EXPRESSION, LOCALIZATION AND FUNCTIONAL CHARACTERISTICS OF
BREAST CANCER RESISTANCE PROTEIN IN CACO-2 CELLS

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ABBREVIATIONS:
A-to-B: apical-to-basolateral; B-to-A: basolateral-to-apical; BCRP: breast cancer resistance protein; E3S: estrone-3-sulfate; EVOM: epithelial volt ohm meter; FBS: fetal bovine serum; HBSS: Hank’s balanced salt solution; HEPES: 4-(2-hydroxy)-1-piperazineethanesulfonic acid; MDR-1: Human multidrug resistance gene 1; MRP: multidrug resistance-associated protein; MTX: methotrexate; $P_{app}$: apparent permeability coefficient; P-gp: P-glycoprotein; SEM: standard error of mean; TEER: transepithelial electrical resistance
ABSTRACT

The function of breast cancer resistance protein (BCRP) and its role in drug absorption, distribution and elimination has recently been evaluated. The objective of the present study was to examine the expression, localization and functional characteristics of BCRP in Caco-2 cells, a widely used human intestinal epithelial cell model for investigating intestinal drug absorption. The expression of BCRP in Caco-2 cells was measured by Western blotting using the antibody BXP-21. Localization of BCRP was determined by an immunofluorescence technique using both antibodies BXP-21 and BXP-34. The drug efflux function of BCRP was evaluated via the epithelial transport of methotrexate (MTX) and estrone-3-sulfate (E3S) across Caco-2 cell monolayers (Transwell™) in the presence or absence of the BCRP inhibitors Ko143 or GF120918. Results from western blot assay indicated that Caco-2 cells in the late passage (p56) expressed a higher level of BCRP as compared to the level in the early passages (p33). The total amount of BCRP protein did not change after the cells were confluent. Immunofluorescence studies revealed the positive staining of BCRP on the apical membrane of Caco-2 cells but not on the basolateral membrane after cell confluence. MTX and E3S showed a preferential basolateral-to-apical (B-to-A) transport across Caco-2 cell monolayers. Both BCRP inhibitors Ko143 and GF120918 increased the apical-to-basolateral (A-to-B) transport but decreased the B-to-A transport of MTX and E3S. Caco-2 cells may therefore be used as an in vitro model to study the transport characteristics of BCRP.
INTRODUCTION

Breast cancer resistance protein (BCRP) was originally cloned and sequenced from genomic DNA, from highly mitoxantrone-resistant S1-M1-80 human colon carcinoma cells and from MCF7 AdVp human breast cancer cells selected in doxorubicin (Doyle et al., 1998; Miyake et al., 1999). BCRP is a member of the ATP-binding cassette transporter G family and is also known as ABCG2 or ABCP or MXR (Ejendal and Hrycyna, 2002; Doyle and Ross, 2003). It is a 655 amino-acid polypeptide (72 kDa), containing six putative transmembrane domains and four potential N-glycosylation sites. BCRP is similar to one-half of the duplicated P-glycoprotein (P-gp) or multi-drug resistance protein 1 (MRP1) molecule and functions as a homodimer bridged by disulfide bonds (Doyle et al., 1998; Kage et al., 2002).

BCRP is endogenously expressed at high level in human placenta and to a lesser extent in liver, small intestine and colon, ovary, veins, capillaries, kidney, adrenal and lung, with little to no expression in brain, heart, stomach, prostate, spleen and cervix (Maliepaard et al., 2001) (Litman et al., 2001) (Doyle et al., 1998; Scheffer and Scheper, 2002). Importantly, BCRP is expressed in the human jejunum at levels considerably higher than many other ABC transporters (Taipalensuu et al., 2001). BCRP has been demonstrated to exist on the apical membrane of intestinal epithelium and limited the oral absorption of topotecan in mice and humans (Jonker et al., 2000) (Kruijtzer et al., 2002a). Given the liver and intestinal localization pattern, BCRP, similar to P-gp, may act as a barrier to uptake and absorption and limit the oral bioavailability of drugs as well as mediating hepatobiliary excretion of drugs(Jonker et al., 2000; Jorritsma et al., 2002; Kruijtzer et al., 2002b).
Caco-2 cells are derived from human colonic adenocarcinoma cell line, and exhibit morphological and functional similarities to intestinal enterocytes. It has been widely used as a model of human intestinal epithelium for studies of intestinal drug absorption and metabolism. Many active transport systems such as P-gp (encoded by MDR-1 gene, also named as ABCB1) and multidrug resistance-associated protein 2 (MRP2, or ABCC2) have been characterized in Caco-2 cells (Gutmann et al., 1999) (Doppenschmitt et al., 1999) (Makhey et al., 1998). BCRP mRNA has been detected in Caco-2 cells although its level is 100-fold lower than that in human jejunum (Taipalensuu et al., 2001).

The present study was aimed at characterization of the protein expression, localization and efflux function of BCRP in Caco-2 cells. We have used BXP-21 monoclonal antibody (mAb) to determine the BCRP protein expression and BXP-21 and BXP-34 mAbs to characterize the subcellular distribution of BCRP in Caco-2 cells. In addition, the efflux function of BCRP in Caco-2 cells was determined employing estrone-3-sulfate (E3S) and methotrexate (MTX) as substrates. Knowledge of the properties of BCRP in Caco-2 cells is valuable to investigate the absorption mechanism of drug molecules using this *in vitro* model system.

**MATERIALS AND METHODS**

**Materials**

[^3]H Estrone-3-Sulfate (specific activity, 45 Ci/mmol) and[^3]H Methotrexate (specific activity, 33.5 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). GF120918 and LY335979 were synthesized at Millennium Pharmaceuticals, Inc.
Ko143 was obtained from the Netherlands Cancer Institute (Amsterdam, Netherlands). MK571 was purchased from Alexis Biochemicals (San Diego, CA). Unlabeled estron-3-sulfate (E3S), methotrexate (MTX), prazocin, and 2,4-dinitrophenol (2,4-DNP) were purchased from Sigma Chemicals (St. Louis, MO). Cell culture media and supplies were obtained from Gibco (Grand Island, NY). BXP-21 and BXP-34 murine monoclonal antibodies were purchased from Signet (Dedham, MA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was obtained from Abcam (Cambridge, MA). Horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG2a, mouse IgG2a, and mouse polyclonal antibody I19 for β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All supplies for Western blot studies were obtained from Invitrogen Life Technology (Calibad, CA).

Caco-2 Cell Culture

The Caco-2 cells (passage 18) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The stock cells were cultured in 150 cm² tissue culture T-flasks for subsequent plating onto 24-Transwell™ plates (0.33 cm²/well, 0.4 µm pore size, Costar, Corning, NY). Briefly, 1 × 10⁵ cells were suspended in 0.2 mL culture medium (Dulbecco's Modified Eagle's Medium (DMEM) with 0.1 mM nonessential amino acids, 2 mM L-glutamine, 4.5 gm/L Glucose, and 10% Fetal Bovine Serum(FBS)) and added to the upper chamber of each filter membrane of a Transwell™ plate. One mL of cell-free culture medium was added to the lower chamber. The Transwell™ plates were then incubated at 37°C in an atmosphere of 5% CO₂ in air and 90% humidity. The culture media were changed every other day. Confluent cell
monolayers were obtained within 5-7 days after plating. The transepithelial electrical resistance (TEER), as measured by an epithelial voltohm meter (EVOM, World Precision instruments, West Haven, CT), gradually increased and reached a plateau after day 5 indicating the formation of tight junctions. Monolayers with TEER values greater than 250 ohms•cm² were used. Unless otherwise specified, cell passages between 20 and 40 other than specification (approximately 21-25 days in culture) were used in transport studies to ensure the complete enterocyte-like cell differentiation of the Caco-2 cells.

**Western Blotting**

Cells were scraped and subsequently lysed in hypotonic lysis buffer, consisting of 150 mM of NaCl, 50 mM of Tris-HCl (pH 7.4), 1% SDS, 1% Triton X-100 supplemented with protease inhibitors (aprotinin, leupeptin and PMSF) (Yu and Sinko, 1997). Cell lysates were sonicated and centrifuged at 10,000 x g for 2 min. The supernatant was aliquoted and stored at -80°C. Protein levels were determined using the BCA protein assay (Pierce Biotechnology, Rockford, IL). Cell lysates, with and without treatment of reducing agent (7-13% of dithiothreitol) were separated on a 4-12% gradient polyacrylamide gel and subsequently transferred electrophoretically to PVDF membrane. Proteins were hybridized using BXP-21 (1:150 optimal dilution) and HRP-conjugated goat anti-mouse IgG2a (1:1000), and further visualized by enhanced chemiluminescence (ECL) (Amersham Life Science, Piscataway, NJ). The protein bands were semiquantified by NIH image software.
Immunohistochemistry

Cell monolayers cultured on the Transwells™ were washed with PBS, pH 7.4; similar PBS washes were included between each subsequent step. Cell monolayers were fixed in 3.7% (v/v) formaldehyde solution for 10 min at room temperature. Cell membranes were permeabilized by saponin (2%, 2 min at room temperature). Nonspecific binding sites were blocked by incubation in 10% FBS for 45 min. Cells were incubated with anti-human BCRP mAb (BXP-21 or BXP-34 in 1.5% FBS) for 2 h. BCRP staining was revealed by incubation with FITC-conjugated goat anti-mouse antibody for 1 h. The nucleus was stained by propidium iodide for 15 min. After a final wash, cell monolayers were mounted in Vectashield before examination on a Zeiss PASCAL confocal laser scanning system. Control for nonspecific staining was the replacement of BXP-21 or BXP-34 with a nonspecific antibody from the same class, mouse IgG2a.

Transport Studies in Caco-2 cells

Bi-directional transport studies were performed at 37°C in air. Prior to each experiment, the confluent cell monolayers on Transwell™ inserts were washed and equilibrated for 30 minutes with transport media (Hank’s balanced salt solution (HBSS) containing 10 mM of N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid (HEPES) and 10 mM of glucose, pH 7.4). The experiment was initiated by adding a solution containing the test compound to either the apical (for A-to-B transport) or basolateral (for B-to-A transport) compartment. When applicable, inhibitors such as GF120918 (2 µM), prazocin (100 µM), MK571 (50 µM), Ko143 (1 µM), or LY335979 (5 µM) were present.
in the transport medium of the donor side from the preincubation period throughout the permeability study. At 15, 30, 45 and 60 minutes, the sample aliquots of receiving solutions were withdrawn from the basolateral side (for A-to-B transport) or from the apical side (for B-to-A transport), and replaced immediately with an equal amount of fresh transport media except at the 60 min time point (the end of the incubation). The samples were mixed with 5 mL of scintillation cocktail and the radioactivity was determined in a liquid scintillation spectrophotometer (Beckman, Fullerton, CA).

DATA ANALYSIS

The cumulative amount of drug (Q) on the receiver side was plotted as a function of time. The steady-state flux J was then estimated from the slope (dQ/dt). The apparent permeability coefficient (P_{app}) of unidirectional flux for the test compound was estimated by normalizing the flux J (mole/sec), against the nominal surface area A (0.33 cm^2) and the initial drug concentration in the donor chamber C_0 (mol/mL), or P_{app} = J/(A*C_0). The B/A ratio was equal to the P_{app} value for A-to-B transport (P_{app, A-to-B}) divided by the P_{app} value for B-to-A transport (P_{app, B-to-A}). The kinetic parameters for the E3S transport were estimated by fitting the flux against the donor E3S concentration using a non-linear regression with a method of least squares fitting (GraphPad, San Diego, CA).

Data are expressed as mean ± SEM of three individual monolayers. Tests of significances of differences between mean values were made using two-tailed unpaired Student’s t-test. A probability of less than 0.05 (p < 0.05) was considered to be statistically significant.
RESULTS

Expression and Localization of BCRP

BCRP protein expression was determined in Caco-2 cell lysate samples via western blot analysis using BXP-21 as the primary antibody. As shown in Figure 1A, both the half transporter BCRP (~70 kDa) and its dimer (~140 kDa) were observed in lanes 1 and 4, in which the Caco-2 cell lysates were not treated with reducing agent (7-13% of dithiothreitol). Lanes 2, 3, 5, and 6 are the lysates treated with reducing agent which showed a strong band of BCRP monomer (~70 kDa). Lane 6 depicts a trace amount of BCRP dimer when a high concentrated unheated sample was loaded.

When Caco-2 cells were cultured in cell culture T-flasks, the expression of BCRP monomer and dimer in Caco-2 cells was more than 3-fold higher in the late passage (passages 56 (p56) or 59(p59)) than in the early passage (passages 33 (p33) or 36 (p36)) after normalization to the expression of β-actin (Figures 1B and 1C). However, once the Caco-2 cells were grown on the Transwell™ plates, the BCRP monomer expression was about 3-fold higher and the dimer expression was about 10-fold higher in the early passage (p36) than in the late passage (p59). The expression level of BCRP, after normalization to the house keeping protein β-actin, did not change on 5th, 12th and 19th days after the cell seeding on Transwell™ plates (Figure 1B).

Caco-2 cells (p33) cultured on the Transwell™ plates showed positive staining of BCRP in the presence of BXP-21 (upper panel) or BXP-34 (lower panel) under confocal laser-scanning microscopy (Figure 2A). Two days after cell seeding (prior to confluence), BCRP staining was observed not only on both apical and basolateral membranes but also in the cytoplasm. On the 5-day-old culture, cells reached confluence
and showed increased TEER values (indicative of the tight junction formation), BCRP staining was observed only on the apical membrane and there was no detectable staining in the cytoplasm, indicating the localization of BCRP on the apical membrane. The same phenomena were also noted during the cell differentiation period (e.g., 12th day and 19th day after cell seeding). When the late passage cells (p59) were grown on the Transwell™ plates for 12 days, multiple layers were observed as demonstrated by the multiple nuclei staining on the x-z and y-z views (Figure 2B). BCRP staining, however, was still preferentially shown on the apical side of cell membranes (Figure 2B). The absence of any green staining in the cell monolayers with mouse IgG2a and FITC-conjugated second antibody served as a negative control.

**Efflux Function of BCRP**

The function of BCRP as an efflux transporter in Caco-2 cell monolayers was evaluated using a known substrate, E3S (Suzuki et al., 2002; Suzuki et al., 2003). As shown in Table 1, the $P_{\text{app}}$ value of $[^3]$H-E3S (0.02 µM) from B-to-A was $46.1 \times 10^{-6}$ cm/sec and was 8-fold higher than the $P_{\text{app}}$ value ($5.7 \times 10^{-6}$ cm/sec) for A-to-B in the 5-day culture (p33). In 12- and 19-day cultures (p33), the $P_{\text{app}}$ values for A-to-B decreased to 4.1 and $4.5 \times 10^{-6}$ cm/sec, and $P_{\text{app}}$ value for B-to-A increased to 63.0 and $58.0 \times 10^{-6}$ cm/sec, respectively. In the present of Ko143 (a BCRP inhibitor), the A-to-B and B-to-A transport of E3S were almost equal with $P_{\text{app}}$ values around 9 to $11 \times 10^{-6}$ cm/sec. Figure 3 showed the comparison of BCRP-mediated $[^3]$H-E3S (0.02 µM) efflux in both early passage (p36) and late passage (p59) Caco-2 cell monolayers. In the p36, the $P_{\text{app}}$ value for A-to-B transport of E3S was $2.3 \times 10^{-6}$ cm/sec and was 32-fold less than the $P_{\text{app}}$
value for B-to-A transport. The $P_{app}$ value increased to $6.1 \times 10^{-6}$ cm/sec in the presence of Ko143. However, $P_{app}$ values of E3S for A-to-B and B-to-A transports in p59 were 16.2 and $37.1 \times 10^{-6}$ cm/sec, respectively. The difference of the directional E3S transport was abolished by the BCRP inhibitor Ko143. The TEER value was 4-fold higher in p36 than in p59 in the present cell culture system (Figure 3).

When $[3H]$-E3S (0.03 µM) was co-incubated with Ko143 (1 µM), GF120918 (2 µM) (an inhibitor for BCRP and P-gp) or prazocin (100 µM) (an inhibitor for BCRP and P-gp) in Caco-2 cell monolayers, the $P_{app}$ value of E3S for the A-to-B transport increased and the $P_{app}$ value for the B-to-A transport decreased, resulting in the B/A ratio drop from 27 to almost unity (Figure 4). In contrast, the P-gp specific inhibitor LY335979 (5 µM) did not significantly change the directional transport pattern of E3S ($p > 0.05$) (Figure 4). Apical or basolateral presence of 2, 4-DNP at 200 µM (known to inhibit mitochondrial ATP synthesis and deplete cellular ATP (Siekevitz and Potter, 1953)) totally inhibited the efflux of E3S with an increase in the A-to-B transport and a decrease in the B-to-A transport (Figure 4).

Figure 5 showed the unidirectional B-to-A flux ($J_{ba}$) of E3S across cultured Caco-2 cells at various concentrations in the absence or presence of a BCRP inhibitor, Ko143 (1 µM). The net efflux of E3S across Caco-2 cell monolayers in the B-to-A direction, the difference of B-to-A flux in the presence or absence of BCRP inhibitor ($J_{net} = J_{B-to-A} - J_{B(i)-to-A}$), was saturable and followed the Michaelis-Menten kinetics. The estimated apparent $K_m$ and $v_{max}$ were 13.1 µM and 10.8 pmol/sec, respectively.

MTX was chosen to further investigate the BCRP efflux function in the Caco-2 cell monolayers. As shown in Figure 6, the B-to-A transport of MTX was significantly
decreased in the presence of MK571 (50 µM) (a MRP inhibitor), Ko143 (1 µM) and GF120918 (2 µM) (p < 0.05). However, neither $P_{app}$ value for A-to-B nor $P_{app}$ value for B-to-A of MTX was significantly affected after co-incubation with LY335979 (5 µM).

The B-to-A efflux of rhodamine 123, which is a substrate for P-gp and mutant BCRP, was significantly decreased in the presence of LY335979 and GF120918 (p < 0.05), but was not changed by co-incubating with Ko143 (1 µM) (Figure 7).

**DISCUSSION**

Western blot analysis of Caco-2 cell lysates demonstrates the presence of both monomer (~70 kDa) and homodimer (~140 kDa) of BCRP in the Caco-2 cells (Figure 1A). The 140 kDa BCRP complex dissociated to 70 kDa polypeptides in the presence of the reducing agent dithiothreitol indicating that the BCRP dimer was linked by disulfide bonds. This finding is consistent with the recent discovery by Kage et al. (Kage et al., 2002). The same group also demonstrates the necessity of homodimerization for the BCRP function when used a dominant-negative mutation of BCRP with a L554P alteration in the fifth transmembrane domain (Kage et al., 2002).

Immunohistochemical analysis of Caco-2 cells reveals the presence of BCRP on apical and basolateral plasma membranes as well as in the cytoplasm prior to cell confluence (Figure 2). Upon cell confluence, BCRP was sorted to the “apical membrane” consistent with potential intracellular localization and redistribution of BCRP to the plasma membrane shown in BCRP over-expressed MCF-7 AdVp3000 and S1-M1-80 drug-resistant cells (Litman et al., 2000). BCRP transfected MDCK or LLC-PK
epithelial cells also exhibited polarized apical localization of BCRP (Jonker et al., 2000) (Maliepaard et al., 2001), implying that BCRP may contain a certain endogenous sorting signal which was recognized by epithelial cells (Jonker et al., 2000) (Imai et al., 2003). The polarized apical BCRP distribution in Caco-2 cells continues during the cell differentiation period, which is consistent with the proposed role for BCRP as a secretory detoxifying transporter, contributing to the gastrointestinal epithelial barrier. Results from transport function assays using E3S as the BCRP substrate agreed with immunohistochemical study results. The directional transport of E3S across Caco-2 monolayers was observed immediately after cells reached confluency (5-day culture) and continued throughout the differentiation phase (day 12-19 cell culture) (Table 1). In view of the fact that total BCRP protein expression had not changed over day 5, day 12, and day 19 (Figure 1B), the higher A-to-B transport and lower B-to-A transport of E3S across Caco-2 cells on day 5, compared to those on day 12 and day 19, indicated that BCRP continuously redistributed to the apical membrane during the differentiation phase.

Similar to P-gp, MRP, and lung resistant protein (LRP) (Yu and Sinko, 1997), the expression of BCRP is passage dependent. The expression of BCRP increased approximately three fold from the early passage to the late passage when Caco-2 cells were cultured in T-flasks, but decreased about 3-10 fold for monomer and dimer, respectively, from the early passage to the late passage when Caco-2 cells were grown on Transwell™ plates (Figures 1B and 1C). While our findings are novel, they are supported by the previous reports that culture conditions and cell growing matrices may affect efflux pump protein expression (Yu and Sinko, 1997). The BCRP-mediated E3S efflux in the day 12 p59 Caco-2 cells was much lower than that of day 12 p36 Caco-2
cells with the ratio B/A of 2 and 30, respectively (Figure 3), indicating less BCRP expression, particularly the dimer, which is responsible for decreased efflux pump function. The low efflux function of BCRP was also seen in day 19 culture of passages 56, 59 and 72 (data not shown). The increased A-to-B transport of E3S in the p59 was likely due to the decreased tight junctions, reflected by the low TEER value and multiple layers formed as a result of cells grown on Transwell™ plates (Figure 2B).

The polarized expression of BCRP in Caco-2 cells is consistent with the net secretory transport (ratio B/A > 8) of E3S, a prototypical substrate of BCRP. Although there is a good agreement between the morphological and the functional measurements, the vectorial transport of E3S attributed to the BCRP expression in Caco-2 cells was further supported by the reduction of E3S efflux by the BCRP inhibitors Ko143, GF120918 and prazocin (Figure 4). Ko143, a specific inhibitor of BCRP (Allen et al., 2002b), totally inhibited the E3S efflux and resulted in almost equal P_app values for A-to-B and B-to-A of E3S across Caco-2 cell monolayers at the concentration of 1 µM. Under the same experimental conditions, Ko143 did not change the efflux properties of taxol (a P-gp substrate) and vinblastine (a substrate for P-gp and MRP) in Caco-2 cells (data not shown), indicating that Ko143, at the concentration of 1 µM, did not inhibit P-gp and MRP. P-gp and BCRP inhibitors GF120918 and prazocin (Jonker et al., 2000) (Cisternino et al., 2004) completely abolished the efflux of E3S. In contrast, the potent P-gp specific inhibitor, LY335979 (Shepard et al., 2003) did not change A-to-B or B-to-A transport of E3S. Altogether, the modulation of GF120918 and prazocin on the E3S efflux across Caco-2 cells was due to the inhibition of BCRP. The uncoupling agent, 2, 4-DNP, increased the A-to-B transport and decreased the B-to-A transport of E3S,
suggesting that blocking the mitochondrial ATP synthesis and depleting the cellular energy abolished the BCRP efflux activity (Figure 4). Data from the present studies indicated that BCRP mediated efflux of E3S in the Caco-2 cell system and this efflux process was energy dependent. The observed Km of E3S for the BCRP in Caco-2 cells was 13.1 µM (Figure 5), which was close to the Km (16.6 µM) determined by the BCRP vesicle studies (Suzuki et al., 2003), suggesting that Caco-2 cells can be used to assess kinetic constants of BCRP substrates.

The efflux activity of BCRP in Caco-2 cells was further investigated using MTX, a substrate for MRP and BCRP (Volk and Schneider, 2003) (Figure 6). Ko143 (a BCRP inhibitor) and GF120918 (an inhibitor for BCRP and P-gp) but not LY335979 (a P-gp inhibitor) reduced the B-to-A transport of MTX across Caco-2 cell monolayers, implying that BCRP is present in Caco-2 cells. The decreased B-to-A transport of MTX in the presence of MK571 (a MRP inhibitor (Leie et al., 1994)) was due to the inhibition of MRP2. All MRP or BCRP inhibitors affected the secretory transport (B-to-A) of MTX or E3S more than the absorptive transport (A-to-B), suggesting the presence of other active transporter(s), such as OAT or OATP on the basolateral side responsible for the uptake of MTX or E3S (Cha et al., 2001; Nezu et al., 2001; Troutman and Thakker, 2003). Since Ko143 did not inhibit E3S uptake in hOATP2 and hOAT3 expressed xenopus oocytes up to 10 µM (data not shown), Ko143 mediated E3S and MTX efflux in Caco-2 cells could be totally attributed to BCRP.

BCRP mutations have been found in some cancer cell lines (Honjo et al., 2001). The mutation has either threonine (T) or glycine (G), instead of arginine (R), at the amino acid position 482. The R482 of BCRP locates in the third transmembrane domain and
may alter the substrate specificity upon mutation. The BCRP-mediated efflux of MTX in our Caco-2 cells indicated that the BCRP expressed on Caco-2 cells might be the wild type because R482T and R482G mutation were unable to transport MTX to any extent (Chen et al., 2003). The conclusion is further demonstrated by the efflux of rhodamine 123, to which BCRP-conferred resistance is observed in R482T or R482G mutation but not the wild type expressed cell lines (Allen et al., 2002a). In Caco-2 cells, the efflux of rhodamine 123 could be totally abolished by P-gp inhibitors, LY335979 and GF120918, but not affected by BCRP inhibitor, Ko143 (Figure 7).

In conclusion, both BCRP monomer and dimer are expressed in the Caco-2 cells. BCRP is polarized located at the apical side of Caco-2 cells and can efficiently transport its substrate such as E3S and MTX out of cells. Therefore, besides BCRP transfected cell lines, Caco-2 cells can also be used as an in vitro model to study the transport function of BCRP.

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REFERENCE


and fetal penetration of topotecan. *Journal of the National Cancer Institute* 92:1651-1656.


DMD #3442


Yu H and Sinko PJ (1997) The Influence of the Microporous Substratum and Hydrodynamics on Resistances to Drug Transport in Cell Culture Systems:
DMD #3442

LEGENDS FOR FIGURES

Figure 1: Western blotting analysis of BCRP in Caco-2 cell lysate samples. Blots were hybridized using BXP-21 as the primary antibody. BXP-21 did not cross-react with MRP1, MRP2 or MDR1 P-gp. Bands were visualized using ECL. (A) Lanes 1 and 4: samples without reducing agent sat at room temperature for 2 min; lanes 2 and 5: samples with reducing agent heated at 85 °C for 2 min; lanes 3 and 6: samples with reducing agent stayed at room temperature for 2 min. (B) p33 and p55: Caco-2 cells at passages 33 and 55, respectively. FC: 7-day Caco-2 cell culture in T-flasks. TC: Caco-2 cells cultured on Transwell™ plates and collected on day 5 (lane 3), 12 (lane 4) and 19 (lane 5) after seeding. (C) p36 and p59: Caco-2 cells at passages 36 and 59, respectively. FC: 7-day Caco-2 cell culture in T-flasks. TC: 12-day Caco-2 cell culture on Transwell™ plates. Lanes 1, 2, 3 and 4: Cell lysate samples were neither reducing agent nor heat treated. Lanes 5, 6, 7, and 8: Cell lysate samples were incubated with reducing reagent and heated at 85 °C for 2 min.

Figure 2: Immunolocalization of BCRP in the Caco-2 cells cultured on Transwell™ plates by confocal laser-scanning microscopy. (A) Caco-2 cells of p33. BCRP was demonstrated by primary antibodies BXP-21 (upper panel) and BXP-34 (lower panel), and was further identified by the FITC-conjugated anti-mouse IgG2a (green). (B) Caco-2 cells of p59 (cultured for 12 days). The nucleus was stained by propidium iodide (red). Cells in control panel were only treated with mouse IgG2a and 2nd antibody. The upper and right sides of the each image are optical x-z and y-z sections perpendicular to the selected plane (shown in either green or red line on the x-y section image) of the cell.
obtained by confocal laser scanning microscopy. The apical side is toward inside and basal side is toward outside in both x-z and y-z sections.

Figure 3: BCRP-mediated efflux of estrone-3-sulfate (E3S) in Caco-2 cells of passages 36 and 59. Ko143 (an inhibitor for BCRP) was used at the concentration of 1 µM. The transport study was conducted on Caco-2 cell monolayers 12 days after seeding on the Transwell™ Plates. Results obtained from the p36 and p59 Caco-2 cells were labeled as p36 and p59, respectively. (Data expressed as Mean ± SEM, n=3).

Figure 4: The effects of efflux pump inhibitors on the transport of E3S. LY335979 (5 µM), a P-gp specific inhibitor, did not affect the E3S permeability. However, GF120918 (2 µM) (an inhibitor for P-gp and BCRP), Prazocin (100 µM) (an inhibitor for P-gp and BCRP) and Ko143 (1 µM) (a BCRP inhibitor) increased A-to-B transport and decreased B-to-A transport of E3S, indicating that BCRP pumps E3S out of Caco-2 cells. The uncoupling agent 2,4-dinitrophenol (2,4-DNP) changed the ratio of B-to-A over A-to-B of E3S from 26.7 to 0.6, suggesting that blocking the mitochondrial ATP synthesis and depleting the cellular energy abolished the BCRP efflux activity. (Data expressed as mean ± SEM (n = 3)).

Figure 5: B-to-A transport rate of E3S in the presence or absence of Ko143 and net flux of E3S across Caco-2 cells. The efflux of E3S was saturable and the Km of E3S for BCRP in Caco-2 cells was 13.2 µM, which was close to the Km (16.6 µM) determined in BCRP vesicle studies. The v_max was estimated as 10.8 pmol/sec. (Data expressed as mean ± SEM (n = 3)).

Figure 6: The effects of efflux pump inhibitors on the transport of methotrexate (MTX). LY335979 (5 µM), a P-gp specific inhibitor, did not affect the MTX
permeability. However, GF120918 (2 µM) (an inhibitor for P-gp and BCRP), Ko143 (1 µM) (a BCRP inhibitor) and MK571 (50 µM) (a MRP inhibitor) decreased the B-to-A transport of MTX, indicating that both BCRP and MRP mediated the MTX efflux across Caco-2 cells. (Data expressed as mean ± SEM (n = 3)).

**Figure 7: The effects of efflux pump inhibitors on the transport of rhodamine 123.**

LY335979 (5 µM) (a P-gp specific inhibitor) and GF120918 (2 µM) (an inhibitor for P-gp and BCRP) but not Ko143 (1 µM) (a BCRP inhibitor) decreased the B-to-A transport of rhodamine 123, indicating that P-gp but not BCRP mediated the rhodamine efflux across Caco-2 cells. (Data expressed as mean ± SEM (n = 3)).
Table 1: BCRP-mediated efflux of estrone-3-sulfate (E3S) in Caco-2 cells on days 5, 12, and 19 after seeding.

<table>
<thead>
<tr>
<th></th>
<th>A-to-B</th>
<th>B-to-A</th>
<th>ratio (B/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5 days after cell seeding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3H-E3S</td>
<td>5.7 ± 0.3</td>
<td>46.1 ± 1.0</td>
<td>8.1</td>
</tr>
<tr>
<td>3H-E3S+Ko143 1µM</td>
<td>9.2 ± 1.4</td>
<td>8.0 ± 0.3</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>12 days after cell seeding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3H-E3S</td>
<td>4.1 ± 0.3</td>
<td>63.0 ± 1.4</td>
<td>15.6</td>
</tr>
<tr>
<td>3H-E3S+Ko143 1µM</td>
<td>11.2 ± 0.7</td>
<td>11.0 ± 0.9</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>19 days after cell seeding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3H-E3S</td>
<td>4.5 ± 0.1</td>
<td>58.0 ± 0.2</td>
<td>12.8</td>
</tr>
<tr>
<td>3H-E3S+Ko143 1µM</td>
<td>8.7 ± 0.9</td>
<td>6.0 ± 0.4</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM (n = 3)
Figure 1

(A)

<table>
<thead>
<tr>
<th>Loading 1.35 µg of protein</th>
<th>Loading 6.75 µg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

- BCRP dimer
- BCRP
Figure 1
Figure 1
Figure 2 (B)

1st mAB: BXP-21

1st mAB: BXP-34

Control
**Figure 3**

- **Graph:**
  - Label: $P_{\text{app}} \times 10^6$ (cm/sec)
  - Y-axis: 0 to 80
  - X-axis: Various groups of cell passage numbers:
    - $^{3}\text{H-E3S-p36}$
    - $^{3}\text{H-E3S + Ko143-p36}$
    - $^{3}\text{H-E3S-p59}$
    - $^{3}\text{H-E3S + Ko143-p59}$
  - Bars represent $P_{\text{app}}$ for each group:
    - A-to-B (open bars)
    - B-to-A (black bars)

- **Table:**
<table>
<thead>
<tr>
<th>Cell passage No.</th>
<th>P36</th>
<th>P59</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEER (ohm cm$^2$) n=6</td>
<td>461 ± 97</td>
<td>79 ± 12</td>
</tr>
</tbody>
</table>

- Notes: The figure compares the apparent permeability ($P_{\text{app}}$) across different cell passage numbers and treatments, with TEER measurements provided in the table.
Figure 4
Figure 6
Figure 7