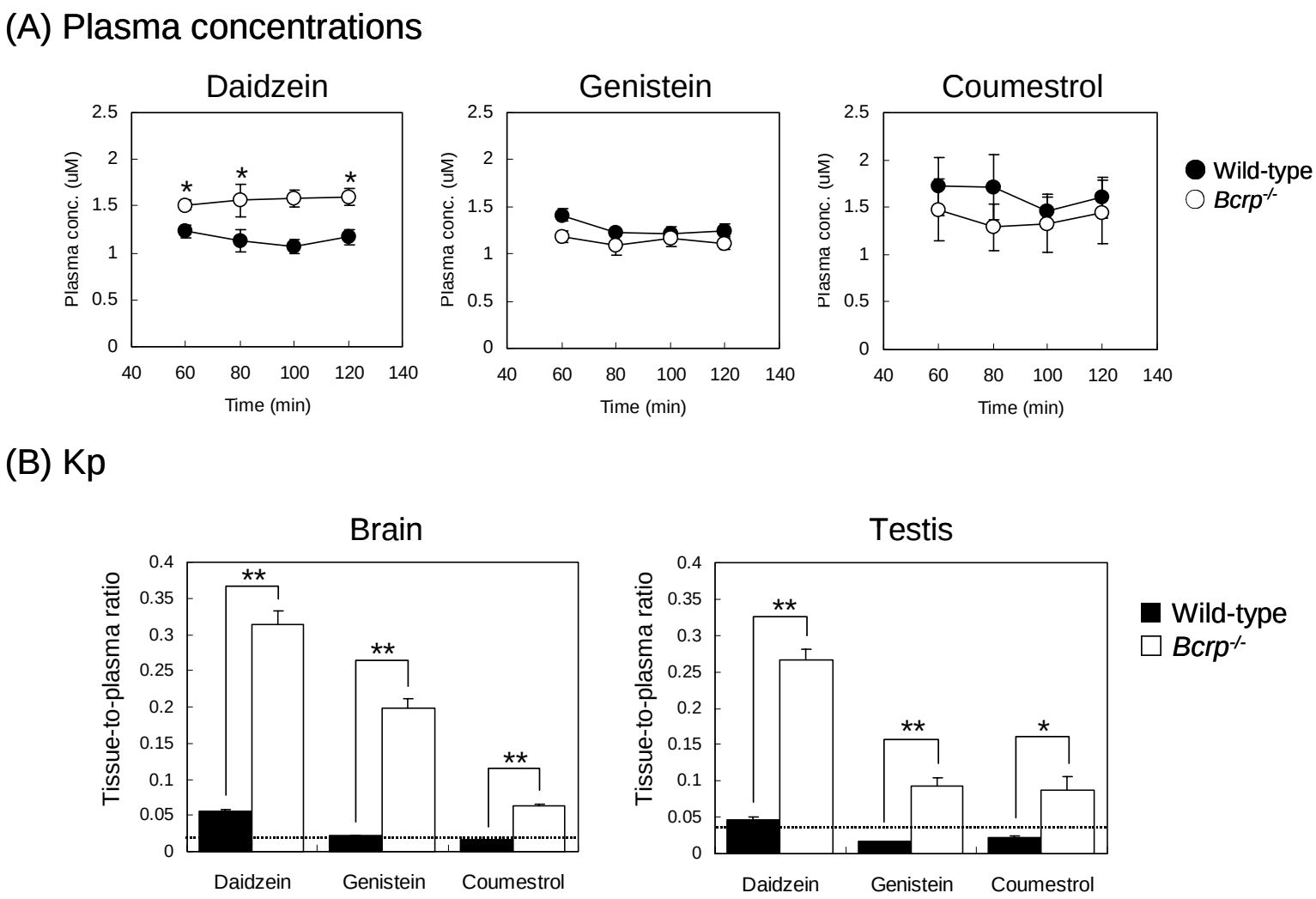

Figure5

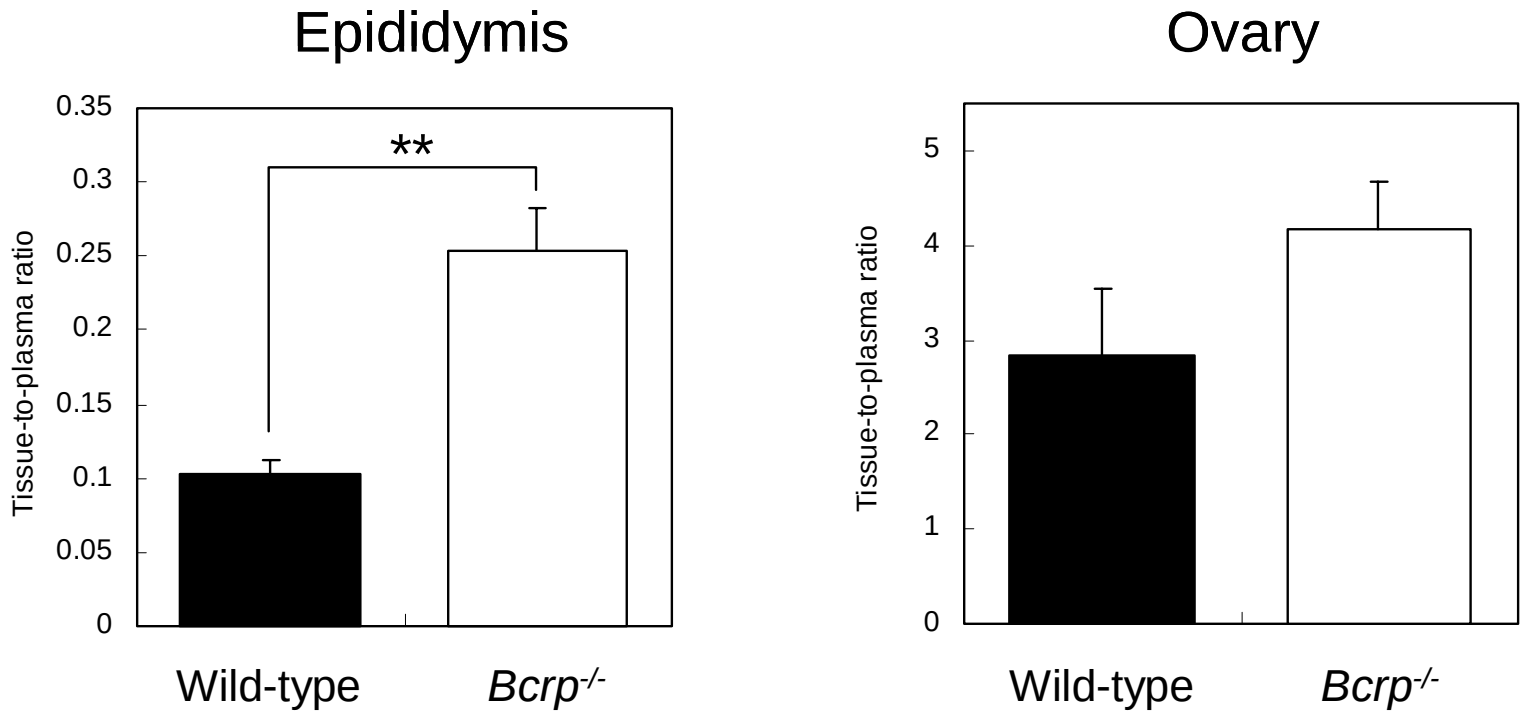

Figure6

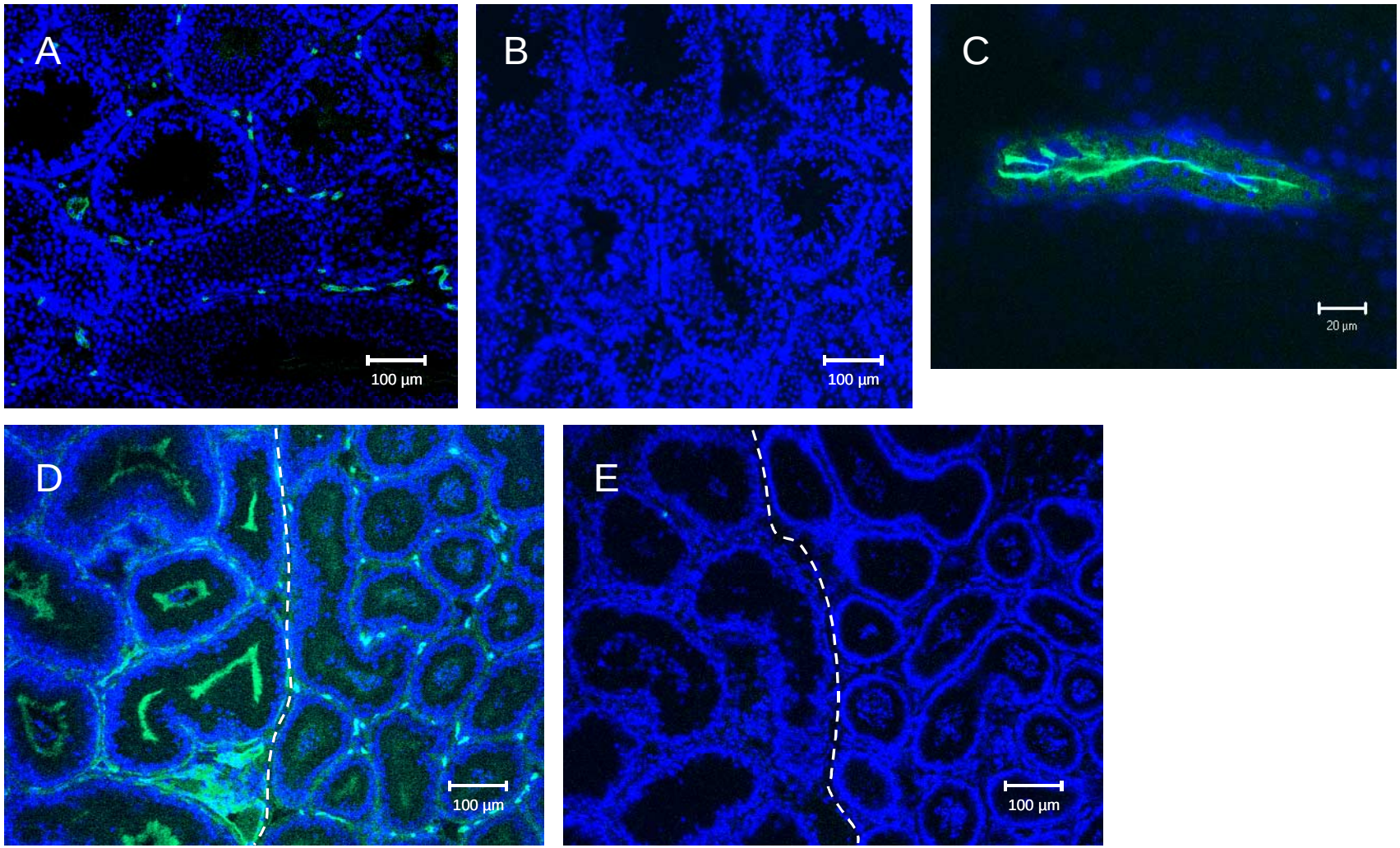

Figure7