Correlation Between Membrane Protein Expression Levels and Transcellular Transport Activity for Breast Cancer Resistance Protein

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ABBREVIATIONS: ABC, ATP-binding cassette; BBB, blood-brain barrier; BCRP, breast cancer resistance protein; CNS, central nervous system; DMEM, Dulbecco's Modified Eagle Medium; ER, efflux ratio; $K_{p,uu}$, unbound brain-to-blood (plasma) concentration ratio; LC, liquid chromatography; MDCK, Madin-Darby canine kidney; MOI, multiplicity of infection; MS, mass spectrometry; P-gp, P-glycoprotein.

Abstract

Emerging evidence indicates an important role for breast cancer resistance protein (BCRP) in limiting brain penetration of substrate drugs. While in vitro Transwell® assays can provide an indication of BCRP substrate potential, the predictability of these to in vivo brain penetration is still under debate. The present study examined the correlation of BCRP membrane protein expression level and transcellular transport activity across MDCKII monolayers. We expressed human BCRP or murine BCRP1 in MDCKII wild-type cells using BacMam2 virus transduction. The selective P-glycoprotein (P-gp) inhibitor LY335979 (1 μM) was included in transport medium to measure BCRP-mediated transcellular transport for P-gp and BCRP co-substrates. BCRP protein levels in membrane extracts from MDCKII-BCRP or MDCKII-Bcrp1 cells were quantified by liquid chromatography-tandem mass spectrometry. The results are summarized as follows: 1) membrane protein expression levels correlates with corrected efflux ratios of substrates for human BCRP and murine BCRP1 within the efflux ratios investigated; 2) we demonstrate a good concordance in rank order between BCRP and BCRP1 mediated efflux ratios for 12 drugs; 3) we propose an approach to contextualize in vitro BCRP transport data of discovery compounds by comparing to the in vitro and in vivo transport data of reference drug dantrolene and taking into account inter-batch variation in BCRP protein expression. This approach correctly predicted compromised brain penetration for 25 discovery compounds in rodents, which were BCRP but not, or weak P-gp substrates. These results suggest that BCRP-expressing MDCKII cells are useful for predicting the in vivo role of BCRP in brain penetration.

Introduction

Drugs designed for brain disorders should be able to the cross blood-brain barrier (BBB) at appropriate concentrations, to exert their desired pharmacology. The strength of the BBB is the result of the very tight junctions between the endothelial cells, polarized active efflux transporters and limited pinocytosis. The major efflux transporters expressed at the luminal side of the BBB are ATP-binding cassette (ABC) family of transporters including P-glycoprotein (P-gp; ABCB1) and breast cancer resistance protein (BCRP; ABCG2). The prominent role of P-gp in limiting entry of various drugs into the central nervous system (CNS) has been well appreciated (Doran et al., 2005; Schinkel et al., 1996; Shen and Zhang, 2010). Thus polarized cell monolayers such as MDCK or Caco2 with expression of human P-gp were deployed early by the pharmaceutical industry to triage drug candidates in CNS drug discovery. The predictive value of these in vitro assays was widely recognized and strong P-gp substrates were unarguably de-prioritized for centrally acting compounds (Feng et al., 2008; Kikuchi et al., 2013; Mahar Doan et al., 2002; Yamazaki et al., 2001).

BCRP (ABCG2) is the second member of the G subfamily of ABC transporter proteins. BCRP is constitutively expressed in human tissues such as CNS (luminal membrane of BBB endothelia), intestine, kidney, liver, placenta, testis, and stem cells, contributing to the absorption, distribution, and elimination of drugs and endogenous compounds (Schnepf and Zolk, 2013). Since its discovery in 1998, many drugs and endogenous substances have been identified to be transported by BCRP (Doyle et al., 1998; Lee et al., 2015). Brain penetration studies in naïve, *Mdr1a/b*(–/–), *Bcrp1*(–/–), *Mdr1a/b*(–/–)*Bcrp1*(–/–) mice or rats clearly illustrated the restrictive effect of BCRP1 on brain disposition for substrate drugs, such as cancer therapeutics, dantrolene and phytoestrogens (Enokizono et al., 2007; Schnepf and Zolk, 2013).

Recent liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based proteomic analyses revealed that BCRP is the most abundant transporter in human BBB and shows approximately 2-fold higher expression in human than in rodent. This is in contrast to about 2-3-fold lower P-gp expression at BBB in human than rodent (Hoshi et al., 2013; Kamiie et al., 2008; Uchida et al., 2011b). In addition, BCRP expression is up-regulated in some neurodegenerative diseases such as Alzheimer disease and amyotrophic lateral sclerosis patients (Jablonski et al., 2012; Xiong et al., 2009). These data could imply a more prominent role of BCRP in brain penetration in diseased human as compared with mice and rats.

Despite this emerging evidence, BCRP is largely overlooked in CNS drug discovery programs due to several reasons. First, BCRP and P-gp share largely overlapping substrate specificity. These transporters cooperate to limit the brain distribution of their co-substrates and compensate for each other when the function of one of these is impaired. Teasing out BCRP specific impact on drug brain penetration in intact systems is not an easy task, in part due to the limited specificity of substrates and inhibitors. In addition, utilization of mouse or rat as the most-evaluated preclinical species to examine the role of efflux transporters at BBB in brain penetration may underestimate and reinforce the contribution of BCRP and P-gp, respectively, due to species difference in protein expression. Second, BCRP is distinct from P-gp and MRPs, as it is a half-transporter that functions as a dimer or oligomer (Poguntke et al., 2010). The structural understanding of drug binding and transport by BCRP is still incomplete. Structural and physicochemical properties governing the interaction with BCRP remain largely unexplored. This complexity poses a lot of uncertainties for the project teams to know how to walk away from this liability. Third, predictive value of in vitro BCRP transport assays remains controversial. BCRP was reported to play a

minimal role at the BBB in vivo in spite of mediating transport of various compounds in vitro (Zhao et al., 2009). Another study did not demonstrated a positive relationship between in vivo and in vitro BCRP activities in brain and testis penetration for eight compounds (Enokizono et al., 2008). These knowledge gaps cast doubt on the benefit of compound screening against BCRP in CNS discovery projects.

In the present study, we aimed to examine the relationship between BCRP protein expression and transcellular transport activity using newly constructed human and murine BCRP expressing MDCKII cells. We have also examined species difference in mouse and human BCRP transport activities for 12 drugs. Finally, we examined the relationship between BCRP efflux ratio and rodent brain-to-blood unbound concentration ratio for 25 compounds, which were BCRP but not, or weak P-gp substrates.

Materials and Methods

Materials

Axitinib and flavopiridol were purchased from Toronto Research Chemicals (Toronto, Canada). Dantrolene sodium salt, coumestrol, prazosin hydrochloride, and Ko143 were obtained from Sigma (St. Louis, MO). Daidzein was procured from J&K Chemical Ltd (Shanghai, China); genistein from Acros Organics; erlotinib hydrochloride and sunitinib maleate from BIONET (UK); dasatinib and gefitinib from Nanjing Chemlin Chemicals Co., Ltd (Nanjing, Jiangsu, China); imatinib tosylate and sorafenib tosylate from Far Top Limited Co., Ltd. (Nanjing, Jiangsu, China). LY335979 hydrochloride was purchased from Shanghai Haoyuan Chemexpress Co., Ltd (Shanghai, China). Amprenavir was obtained from GSK compound library. Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). All other reagents used were of bioanalytical grade or higher. For BCRP and BCRP1 quantification, signature peptide (SSLLDVLAAR) standard and corresponding stable isotope labeled ([$^{13}C^{15}N$]-leucine, SSLLDVL*AAR) internal standard were obtained from GL Biochem (Shanghai) Ltd. (Shanghai, China). The ProteoExtract native membrane protein extraction kit was procured from Calbiochem (Temecula, CA). The protein quantification bicinchoninic acid assay (BCA) kit was purchased from Thermo Fisher Scientific (Waltham, MA). TPCK treated trypsin from bovine pancreas was purchased from Sigma (St. Louis, MO) and used for protein digestion. Iodoacetamide was obtained from Sigma (St. Louis, MO) and dithiotrietol was purchased from Fluka (Buchs, Switzerland).

Generation of BacMam2 Virus Carrying Human BCRP and Murine Bcrp-1 cDNA

ABCG2 human cDNA was amplified from brain, placenta and testis cDNA using PCR and then cloned into pCDNA3.2TOPO. The BCRP sequence was verified in house. Abcg2 murine cDNA clone was purchased from Origene (Rockville, MD). PCR cloning of human BCRP or murine Bcrp1 cDNA into pHTBV1mcs3 vector between BamHI and XhoI sites was verified. The BacMam plasmid pHTBV-BCRP or pHTBV-Bcrp1 was transformed into DH10Bac E.coli competent cells (Invitrogen, Carlsbad, CA, USA), and a single colony from a freshly streaked selective plate was picked and inoculated into 5.0 ml of medium containing the appropriate selective antibiotic. After ~16 h incubation at 37℃ with vigorous shaking (200rpm), bacmid DNA was extracted. The purified BCRP or Bcrp1 bacmid DNA was transfected into Sf9 cells in 6-well dishes at 9×10^5 cells/well using Cellfectin (Invitrogen, Carlsbad, CA, USA). After 3-4 days when cytopathic effect (inhibition of cell growth and lysis of cells) is evident, the culture was collected and centrifuged to pellet the cell debris. The supernatant containing the virus (P0 virus) was transferred to a sterile 15-mL screw cap tube, and stored at 4℃ in the dark. The virus stock was further amplified by infecting 200 ml of Sf9 cells (2×10^6 cells/ml) at a multiplicity of infection (MOI) ranging from 0.05 to 0.1. The P1 virus was harvested 4 days later, and titration was performed in a viral plaque assay using BacPAK Baculovirus Rapid Titer Kit (Clontech, Palo Alto, CA, USA). The recombinant virus was confirmed to contain the human BCRP or murine Bcrp1 gene insert by PCR using extracted virus DNA as template and referred to as BacMam2-BCRP or BacMam2-Bcrp1.

Construction of Human BCRP and Murine BCRP1 Expressing MDCKII Cells.

Polarized Madin-Darby canine kidney wild-type (MDCKII-WT) cells were obtained from The Netherlands Cancer Institute (Amsterdam, Netherlands). MDCKII cells were seeded at a density of 0.1 million/Transwell® insert in Dulbecco's Modified

Eagle Medium (DMEM) with 10% fetal bovine serum. BacMam2-BCRP or BacMam2-Bcrp1 at an appropriate multiplicity of infection (MOI) was administered to the apical side of 24-well Transwell® inserts (Millipore, Bedford, MA) 24 h post MDCKII cell seeding. The cells were cultured for another 48 h before transport experiments.

Transport studies across BacMam2-BCRP or BacMam2-Bcrp1 Cells.

On the day of transport experiments, donor solutions were prepared by diluting test compounds in transport medium (DMEM supplemented with 4500 mg/L D-glucose, L-glutamine, 25 mM HEPES but without sodium pyruvate and phenol red, pH 7.4). Receiver solution was the transport medium. The transport of test compounds was measured in both directions [apical to basolateral (A→B) and basolateral to apical (B →A)] for 90 min in the absence and presence of transporter inhibitor. Transepithelial electrical resistance (TEER) was measured using a Millipore Millicell-ERS system with "chopstick" electrodes (Millipore Corporation, Bedford, MA). Lucifer yellow was used as a paracellular marker to determine the integrity of MDCKII monolayers and measured by a SpectraMax Gemini cytofluorimeter (Molecular Devices, CA) set to an excitation wavelength of 430 nm and an emission wavelength of 540 nm. The exact permeability (P_{exact}, nm/s) and mass balance (MB, %) for test compound across MDCKII monolayers were determined using the following equation:

$$P_{exact} = -\left(\frac{V_D V_R}{(V_D + V_R)At}\right)\ln\left\{1 - \frac{(V_D + V_R)C_R(t)}{(V_D C_D(t) + V_R C_R(t))}\right\} \times 10^7 \quad (1)$$

$$MB(\%) = \frac{V_D C_D(t) + V_R C_R(t)}{V_D C_D(t0)} \times 100 \quad (2)$$

where V_D and V_R are donor and receiver well volumes in mL, respectively; A is the membrane surface area in cm^2; t is transport time in seconds; $C_R(t)$ and $C_D(t)$ are the measured mass spectrometric response or concentration of test compound in the

receiver and donor well at time t (90 min), respectively; C_D (t0) is the measured mass spectrometric response or concentration of test compound in the initial donor solution at time 0. This equation was also used to determine the membrane permeability of lucifer yellow.

Efflux ratio (ER) is defined as permeability ratio of basolateral-to-apical to apical-to-basolateral direction. Corrected efflux ratio is ratio of ER in the absence of inhibitor to presence of inhibitor completely abolishing transporter activity. This is introduced to account for any involvement of endogenous transporters in MDCKII-WT cells in transcellular transport of test compounds.

Quantification of Test Compounds in Transport Samples by LC-MS/MS.

Quantification of drugs in MDCKII transport samples was performed by Waters ACQUITY UPLC™ system coupled with AB Sciex 4000 Q-Trap or API 5000 triple-quadrupole mass spectrometer (AB Sciex, Foster City, CA). The samples were processed by deproteination with the same volume of acetonitrile containing an appropriate internal standard. The chromatographic separation was achieved on a Waters ACQUITY UPLC™ BEH C_{18} or UPLC HSS T3 (50 × 2.1 mm, 1.7 μm, Waters, Milford, MA) analytical column at 40 ℃, using a gradient of aqueous (solvent A: 1 mM ammonia acetate in water or 0.1% formic acid in water) and organic (solvent B: CH3CN-CH3OH with or without 0.1% FA (4:1, v/v)) mobile phase at a flow rate of 600 μL/min. Run time for each compound was 2.0 min. Key chromatographic and mass spectrometric settings were optimized to yield best sensitivity for each test compound and detailed in Supplemental Table 1.

Protein quantification of human BCRP and murine BCRP1 by LC–MS/MS

Protein quantification of human BCRP in BacMam2-BCRP transduced or murine BCRP1 in BacMam2-Bcrp1 transduced MDCKII membrane extracts was conducted

by mass spectrometry-based targeted proteomics using validated LC–MS/MS methods. Briefly, the cells were harvested from semi-permeable membranes in the Transwell® inserts using the extraction buffer I of ProteoExtract™ membrane extraction kit. The total membrane proteins were isolated and digested using the procedure outlined before (Kamiie et al., 2008; Uchida et al., 2013).

Chromatographic and spectrometric conditions for protein quantification of BCRP and BCRP1 is described below. Trypsin digest (8 μl) was injected onto the column (ACQUITY UPLC™ HSS T3, 2.1 × 100 mm, 1.8 μm). A mobile phase consisting of water containing 0.2% acetic acid (A) and acetonitrile-methanol (1:1, v/v) containing 0.2% acetic acid (B) was employed. A flow rate of 0.5 mL/min was used with elution starting at 20% B for 0.2 min, followed by a linear gradient increasing to 60% B (0.2-4.5 min). This was followed by another steep linear gradient increasing to 95% B (4.5-4.55 min) and then eluting the column with 95% mobile phase B for 0.65 min, and re-equilibrating it at 20% B for 1.25 min. MS/MS analysis of BCRP and BCRP1 was performed by monitoring the signature peptide (522.5 to 644.7) and the internal standard (526.8 to 651.7) using an optimized cone voltage and collision energy. Representative LC-MS/MS chromatograms and calibration curve of the surrogate peptide are shown in Supplemental Figure 1.

Accuracy and precision of the assay was >80% and <20% respectively. BCRP and BCRP1 protein expression data (picomoles per milligram) were expressed relative to the total protein content of the isolated membrane, as determined by the Pierce® BCA Protein Assay Kit. All samples were digested and measured in duplicate.

Relationship between efflux ratio (ER) and the efflux activity

Relationship between efflux ratio (ER) and the efflux activity (PS_{efflux}) for BCRP across MDCKII monolayers was adapted from a three-compartment model (apical, cellular, and basolateral) described previously (Kalvass and Pollack, 2007):

$$PS_{efflux} = 2\ PS\ (ER - 1) \quad (3)$$

where PS_{efflux} is BCRP-mediated efflux activity; PS is the passive permeability-surface area product; ER is permeability ratio of basolateral-to-apical to apical-to-basolateral direction. Corrected efflux ratio was also used herein to account for any influence introduced by endogenous transporters in MDCKII-WT cells. Based on eq. (3), PS_{efflux} is quantitatively proportional to ER-1 multiplied by the passive permeability.

Dantrolene is a specific BCRP substrate and was used as a reference compound in each in vitro experiment. Under the condition of linear relationship between BCRP expression and in vitro or in vivo transcellular transport activity, the following equation is derived:

$$\frac{PS_{\text{in vitro,test compound}}}{PS_{\text{in vivo,test compound}}} = \frac{PS_{\text{in vitro,Dan}}}{PS_{\text{in vivo,Dan}}} \quad (4)$$

Substitution of eq.3 into eq. 4 and rearrangement yield eq.5:

$$\frac{ER_{\text{in vitro,test compound}} - 1}{ER_{\text{in vitro,Dan}} - 1} = \frac{ER_{\text{in vivo,test compound}} - 1}{ER_{\text{in vivo,Dan}} - 1} \quad (5)$$

In vivo efflux ratio ($ER_{in\ vivo}$) was defined as the brain-to-plasma (blood) ratio in Bcrp-deficient animals divided by the brain-to-blood (plasma) ratio in Bcrp-competent animals at steady state. Unbound brain-to-blood (plasma) concentration ratio ($K_{p,uu}$) of a discovery compound can be described by reciprocal of in vivo efflux ratio (Uchida et al., 2011a).

$$K_{p,uu} = \frac{1}{ER_{\text{in vivo,test compound}}} \quad (6)$$

Statistical Analysis.

Permeability and efflux ratios are presented as mean ± standard deviation (S.D.) of technical or experimental replicates. Protein levels were mean values of duplicate measurements. Linear regression analysis was performed with Microsoft Excel 2007. In all cases, $p < 0.05$ was considered to be statistically significant.

Results

Construction of MDCKII Monolayers Expressing BCRP or BCRP1

BacMam2 viruses carrying BCRP or Bcrp1 cDNA in DMEM medium was dosed into the apical side of Transwell® inserts at a designated multiplicity of infection (MOI). Transport studies were carried out two days after transduction of BacMam2. TEER values of MDCKII monolayers were elevated after BacMam2 virus transduction. The extent of increase in TEER values correlated positively with MOI of BacMam2 (Fig. 1A). Low permeability of paracellular marker lucifer yellow (typically < 20 nm/s) confirmed tightness of MDCKII-Bcrp1 or MDCKII-BCRP monolayers. MDCKII-WT cells expressed endogenous canine P-gp, transport activity of which was probed by amprenavir, a P-gp specific substrate. ER of amprenavir was reduced along with increasing transport of dantrolene, a specific probe substrate of BCRP1 (Fig. 1B). This was consistent with reverse correlation of canine P-gp and BCRP mRNA levels in BacMam2-BCRP transduced MDCKII cells (data not shown). Inclusion of 1 μM of P-gp specific inhibitor LY335979 in transport medium completely inhibited endogenous P-gp activity (Shepard et al., 2003), with no effect on BCRP1 activity in MDCKII cells (Fig. 1C). Inter-batch variability in BCRP transport activity was notable (Fig. 1D). ER of dantrolene was 15.9 ± 8.9 in 16 consecutive experiments with coefficient of variation of 56% in MDCKII-BCRP cells. ER of dantrolene collapsed to around unity (0.935 ± 0.307) in the presence of 0.2 μM of BCRP specific inhibitor Ko143 in transport medium (Allen et al., 2002).

Correlation between BCRP Membrane Protein Concentration and Transcellular Transport Activity across MDCKII Monolayers

BCRP and BCRP1 protein levels in membrane extracts of MDCKII cells transduced by BacMam2 viruses were quantified by a validated LC-MS/MS method. While the

multiple surrogate peptides for BCRP and BCRP1 were observed to have different absolute quantitative values, these correlated strongly (data not shown, R^2 typically greater than 0.9). The difference could be the result of differential digestion efficiency of signature peptides, unexpected post-translational modifications of signature peptides, or inaccurate quantification of synthetic signature peptides. We report herein, protein expression levels measured by a surrogate peptide consistently providing the highest protein quantification results, which may more accurately represent the true protein abundance, as reported previously (Balogh et al., 2013; Peng et al., 2015).

MDCKII cells with different levels of protein expression of BCRP or BCRP1 were generated by titrating MOI of BacMam2-BCRP or BacMam2-Bcrp1 virus in Transwell® apical compartments. Linear correlation between mRNA level and protein expression for BCRP was observed for the initial five MOI investigated. Higher BacMam2-BCRP or BacMam2-Bcrp1 virus load led to reduction in mRNA level (data not shown). This might be due to interference in transcription and translation pathway of BCRP by high BacMam2 virus load and therefore this specific data was not included in the following correlation analysis.

Dantrolene, coumestrol, daidzein, and genistein are BCRP specific substrates (Enokizono et al., 2007; Fuchs et al., 2014). Transport of these four compounds across MDCKII monolayers with different level of BCRP expression was examined (Table 1 and Supplemental Table 2). ERs of coumestrol, daidzein, and genistein were collapsed to 0.5 or below in the presence of BCRP inhibitor Ko143, indicating their transcellular transport across MDCKII monolayers was impacted by endogenous canine transporters. Thus corrected efflux ratio was used herein to represent BCRP-mediated transport activity. Three micromolar donor concentration was used in this investigation. A plot of the human BCRP protein levels in membrane extracts versus

in vitro corrected efflux ratios in BacMam2-BCRP transduced MDCKII cells showed a significant correlation for dantrolene (R^2 = 0.89, p < 0.05) and genistein (R^2 = 0.85, p < 0.05) and a moderate correlation for coumestrol (R^2 = 0.64, p = 0.1) and daidzein (R^2 = 0.64, p = 0.1) (Fig. 2).

We also investigated the relationship between BCRP1 protein expression levels in membrane extract versus in vitro corrected efflux ratios in BacMam2-Bcrp1 transduced MDCKII cells. A strong linear correlation between protein level and in vitro corrected efflux ratio was observed for dantrolene (R^2 = 0.83, p < 0.05), daidzein (R^2 = 0.79, p < 0.05), genistein (R^2 = 0.99, p < 0.01), and prazosin (R^2 = 0.94, p < 0.01) (Table 2, Fig. 3, Supplemental Table 3). The P-gp specific inhibitor LY335979 was included in transport medium with prazosin, a known P-gp and BCRP co-substrate (Zhou et al., 2009). According to eq. 3, these results indicated a linear correlation between expression level and transcellular transport activity for BCRP (PS_{efflux}) at the efflux ratios investigated.

Correlation between BCRP and BCRP1 Efflux Ratios across MDCKII Monolayers

The in vitro BCRP1 and BCRP efflux ratios were assessed using a set of drugs including P-gp and BCRP co-substrates across MDCKII monolayers. Endogenous canine P-gp activity was inhibited by including LY335979 (1 μM) in transport medium. To avoid data difference due to any inter-batch variation in BCRP expression, the 12 drugs were evaluated in a single experiment. One micromolar donor concentration was used for all drugs. All the selected drugs were identified to be murine and human BCRP substrates. ERs for those drugs were greater than 2 and collapsed to around or below unity in the presence of the BCRP specific inhibitor Ko143 (Table 3 and Table 4). When the impact of BCRP on directional transport was

ranked across all tested drugs, considerable overlap between human and mouse was observed. Corrected efflux ratios of BCRP1 and BCRP were within two-fold for all drugs except flavopiridol, which had 2.2-fold higher efflux in human (Fig. 4).

Discussion

Screening for BCRP substrates in CNS drug discovery is mainly issue-driven and this typically takes place when a discovery compound with high passive permeability has a low unbound brain-to-blood (plasma) ratio ($K_{p,uu}$) and is not a strong P-gp substrate (Di et al., 2013; Poirier et al., 2014). A challenge facing project teams is how to contextualize the in vitro MDCKII-BCRP transport data. To predict transporter-mediated disposition at BBB, physiologically-based pharmacokinetic (PBPK) approaches use in vitro data from over-expressing cell lines and scale these to the in vivo, by correcting the differences in transporter protein expression via in vitro–in vivo extrapolation. This approach requires making two assumptions. First, passive permeability is similar across epithelial (MDCK) and endothelial (BBB) membrane. Second, there is a linear correlation between expression and activity of the transporter protein (Kumar et al., 2015). Available evidence supports the hypothesis that there is no substantial difference in passive permeability across epithelial and endothelial membranes (Liu et al., 2014; Uchida et al., 2011a). The current study aims to examine the validity of the second assumption for BCRP, using MDCKII-BCRP and MDCKII-Bcrp1 monolayers. Our data demonstrate a linear correlation between BCRP membrane protein expression and BCRP-mediated transcellular transport activity across MDCKII monolayers, within the efflux ratios investigated. This implies that it is feasible to predict BCRP-mediated in vivo impact on brain penetration using a BCRP expressing MDCKII Transwell® system. This was earlier exemplified by P-gp substrates that showed transcellular transport activity was proportional to the transporter expression levels (Tachibana et al., 2010). Successful reconstruction of in vivo brain distribution of P-glycoprotein substrates has been based on the BBB transporter protein concentration, in vitro intrinsic transport activity, and in vitro

transporter expression in mice and monkey (Uchida et al., 2011a; Uchida et al., 2014).

Dantrolene is a specific BCRP substrate with well characterized BBB transport profiles in preclinical species. We use this reference drug as an indicator of BCRP competency in Transwell® system. A review of literature showed that the in vivo brain penetration efflux ratios of dantrolene in *Bcrp1*(–/–) to wild type mice and rats were 6.30 and 3.30 at steady state, respectively, and mean Bcrp expression at mouse, rat, and human BBB was 3.37, 4.15, and 7.15 fmol/μg protein, respectively (Table 5). By averaging the Bcrp expression and in vivo brain penetration efflux ratios in mice and rats and assuming no species difference in BCRP transport activity, we predicted the human in vivo efflux ratio ($ER_{in\ vivo,\ Dan}$) to be 9.1 and unbound brain-to-plasma concentration ratio 0.11 (Table 4). The predicted human BCRP in vivo efflux ratio of dantrolene is useful to contextualize the BCRP in vitro transport data for discovery compounds. $K_{p,uu}$ greater than 0.3 is typically used as a starting point of acceptable brain penetration in CNS drug discovery programs. Based on eq. 5, this would require $(ER_{in\ vitro,\ test\ compound} - 1)/(ER_{in\ vitro,\ Dan} - 1) < (1/0.3\text{-}1)/(9.1\text{-}1) = 0.29$ in MDCKII-BCRP transport studies. Conversely, $(ER_{in\ vitro,\ test\ compound} - 1)/(ER_{in\ vitro,\ Dan} - 1) > 0.29$ would predict $K_{p,uu}$ smaller than 0.3 and raise a flag for BCRP-mediated compromise in brain penetration at human BBB. Verification of the strength of our predictions requires the clinical efflux ratio of dantrolene. This will now be possible as the positron emission tomography BCRP probe [^{13}N]dantrolene has been successfully synthesized (Kumata et al., 2012).

To verify this contextualization approach, we applied this method to twenty-two proprietary discovery compounds and three phytoestrogens including coumestrol, daidzein, and genistein, which are BCRP but not or weak P-gp substrates

(Supplemental Table 4). We observed good correlation of restricted brain penetration with benchmarked in vitro efflux ratios in rodents (Fig. 5). Compromised brain penetration ($K_{p,uu}$ < 0.3) is correctly predicted when ($ER_{in\ vitro,\ test\ compound}$–1)/($ER_{in\ vitro,\ Dan}$–1) is greater than 0.6 (Quadrant I, 0.6 is derived from mean in vivo efflux ratio of dantrolene in rat and mouse). Some compounds show greater restriction in brain penetration than expected (Quadrant IV), likely due to additional BBB efflux mechanisms or species difference in BCRP or P-gp transport. In our experience, it is not unusual for a compound to have limited brain penetration (e.g. $K_{p,uu}$ < 0.3), even though it is not a P-gp and BCRP substrate and has high passive permeability. It is possible that member(s) of the MRP family of transporters are involved, or some other yet-to-be-identified efflux pathways. This highlights the gap in our understanding of the very intricate BBB. Empirically, efflux ratio of BCRP greater than 5 was used as a "red flag" of brain penetration impairment. However, this empirical approach does not consider inter-batch BCRP expression variability and may only be applied in our lab. The proposed contextualization approach has been improved by addressing this issue and may be transferable to other labs.

On a simpler scale, we are able to manipulate the in vitro system to minimize impact of other transporters, as the endogenous canine transporters expressed on MDCKII cells may confound the transport data of P-gp and BCRP co-substrates. MDCKII-WT cells express canine P-gp but not BCRP. In addition, endogenous P-gp expression can be impacted by BacMam virus load (Liu et al., 2015). In prior studies, BCRP-expressing in vitro Transwell® system was not found to be predictive of in vivo transport activity (Enokizono et al., 2008; Zhao et al., 2009), likely because P-gp/BCRP co-substrates were included and the activity of endogenous P-gp was ignored. In this study, inclusion of P-gp specific inhibitor LY335979 in transport

medium inhibited the endogenous P-gp activity. Quantitative proteomics combined with transport studies in this relatively "pure" BCRP functioning in vitro system may be a very useful method to predict the fraction of drug transported by BCRP in vivo across BBB. This parameter is critical when predicting the $K_{p,uu}$ of a drug and the significance of transporters in determining brain penetration of a drug (Prasad and Unadkat, 2015). In theory, the additive effect of P-gp and Bcrp on brain-to-plasma ratio for co-substrates can be reconstructed by their respective contribution to the net efflux at the BBB (Kodaira et al., 2010).

The mouse BCRP1 and human BCRP amino-acid sequences are 81% identical and 86% homologous, with very high homology in the ATP binding region (Doyle and Ross, 2003). Despite broad use of Bcrp1 knockout mice in exploring the effect of this transporter on drug disposition, little is known about differences in the kinetics of BCRP-mediated drug transport between human and mouse. Use of newly introduced BCRP expressing MDCKII monolayers without endogenous canine P-gp activity allows us to examine species difference in murine and human BCRP-mediated transcellular transport. In order to control inter-batch variability in BCRP expression, 12 drugs were evaluated simultaneously in a single experiment. Good concordance of murine and human BCRP efflux ratios across MDCKII monolayers was observed. A further comparative study with a range of chemotypes using these systems is required. Meaningful comparison can only be obtained by close monitoring of BCRP expression among different batches of MDCKII cells.

In summary, we have constructed human BCRP and murine BCRP1 expressing MDCKII cells by BacMam2 virus transduction. Inclusion of P-gp specific inhibitor LY335979 in the test system allowed us to create a relatively "clean" system to investigate BCRP-mediated transcellular transport across MDCKII monolayers.

Linear correlation of membrane protein expression levels with transcellular transport activity for BCRP was observed within the efflux ratios investigated. We proposed an approach to contextualize in vitro transport data of discovery compounds by benchmarking the in vitro transport data of reference drug dantrolene and taking into account inter-batch variation in BCRP protein expression. This approach correctly predicted impaired brain penetration for compounds, which are BCRP but not or weak P-gp substrates.

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Authorship Contributions

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Contributed new reagents or analytical tools: Chen.

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Footnotes

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Figure Legends:

Fig. 1. Construction of MDCKII monolayers expressing human BCRP and murine BCRP1. A: TEER values of MDCKII-WT monolayers after transduction of varying multiplicity of infection of BacMam2-Bcrp1 (murine) and BacMam2-BCRP (human) viruses (n=12). B: Efflux ratios of dantrolene and amprenavir (n=3, donor concentration 1 μM) across MDCKII-WT monolayers transduced with varying multiplicity of infection of BacMam2-Bcrp1 viruses. C: Effect of different concentrations of P-gp specific inhibitor LY335979 on apical-to-basolateral (gray bar) and basolateral-to-apical (black bar) permeability of dantrolene (n=3, donor concentration 1 μM) across BacMam2-Bcrp1 transduced MDCKII-WT monolayers. D: Efflux ratio of dantrolene (donor concentration 3 μM) from 16 consecutive transport experiments across BacMam2-BCRP transduced MDCKII-WT monolayers in the absence or presence of 0.2 μM of Ko143; mean ± SD is indicated near the horizontal solid line.

Fig. 2. Correlation of BCRP protein expression level in membrane extracts of BCRP-expressing MDCKII cells with in vitro corrected efflux ratio of dantrolene, coumestrol, daidzein, and genestein. Efflux ratios are presented as mean ± S.D. from three individual Transwells. Protein levels are mean value of duplicate measurements.

Fig. 3. Correlation of BCRP1 protein expression level in membrane extracts of BCRP1-expressing MDCKII cells with in vitro corrected efflux ratio of dantrolene, daidzein, genestein, and prosozin. Efflux ratios are presented as mean ± S.D. from three individual Transwells. Protein levels are mean value of duplicate measurements.

Fig. 4. Correlation of corrected efflux ratio in Transwell® assays using MDCKII-Bcrp1 and MDCKII-BCRP monolayers for 12 drugs. The y-axis is the human BCRP

corrected efflux ratio, and the x-axis is the murine BCRP1 corrected efflux ratio. Data are shown in mean ± S.D. (n = 3). Dashed lines are line of 2-fold deviation from unity.

Fig. 5. Relationship between BCRP efflux ratios and rat/mouse $K_{p,uu}$ for coumestrol, daidzein, genistein, and 22 proprietary discovery compounds. All compounds are BCRP substrates with ER > 2 across MDCKII-BCRP monolayers and ER < 1.8 or apical-to-basolateral permeability ratio across MDCKII-MDR1 monolayers +/- the P-gp inhibitor GF120918 (2 μM). Proprietary discovery compounds have passive permeability > 250 nm/s except for two compounds, which have passive permeability = 71 nm/s. Compromised brain penetration ($K_{p,uu}$ < 0.3) is predicted when $(ER_{in\ vitro,\ test\ compound}-1)/(ER_{in\ vitro,\ Dan}-1) > 0.6$ (eq. 5 & 6 and Table 5). Mean in vivo ER 4.8 in rat and mouse was used.

Quandrant I: $K_{p,uu} < 0.3$ and $(ER_{in\ vitro,\ test\ compound}-1)/(ER_{in\ vitro,\ Dan}-1) > 0.6$;

Quadrant II: $K_{p,uu} > 0.3$ and $(ER_{in\ vitro,\ test\ compound}-1)/(ER_{in\ vitro,\ Dan}-1) > 0.6$;

Quadrant III: $K_{p,uu} > 0.3$ and $(ER_{in\ vitro,\ test\ compound}-1)/(ER_{in\ vitro,\ Dan}-1) < 0.6$;

Quadrant IV: $K_{p,uu} < 0.3$ and $(ER_{in\ vitro,\ test\ compound}-1)/(ER_{in\ vitro,\ Dan}-1) < 0.6$.

TABLE 1

Protein expression of BCRP in membrane extracts of and corrected efflux ratios of dantrolene, coumestrol, daidzein, and genistein across MDCKII monolayers transduced with varying titres of BacMam2-BCRP viruses

Protein level of BCRP (fmol/μg protein)[a]	Corrected efflux ratio[b]			
	Dantrolene	Coumestrol	Daidzein	Genistein
0	0.63 ±0.22	1.09 ±0.58	0.80 ±0.10	0.52[c]
3.47	1.09 ±0.15	3.00[b]	1.86 ±0.36	1.32 ±0.32
13.4	2.62 ±0.31	3.99 ±1.17	5.95 ±0.61	1.59 ±0.75
34.4	3.56 ±0.77	7.67 ±1.86	10.9 ±3.0	7.21 ±3.86
60.6	6.39 ±1.59	10.7 ±2.4	10.5 ±6.3	8.09[b]
50.1[d]	7.24 ±0.75	18.6 ±4.0	18.4 ±2.3	10.0 ±2.2

[a] Protein levels are mean value of duplicate measurements.

[b]Data are presented as mean ± S.D. from three individual Transwells unless otherwise indicated.

[c] Corrected efflux ratio was calculated from permeability with at least one averaged from two individual Transwells.

[d] The MDCKII cells were transduced with highest titres of BacMam2-BCRP. Protein level of BCRP from the membrane extract was likely underestimated as these cells had highest mRNA level (assayed by qPCR) and BCRP efflux activity. These cells also had highest protein level of BCRP when assayed by LC-MS/MS technique with another different surrogate peptide (ATEIIEPSK).

TABLE 2

Protein expression of BCRP1 in membrane extracts of and efflux ratios of dantrolene, daidzein, genistein, and prazosin across MDCKII monolayers transduced with varying titres of BacMam2-Bcrp1 viruses

Protein level of BCRP1 (fmol/μg protein) [a]	Corrected efflux ratio [b]			
	Dantrolene	Daidzein	Genistein	Prazosin [c]
0	0.79 ±0.27	1.07 ±0.23	0.62 ±0.17	0.71 ±0.20
11.5	1.47 ±0.24	2.62 ±0.20	3.21 ±0.47	2.12 ±0.32
36.5	2.89 ±0.69	4.07 ±0.82	3.37 ±0.97	2.95 ±0.67
65.7	4.10 ±1.06	6.31 ±1.82	4.50 ±0.77	3.89 ±0.51
232	4.51 ±1.54	11.0 ±3.1	6.71 ±1.92	5.11 ±1.14
342	6.42 ±1.75	9.66 ±1.34	9.26 ±1.66	7.94 ±1.44

[a] Protein levels are mean value of duplicate measurements.

[b] Data were presented as mean ±S.D. from three individual Transwells.

[c] One micromolar of LY335979 was included in transport medium of prazosin.

TABLE 3

Permeability and efflux ratios of drugs across MDCKII monolayers transduced with BacMam2-BCRP viruses

Permeability across MDCKII-BCRP was determined with 1 μM of donor concentration and 90 min of transport time. One micromolar of LY335979 was included in transport medium. Data are presented as mean ± S.D. from three individual Transwells.

Drugs	Transport medium without Ko143 and with 1 μM of LY335979					Transport medium with 0.2 μM of Ko143 and 1 μM of LY335979					Corrected efflux ratio
	$P_{A\rightarrow B}$ (nm/s)[a]	MB (%, A→B)[a]	$P_{B\rightarrow A}$ (nm/s)[a]	MB (%,B→A)[a]	Efflux Ratio	$P_{A\rightarrow B}$ (nm/s)	MB (%, A→B)	$P_{B\rightarrow A}$ (nm/s)	MB (%,B→A)	Efflux Ratio	
Dantrolene	54.0 ± 4.9	92.6	484 ± 179	95.7	8.98 ± 3.42	453 ± 24	95.0	228 ± 4	91.5	0.503 ± 0.028	17.8 ± 6.9
Coumestrol	29.3 ± 1.0	85.9	287 ± 36	97.6	9.80 ± 1.29	143 ± 25	47.7	49.4 ± 2.5	88.7	0.345 ± 0.063	28.4 ± 6.4
Daidzein	26.9 ± 6.8	95.9	745 ± 155	96.4	27.7 ± 9.0	224 ± 19	78.9	147 ± 25	96.6	0.657 ± 0.125	42.1 ± 15.9
Axitinib	114 ± 4	92.6	353 ± 9	91.3	3.10 ± 0.14	324 ± 26	87.3	224 ± 25	103	0.691 ± 0.095	4.49 ± 0.65
Dasatinib	8.94 ± 0.51	110	268 ± 16	98.8	29.9 ± 2.5	110 ± 10	92.3	71.2 ± 5.1	113	0.646 ± 0.077	46.4 ± 6.7
Erlotinib	53.3 ± 8.5	117	423 ± 89	117	7.93 ± 2.08	304 ± 22	97.6	228 ± 39	100	0.749 ± 0.139	10.6 ± 3.4
Flavopiridol	20.3 ± 1.8	103	610 ± 43	107	30.1 ± 3.4	274 ± 6	94.2	176 ± 24	130	0.642 ± 0.090	46.9 ± 8.4
Gefitinib	19.4 ± 1.2	90.9	471 ± 36	87.7	24.3 ± 2.4	177 ± 18	71.8	106 ± 6	99.1	0.596 ± 0.067	40.8 ± 6.1
Imatinib	22.8 ± 2.3	73.6	301 ± 30	74.8	13.2 ± 1.9	201 ± 42	72.0	113 ± 10	79.4	0.565 ± 0.130	23.4 ± 6.3
Prazosin	28.6 ± 3.5	106	395 ± 13	94.4	13.8 ± 1.8	310 ± 9	104	212 ± 62	98.3	0.684 ± 0.200	20.2 ± 6.4
Sorafenib	17.7 ± 5.6	43.8	53.8 ± 4.3	81.3	3.05 ± 1.00	20.3 ± 3.5	42.6	3.66 ± 0.23	79.2	0.180 ± 0.033	16.9 ± 6.3
Sunitinib	34.8 ± 7.1	88.0	418 ± 18	82.1	12.0 ± 2.5	158 ± 10	63.8	84.9 ± 11.7	76.3	0.536 ± 0.082	22.4 ± 5.8

[a] $P_{A\rightarrow B}$, apical-to-basolateral permeability; MB (%, A→B), apical-to-basolateral mass balance in percentage; $P_{B\rightarrow A}$, basolateral-to-apical permeability; MB (%, B→A), basolateral-to-apical mass balance in percentage.

TABLE 4

Permeability and efflux ratios of drugs across MDCKII monolayers transduced with BacMam2-Bcrp1 viruses

Permeability across MDCKII-Bcrp1 was determined with 1 μM of donor concentration and 90 min of transport time. One micromolar of LY335979 was included in transport medium. Data are presented as mean ± S.D. from three individual Transwells.

Drugs	Transport medium without Ko143 and with 1 μM of LY335979					Transport medium with 0.2 μM of Ko143 and 1 μM of LY335979					Corrected efflux ratio
	$P_{A\rightarrow B}$ (nm/s)[a]	MB (%, A→B)[a]	$P_{B\rightarrow A}$ (nm/s)[a]	MB (%,B→A)[a]	Efflux Ratio	$P_{A\rightarrow B}$ (nm/s)	MB (%, A→B)	$P_{B\rightarrow A}$ (nm/s)	MB (%,B→A)	Efflux Ratio	
Dantrolene	32.9 ± 5.4	103	822 ± 85	112	25.0 ± 4.9	387 ± 12	102	361 ± 52	94.9	0.931 ± 0.137	26.9 ± 6.5
Coumestrol	24.2 ± 1.9	95.8	407 ± 89	117	16.8 ± 3.9	156 ± 8	47.5	45.9 ± 6.6	108	0.295 ± 0.045	57.0 ± 15.8
Daidzein	20.4 ± 2.5	100	673 ± 86	97.4	33.0 ± 5.8	246 ± 19	68.6	151 ± 18	92.5	0.614 ± 0.088	53.8 ± 12.2
Axitinib	100 ± 8	103	294 ± 43	104	2.94 ± 0.49	336 ± 21	80.7	227 ± 29	87.3	0.678 ± 0.096	4.34 ± 0.95
Dasatinib	10.0 ± 0.4	99.3	218 ± 34	95.0	21.8 ± 3.5	157 ± 11	80.1	98.9 ± 2.6	91.8	0.631 ± 0.049	34.5 ± 6.1
Erlotinib	65.4 ± 3.9	92.7	842 ± 125	93.2	12.9 ± 2.1	368 ± 18	106	332 ± 13	93.3	0.902 ± 0.056	14.3 ± 2.4
Flavopiridol	23.0 ± 0.5	95.9	388 ± 34	96.6	16.9 ± 1.6	319 ± 10	96.5	252 ± 23	97.7	0.789 ± 0.076	21.4 ± 2.9
Gefitinib	18.4 ± 2.5	95.4	309 ± 57	93.9	16.8 ± 3.9	206 ± 4	80.2	92.0 ± 13.8	102	0.448 ± 0.068	37.5 ± 10.3
Imatinib	31.9 ± 3.7	97.5	644 ± 42	96.6	20.2 ± 2.7	277 ± 15	101	187 ± 17	99.2	0.674 ± 0.071	29.9 ± 5.1
Prazosin	27.5 ± 3.5	99.0	596 ± 53	96.7	21.6 ± 3.4	414 ± 11	110	508 ± 27	100	1.23 ± 0.07	17.6 ± 2.9
Sorafenib	14.2 ± 4.1	46.3	65.3 ± 8.2	65.3	4.60 ± 1.45	47.2 ± 3.3	29.9	24.9 ± 2.9	77.3	0.528 ± 0.071	8.70 ± 2.98
Sunitinib	30.7 ± 4.0	87.3	418 ± 97	87.3	13.6 ± 3.6	156 ± 6	66.4	106 ± 34.7	77.2	0.678 ± 0.224	20.0 ± 8.5

[a] $P_{A\rightarrow B}$, apical-to-basolateral permeability; MB (%, A→B), apical-to-basolateral mass balance in percentage; $P_{B\rightarrow A}$, basolateral-to-apical permeability; MB (%, B→A), basolateral-to-apical mass balance in percentage.

TABLE 5

Prediction of in vivo BCRP efflux ratio of dantrolene in human based on in vivo BCRP efflux ratio of dantrolene in preclinical species and BCRP protein expression at BBB

Species (Strain)	BCRP protein expression at BBB (fmol/μg protein)	In vivo efflux ratio of dantrolene	Predicted $K_{p,uu}$ [a]	Reference
Mouse (FVB)	3.53, 3.21 (mean 3.37)	6.30	0.16	(Enokizono et al., 2008) (Uchida et al., 2013) (Agarwal et al., 2012)
Rat (Sprague Dawley)	4.15	3.30	0.30	(Hoshi et al., 2013) (Fuchs et al., 2014)
Human	6.15, 8.14 (mean 7.15)	9.1 (predicted)[b]	0.10	(Shawahna et al., 2011) (Uchida et al., 2011b)

[a] $K_{p,uu}$ was predicted by reciprocal of in vivo efflux ratio.

[b] Human in vivo efflux ratio of dantrolene was predicted by [mean in vivo efflux ratio of dantrolene in mouse and rat–1] divided by ratio of mean Bcrp protein expression at mouse and rat to human BBB + 1. BCRP transport capacity was thereafter assumed to be similar in human as has been reported for mouse and rat.

Figure 1

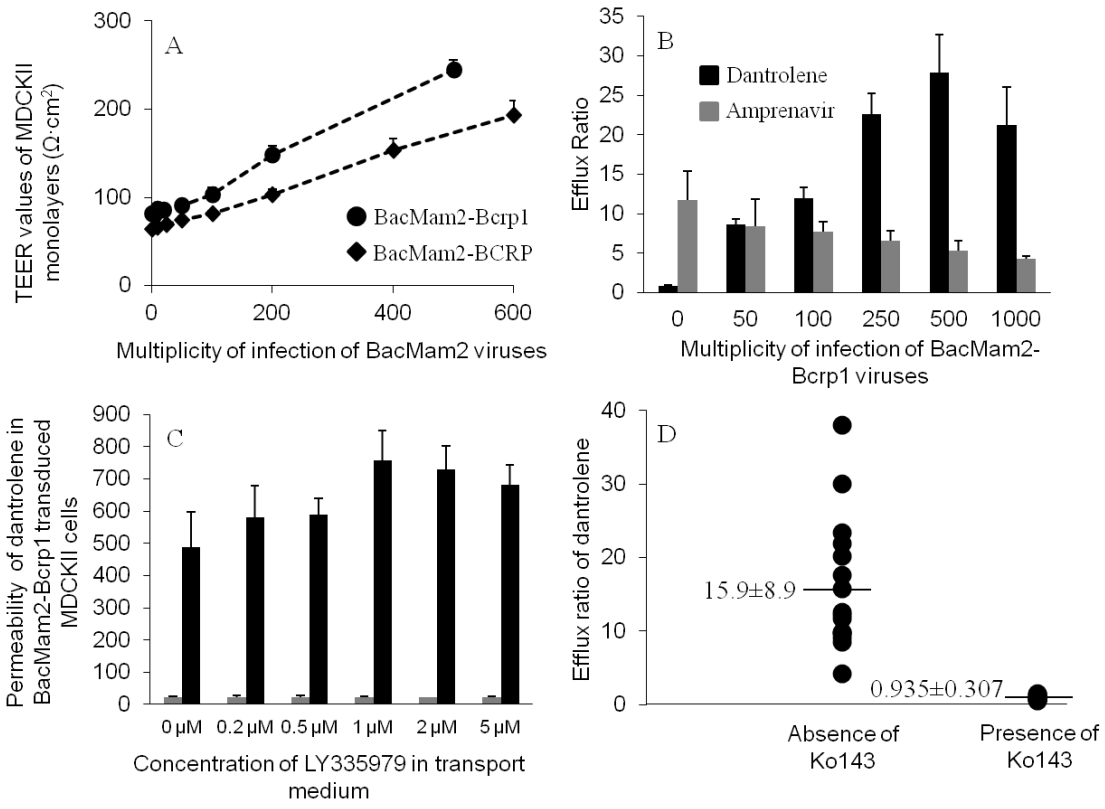

A
TEER values of MDCKII monolayers (Ω·cm²)
300
200
100
0
0
200
400
600
BacMam2-Bcrp1
BacMam2-BCRP
Multiplicity of infection of BacMam2 viruses
B
Efflux Ratio
35
30
25
20
15
10
5
0
Dantrolene
Amprenavir
0
50
100
250
500
1000
Multiplicity of infection of BacMam2-Bcrp1 viruses
C
Permeability of dantrolene in BacMam2-Bcrp1 transduced MDCKII cells
900
800
700
600
500
400
300
200
100
0
0 μM
0.2 μM
0.5 μM
1 μM
2 μM
5 μM
Concentration of LY335979 in transport medium
D
Efflux ratio of dantrolene
40
30
20
10
0
15.9±8.9
0.935±0.307
Absence of Ko143
Presence of Ko143

Figure 2

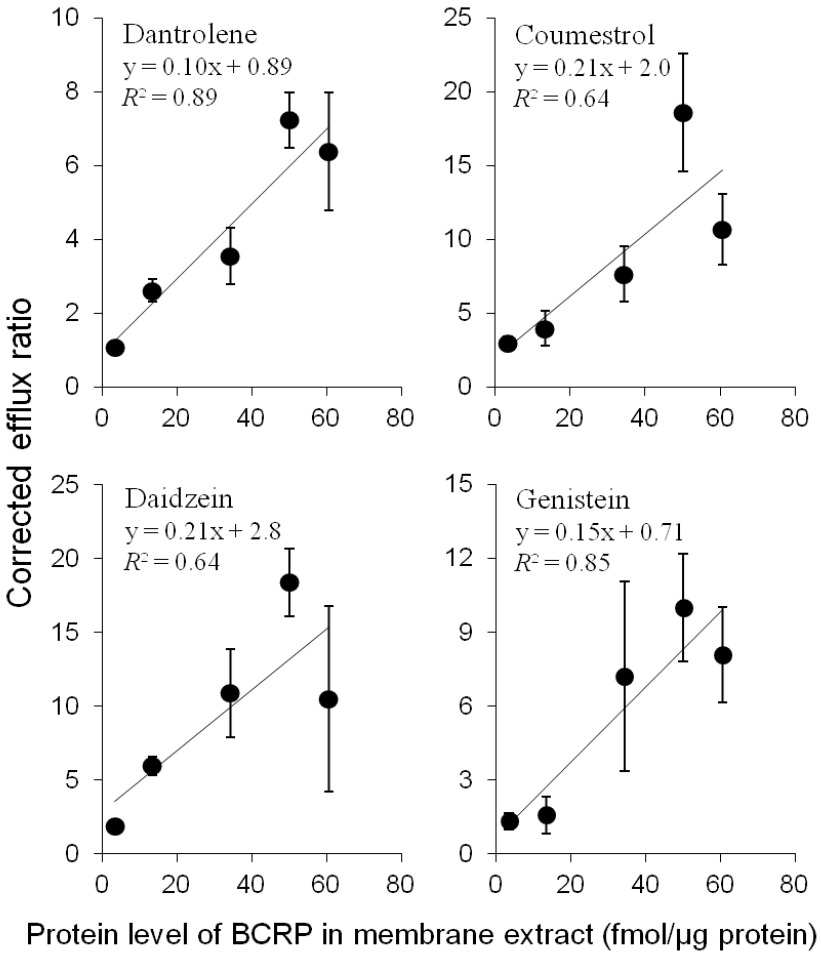

Dantrolene
y = 0.10x + 0.89
R² = 0.89
Coumestrol
y = 0.21x + 2.0
R² = 0.64
Daidzein
y = 0.21x + 2.8
R² = 0.64
Genistein
y = 0.15x + 0.71
R² = 0.85
Corrected efflux ratio
Protein level of BCRP in membrane extract (fmol/µg protein)

Figure 3

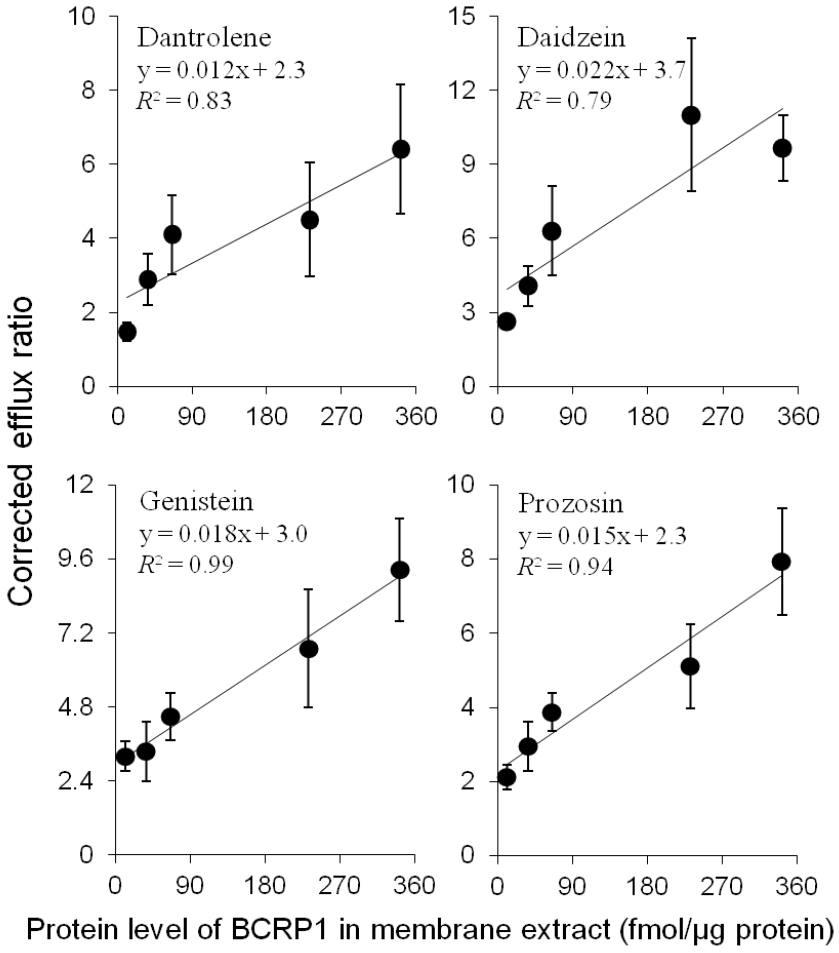

Dantrolene
y = 0.012x + 2.3
R² = 0.83
Daidzein
y = 0.022x + 3.7
R² = 0.79
Genistein
y = 0.018x + 3.0
R² = 0.99
Prozosin
y = 0.015x + 2.3
R² = 0.94
Corrected efflux ratio
Protein level of BCRP1 in membrane extract (fmol/μg protein)

Figure 4

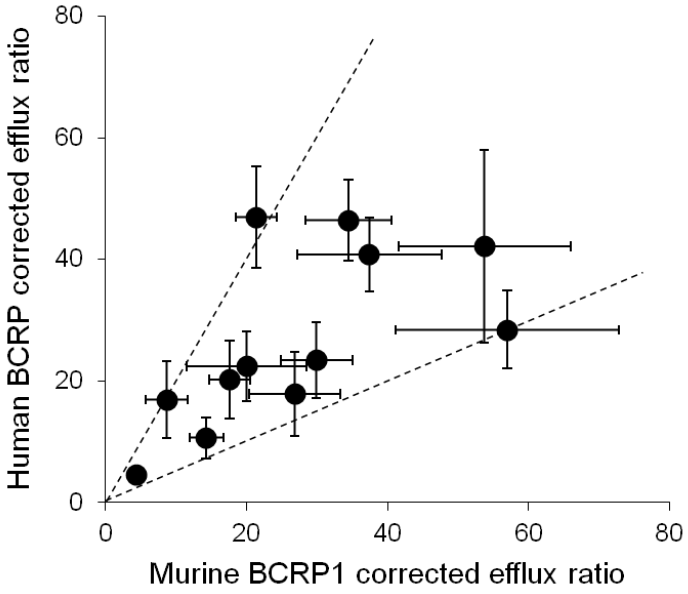
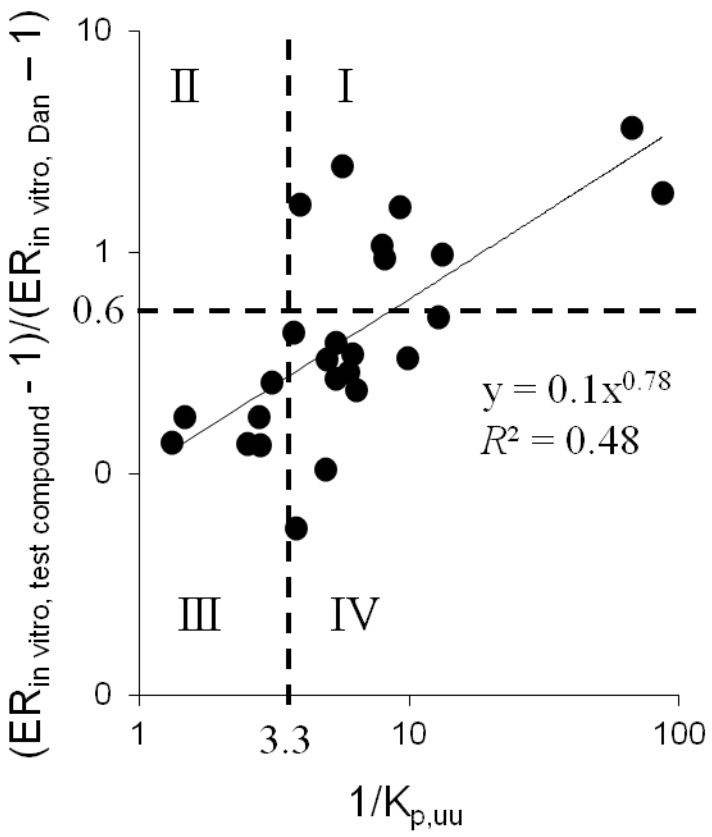

Human BCRP corrected efflux ratio
Murine BCRP1 corrected efflux ratio
0
20
40
60
80

Figure 5


10
1
0.6
0
0
II
I
III
IV
1
3.3
10
100
$(ER_{in\ vitro,\ test\ compound} - 1)/(ER_{in\ vitro,\ Dan} - 1)$
$1/K_{p,uu}$
$y = 0.1x^{0.78}$
$R^2 = 0.48$