DMD Fast Forward. Published on March 19, 2020 as DOI: 10.1124/dmd.119.088674
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Title:

The impact of breast cancer resistant protein (BCRP/ABCG2) on drug transport across Caco-2 cell monolayers

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Running Title Page:

Running Title: Impact of BCRP on drug transport in Caco-2 cells.

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Number of text pages: 42

Number of figures: 7

Number of references: 43

Number of words in abstract: 250 words

Number of words in introduction: 751 words

Number of words in discussion: 1536 words

DMD Fast Forward. Published on March 19, 2020 as DOI: 10.1124/dmd.119.088674
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ABBREVIATIONS: ABC, ATP-binding cassette; AP, apical; AQ, absorptive quotient; AUC, area under the plasma concentration-time curve; BA, bioavailability; BCRP, breast cancer resistance protein; BL, basal; DMEM, Dulbecco's modified Eagles medium; CYP, cytochrome P450; E3S, estrone-3-sulfate; ER, efflux ratio; GF120918, *N*-(4-(2-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl)phenyl)-5-methoxy-9-oxo-9,10-dihydroacridine-4-carboxamide; HBSS, Hank's balanced salt solution; HTS, high-throughput screening; LY, lucifer yellow; LY335979, (*R*)-1-(4-((1a*R*,6s,10b*S*)-1,1-difluoro-1,1a,6,10b-tetrahydrodibenzo[a,e]cyclopropa[c][7]annulen-6-yl)piperazin-1-yl)-3-(quinolin-5-yloxy)propan-2-ol trihydrochloride; MDCK, Madin-Darby canine kidney; MDR1, multidrug resistance protein 1; NCEs, new chemical entities; OATP, organic anion transporting polypeptide; PAMPA, parallel artificial membrane permeation assay; P-gp, P-glycoprotein; TEER, transepithelial electrical resistance; WK-X-34, *N*-(2-{4-[2-(6,7-Dimethoxy-3,4-dihydro-1*H*-isochinolin-2-yl)-ethyl]phenylcarbamoyl}phenyl)-3,4-dimethoxybenzamide.

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ABSTRACT

Breast cancer resistant protein (BCRP) is expressed on the apical membrane of small intestinal epithelial cells and functions as an efflux pump with broad substrate recognition. Therefore, quantitative evaluation of the contribution of BCRP to the intestinal permeability of new chemical entities is very important in drug research and development. In this study, we assessed the BCRP-mediated efflux of several model drugs in Caco-2 cells using WK-X-34 as a dual inhibitor of P-glycoprotein (P-gp) and BCRP and LY335979 as a selective inhibitor of P-gp. The permeability of daidzein was high with an apparent permeability coefficient (P_{AB}) for apical-to-basal transport of 20.3 × 10^{-6} cm/sec. In addition, its efflux ratio (*ER*) was 1.55, indicating that the contribution of BCRP to its transport is minimal. Estrone-3-sulfate and ciprofloxacin showed relatively higher *ER* values (>2.0), whereas their BCRP-related absorptive quotient (AQ_{BCRP}) was 0.21 and 0.3, respectively. These results indicate that BCRP does not play a major role in regulating the permeability of estrone-3-sulfate and ciprofloxacin in Caco-2 cells. Nitrofurantoin showed a P_{AB} of 1.8 × 10^{-6} cm/sec, and its ER was 7.6. However, the AQ_{BCRP} was 0.37, suggesting minimal contribution of BCRP to nitrofurantoin transport in Caco-2 cells. In contrast, topotecan, SN-38, and sulfasalazine had low P_{AB} values (0.81, 1.13, and 0.19 × 10^{-6} cm/sec, respectively), and each AQ_{BCRP} was above 0.6, indicating that BCRP significantly contributes to the transport of these compounds in Caco-2 cells. In conclusion,

Caco-2 cells are useful to accurately estimate the contribution of BCRP to intestinal drug absorption.

Significance statement

We performed an *in vitro* assessment of the contribution of BCRP to the transport of BCRP and/or P-gp substrates across Caco-2 cell monolayers using absorptive quotient (AQ), which has been proposed to represent the contribution of drug efflux transporters to the net efflux. The present study demonstrates that the combined use of a BCRP/P-gp dual inhibitor and a P-gp selective inhibitor is useful to estimate the impact of BCRP and P-gp on the permeability of tested compounds in Caco-2 cells.

INTRODUCTION

The oral route is the most desirable for drug administration because of its convenience, minimal invasiveness, and good patient compliance. The adequate bioavailability of drug candidates is required for them to be manufactured as orally available drugs. Intestinal permeability is an important factor that determines the bioavailability of drugs, and in many cases, it depends on the physicochemical properties of drugs, such as lipophilicity, molecular size, hydrogen bonding, and ionization (Jambhekar and Breem, 2013; Shekhawat and Pokharkar, 2017). However, there are several examples that do not comply with this theory. This is in part because of the involvement of transporters expressed in the intestine (Shekhawat and Pokharkar, 2017). Although various *in silico, in vitro,* and *in vivo* approaches have been developed (Artursson et al., 1991; Fagerholm, 2007; Keldenich, 2009; Larregieu and Benet, 2013; Smith et al., 2014), it is still difficult to predict the oral absorption of new chemical entities (NCEs) in humans, especially for those whose absorption is mediated by transporters.

In the mid-1990s, advances in molecular biology and genomics, such as the development of high-throughput screening (HTS) technology, allowed the identification of several new biological targets. For the most part, drug candidates obtained from HTS have poor physicochemical properties, including high molecular weight, high lipophilicity, and low solubility (Lipinski, 2000), which limit their use as orally administered drugs. Moreover, they

tend to be recognized by drug efflux transporters, such as P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), which are expressed in intestinal epithelial cells. We previously demonstrated the contribution of P-gp to the intestinal absorption of drugs using Caco-2 cell monolayers and mdr1a/1b knockout mice (Fujita *et al.*, unpublished observation).

The BCRP expression level in human intestines has been reported to be equal to or even higher than that of multidrug resistance protein 1 (MDR1) (Doyle et al., 1998; Taipalensuu et al., 2001; Hilgendorf et al., 2007). BCRP is a member of the ATP-binding cassette (ABC) family of transporters. BCRP was discovered in multidrug-resistant cancer cells (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999), but it is also expressed in normal cells, such as in the placenta, liver, brain, and intestine (Maliepaard *et al.*, 2001). Because BCRP has one ATP-binding cassette and six transmembrane domains, it is known as a half-ABC transporter, which forms homodimers to create a functional transporter (Kage et al., 2002; Takada et al., 2005). BCRP and P-gp show broad substrate specificity for various endogenous substrates (bile acids, porphyrins, and folates), sulfate conjugates (estrone-3-sulfate), and anticancer drugs (mitoxantrone and topotecan) (Suzuki et al., 2003; Mao and Unadkat, 2005; Xiao et al., 2006). The role of BCRP in drug disposition in humans has been demonstrated in clinical studies. The oral bioavailability of topotecan in cancer patients increased from 40% to 97% by co-administration of GF120918, a dual inhibitor of P-gp and BCRP (Kruijtzer et al., 2002),

indicating that the role of BCRP in drug intestinal absorption cannot be ignored. The analysis of expression systems is useful to determine whether or not each drug is a substrate for BCRP, but it is difficult to evaluate the extent of its contribution to intestinal absorption. Therefore, it is necessary to quantitatively assess the impact of BCRP and P-gp on the intestinal absorption of drugs.

The Caco-2 cell line is widely used as an *in vitro* intestinal model system to evaluate the intestinal permeability of NECs. Importantly, Caco-2 cells exhibit many of the morphological and functional properties of human intestinal epithelial cells. In addition to metabolic enzymes (Fisher et al., 1999; Siissalo et al., 2008), they express many nutrient transport systems, such as various amino acid transporters (Frølund et al., 2010; Nielsen et al., 2012; Voigt et al., 2013), water-soluble vitamin transporters (Wang et al., 1999; Ramanathan et al., 2001), and the H^+-coupled di/tripeptide transporter (PEPT1) (Dantzig and Bergin, 1990; Thwaites et al., 1994). Various ABC transporters, such as P-gp and BCRP, are also expressed in Caco-2 cells (Volpe., 2008; Taipalensuu et al., 2001).

In the present study, we assessed the potential of efflux transporter inhibitors using paclitaxel and mitoxantrone. Among efflux transporter inhibitors, GF120918 and WK-X-34 are dual inhibitors of P-gp and BCRP, and LY335979 is a selective inhibitor of P-gp. Seven model compounds were selected to estimate the impact of BCRP in Caco-2 cells. The contribution of

BCRP to the permeability of tested compounds across Caco-2 cell monolayers, which express both ABC transporters, was assessed by using the subtraction of permeability method in the presence of a dual inhibitor or a P-gp selective inhibitor.

MATERIALS AND METHODS

Chemicals and reagents

Caffeine, Sepazol-RNA I Super®, Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic mixed stock solution (100X), and 0.25% trypsin/1 mM EDTA solution were purchased from Nacalai Tesque (Kyoto, Japan). Hank's Balanced Salt Solution (HBSS), daidzein , sulfasalazine, and *N*-(4-(2-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl)phenyl)-5-methoxy-9-oxo-9,10-dihydroacridine-4-carboxamide (GF120918; elacridar) were obtained from Sigma-Aldrich (St. Louis, MO). SN-38 was purchased from Wako Pure Chemicals (Osaka, Japan). Ciprofloxacin was purchased from LKT Laboratories, Inc. (St. Paul, MN). Nitrofurantoin was purchased from MP Biomedicals, Inc. (Tokyo, Japan). Topotecan HCl was purchased from ALEXIS CORPORATION (Lausen, Switzerland). *N*-(2-{4-[2-(6,7-Dimethoxy-3,4-dihydro-1*H*-isochinolin-2-yl)-ethyl]phenylcarbamoyl}phenyl)-3,4-dimethoxybenzamide (WK-X-34) was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan).

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(*R*)-1-(4-((1a*R*,6s,10b*S*)-1,1-difluoro-1,1a,6,10b-tetrahydrodibenzo[a,e]cyclopropa[c][7]annulen-6-yl)piperazin-1-yl)-3-(quinolin-5-yloxy)propan-2-ol trihydrochloride (LY335979; zosuquidar) was purchased from Funakoshi (Tokyo, Japan). D-[1-^{14}C]Mannitol (specific radioactivity: 8 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). [6,7-^{3}H(*N*)]Estrone sulfate, ammonium salt (specific radioactivity: 57.3 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). The Platinum® Quantitative PCR SuperMix-UDG was purchased from Thermo Fisher Scientific K.K. (Kanagawa, Japan). Real-time (RT)-PCR primer sets were obtained from Eurofin Genomics (Louisville, KY). RT (5X) buffer, dNTP mix, random primers, oligos(dT)$_{20}$, and ReverTra Ace were purchased from TOYOBO (Osaka, Japan). The Transwell® membrane was purchased from Corning (Corning, NY). Other chemicals used were of the highest purity available.

Cell culture

The human colon adenocarcinoma cell line Caco-2 was obtained from DS Pharma Biomedical (Osaka, Japan). Caco-2 cells were maintained by serial passage in 75 cm^2 plastic culture flasks. Caco-2 cells were routinely cultured with DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Life Technologies, Waltham, USA), 1% non-

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essential amino acids (Nacalai Tesque), and 100 U/mL penicillin G and 100 μg/mL streptomycin (Fujita et al., 1999; Kono et al, 2017). Cells were passaged once they reached approximately 80% confluency using 0.02% EDTA and 0.05% trypsin. For transport experiments, Caco-2 cells were seeded on a polyethylene terephthalate insert (0.4 μm pore size, 12 mm diameter) at a density of 1.0×10^5 cells/well and cultured for 3 weeks. The medium was replaced once every 3 days.

Real-time RT-PCR

Total RNA was isolated from Caco-2 cells after 3 weeks of culture using the Sepasol RNA I reagent according to the manufacturer's instructions. Gene-specific RT-PCR was performed using total RNA that was isolated from each cell culture using a master mix based on the Platinum® Quantitative PCR SuperMix-UDG, primer pairs, and Taqman probes in a Taqman 7500 Sequence Detector according to the manufacturer's protocol. Data were normalized to the amount of GAPDH mRNA in each sample. The primers used for RT-PCR are listed in Supplementary Table 1.

Transport study

The confluent Caco-2 cells grown on Transwell membranes were washed with HBSS,

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followed by the addition of HBSS with or without the indicated concentration of inhibitors to both the apical (AP) and basal (BL) sides of the cell monolayer. After preincubation for 10 min, the experiment was initiated by replacing the HBSS at either the AP or BL side of the cell monolayer with HBSS containing each model compound and the corresponding inhibitor. The cells were incubated at 37°C, and an aliquot of the medium was collected from each compartment at specified times. The amount of each model compound in the samples was measured. In addition, paracellular flux was monitored by the appearance of [^{14}C]mannitol in the acceptor compartment.

Analytical methods

To measure the radiolabeled compounds ([^{3}H]estrone-sulfate and [^{14}C]mannitol), the samples were transferred to counting vials, mixed with a scintillation cocktail (Clearsol I; Nacalai Tesque), and placed in a liquid scintillation counter (LS-6500; Beckman Instruments, Fullerton, CA). Other compounds were analyzed by an HPLC system (Shimadzu LC-10AS pump, Shimadzu SIL-10A autosampler) equipped with a reverse-phase column (COSMOSIL 5C_{18}-AR-II, 3.5 μm inner diameter, 4.6×150 mm). The flow rate was 1.0 mL/min. The compositions of mobile phases were as follows: caffeine, 10 mM phosphate buffer (pH 3.0) with methanol (75:25, v/v); daidzein, 0.1% formic acid with acetonitrile (73:27, v/v);

nitrofurantoin, 10 mM phosphate buffer (pH 3.0) with acetonitrile (83:17, v/v); sulfasalazine, 5 mM phosphate buffer (pH 6.0) with acetonitrile (78:22, v/v); ciprofloxacin, 10 mM formate buffer (pH 3.0) with methanol and acetonitrile (82:9:9, v/v); SN-38, 10 mM phosphate buffer (pH 3.0) with acetonitrile (74:26, v/v); and topotecan, 10 mM phosphate buffer (pH 3.7) with methanol (76:24, v/v). Caffeine, daidzein, nitrofurantoin, and sulfasalazine were detected by absorbance at 273 nm, 250 nm, 366 nm, and 357 nm, respectively, using a Shimadzu SPD-20A UV spectrophotometric detector. Ciprofloxacin, SN-38, and topotecan were analyzed by measuring fluorescence with a Shimadzu RF-10A XL fluorescence detector. Ciprofloxacin, (excitation: 280 nm, emission: 460 nm); SN-38 (excitation: 380 nm, emission: 550 nm); and topotecan, (excitation: 361 nm, emission: 527 nm).

Data analysis

The apparent permeability coefficient (P) was calculated using Eq.1,

$$P=\Delta Q/\Delta t \times 1/\mathrm{A} \cdot C_0 \qquad (1)$$

where $\Delta Q/\Delta t$ is the transported flux of a compound, A is the surface area of the porous membrane (1.13 cm^2), and C_0 is the initial concentration of a test compound added to the donor compartment.

The inhibitory potency for BCRP was determined for each inhibitor. The following equation

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(Eq.2) was fitted to the collected permeability data:

$$P_{BA} = \text{Range}/[1+(C/IC_{50})^{\gamma}] + \text{Background} \quad (2)$$

where P_{BA} is the P value for BL-to-AP transport, C is the concentration of an inhibitor, γ is the Hill coefficient, Range is the arithmetic difference of the P_{BA} value between complete inhibition and in the absence of inhibitors, Background is the P_{BA} in the absence of inhibitors, and IC_{50} is the inhibitor concentration that achieves 50% inhibition.

The efflux ratio (*ER*), a parameter that is widely used as an index of efflux transporter activity, was calculated using Eq.3:

$$ER=P_{BA}/P_{AB} \quad (3)$$

where P_{AB} is the P value for AP-to-BL transport.

Absorptive quotient (*AQ*):

AQ has been proposed as an experimental parameter to quantify the contribution of P-gp to intestinal absorption (Troutman and Thakker, 2003). *AQ* was calculated according to the following equation:

$$AQ = (P_{AB,+inhibitor} - P_{AB}) / P_{AB,+inhibitor} \quad (4)$$

where $P_{AB,+inhibitor}$ is the P_{AB} determined in the presence of an inhibitor.

Based on the above theory and Tachibana's report (2010), we determined the *AQ,* which was recently proposed to represent the contribution of efflux transporters to the net efflux, using

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Eqs.5-7,

$$AQ_{efflux} = \frac{P_{AB,+WK-X-34} - P_{AB}}{P_{AB,+WK-X-34}} = \frac{P_{P-gp} + P_{BCRP}}{P_{AP,eff} + P_{BL,eff} + P_{P-gp} + P_{BCRP}} \quad (5)$$

$$AQ_{P-gp} = \frac{P_{AB,+LY335979} - P_{AB}}{P_{AB,+WK-X-34}} = \frac{(P_{AP,eff} + P_{BL,eff}) \cdot P_{P-gp}}{(P_{AP,eff} + P_{BL,eff} + P_{P-gp} + P_{BCRP}) \cdot (P_{AP,eff} + P_{BL,eff} + P_{BCRP})} \quad (6)$$

$$AQ_{BCRP} = \frac{P_{AB,+WK-X-34} - P_{AB,+LY335979}}{P_{AB,+WK-X-34}} = \frac{P_{BCRP}}{P_{AP,eff} + P_{BL,eff} + P_{BCRP}} \quad (7)$$

where AQ_{efflux} is both P-gp- and BCRP-related AQ, $AQ_{P\text{-}gp}$ is P-gp-related AQ, AQ_{BCRP} is BCRP-related AQ, $P_{AP,\ eff}$ is the passive permeability across the AP membrane in a cellular-to-AP direction, $P_{BL,\ eff}$ is the passive permeability across the BL membrane in a cellular-to-BL direction, $P_{\text{p-gp}}$ is the permeability due to P-gp-mediated efflux activity, and P_{BCRP} is the permeability due to BCRP-mediated efflux activity (Fig. 1).

Statistical analysis

Analysis of variance (ANOVA) was used to test the statistical significance of differences between groups. Multiple comparisons between control and other groups were performed with a Dunnett's test.

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RESULTS

Effects of inhibitors on the BL-to-AP transport of P-gp and BCRP substrates

We evaluated the inhibitory effect of GF120918, WK-X-34, and LY335979 on the BL-to-AP transport of paclitaxel (P-gp substrate) and mitoxantrone (BCRP substrate) across Caco-2 cell monolayers to identify the most potent inhibitor of P-gp and BCRP. Prior to transport studies, we determined the gene expression of *MDR1, BCRP,* and *MRP2* in Caco-2 cells, and the expression level of *BCRP* was higher than that of *MDR1* (data not shown). This gene expression pattern in Caco-2 cells was comparable to what is observed in the human small intestine. The cytochrome P450 (*CYP) 3A4* gene was also expressed in Caco-2 cell monolayers, although the expression level was not as high as that in human intestines. As shown in Fig. 2, the transport of paclitaxel and mitoxantrone was inhibited by GF120918 in a concentration-dependent manner with IC_{50} values of 239 nM and 298 nM, respectively. In addition, the respective IC_{50} values of WK-X-34 were 501 nM and 370 nM, and thus both GF120918 and WK-X-34 were identified as a dual inhibitor of P-gp and BCRP. In particular, the IC_{50} value of WK-X-34 for mitoxantrone transport was lower than that for paclitaxel. In addition, it has been reported that WK-X-34 has no inhibitory effect on MRP2-mediated transport of 5-carboxyfluorescein diacetate in MRP2-expressing Madin-Darby canine kidney (MDCK) cells (Jekerle et al., 2006, 2007). Taking these findings into consideration, WK-X-34 inhibits BCRP

more selectively than P-gp and MRP2. In contrast, the IC_{50} value of LY33579 for paclitaxel was 107 nM, whereas its inhibitory effect on mitoxantrone transport was minimal (IC_{50} > 10 μM). Therefore, these results indicate that LY335979 is a selective inhibitor of P-gp. Based on these results, WK-X-34 at 10 μM was used as an inhibitor of both BCRP and P-gp, and LY335979 at 1 μM was used as a selective P-gp inhibitor.

Assessment of the contribution of BCRP to the transport of BCRP substrates across Caco-2 cell monolayers

To determine the contribution of BCRP and P-gp to the permeability of drugs across Caco-2 cell monolayers, we conducted a transport study in the presence of WK-X-34 and LY335979. Initially, we assessed the effect of WK-X-34 and LY335979 on the transport of caffeine and mannitol, which permeate only by passive diffusion. The P_{AB} and P_{BA} values of both caffeine and mannitol did not change in the presence of WK-X-34 and LY335979 (Fig. 3). Next, we investigated the transport of topotecan and estrone-3-sulfate (E3S), which are typical substrates of BCRP, across Caco-2 cell monolayers. Topotecan exhibited an *ER* of 12.7, indicating the involvement of efflux transporters in its transport (Fig. 4A). Moreover, the transport of topotecan was not inhibited in the presence of LY335979, whereas its AP-to-BL transport was increased, and its BL-to-AP transport was decreased in the presence of WK-X-

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34. These results indicate that topotecan is a favored BCRP-specific substrate. Furthermore, E3S exhibited an *ER* of 2.1 (Fig. 4B), suggesting the involvement of efflux transporters. However, its absorptive permeability was relatively high (P_{AB} = 9.88×10^{-6} cm/sec), which was not changed by the addition of WK-X-34. These results suggest that the contribution of BCRP to the absorptive transport of E3S is low. In contrast, the BL-to-AP permeability of E3S was significantly decreased in the presence of WK-X-34, indicating that BCRP plays a role in the secretory transport of E3S in Caco-2 cells.

Assessment of the contribution of BCRP to the transport of model drugs across Caco-2 cell monolayers

We investigated the contribution of BCRP to the permeability of daidzein, ciprofloxacin, nitrofurantoin, SN-38, and sulfasalazine in Caco-2 cells. The absorptive permeability of daidzein was high (P_{AB} = 20.5 ×10^{-6} cm/sec) and saturable (Fig. 5A). Moreover, its *ER* was 1.5, and the effect of inhibitors on daidzein transport was not observed. These data suggest that the contribution of BCRP to daizdein transport is minimal in Caco-2 cell monolayers. The *ER* of ciprofloxacin was 2.3 (Fig. 5B), suggesting the involvement of efflux transporters in its transport. However, the calculated AQ_{efflux} was 0.15. These results show that P-gp and BCRP do not play a major role in the transport of ciprofloxacin. In contrast, nitrofurantoin exhibited

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an *ER* of 7.6, and its AQ_{efflux} was 0.46 (Fig. 6A). Therefore, these data indicate the contribution of efflux transporters to the transport of nitrofurantoin in Caco-2 cell monolayers. We also determined that the AQ_{BCRP} and $AQ_{P\text{-}gp}$ of nitrofurantoin were 0.37 and 0.09, respectively. This indicates that P-gp hardly contributes to nitrofurantoin transport. In the case of SN-38 and sulfasalazine, their *ERs* were 6.73 and 71.6, respectively (Figs. 6B and C). This indicates that the transport of both drugs, particularly sulfasalazine, is mediated by efflux transporters. Moreover, the Caco-2 permeability of SN-38 and sulfasalazine were not changed in the presence of LY33579 ($AQ_{P\text{-}gp}$ = 0 and 0.10, respectively), indicating that their transport is not mediated by P-gp. However, the transport of SN-38 and sulfasalazine were significantly increased in the presence of WK-X-34, and their $AQ_{P\text{-}gp}$ values were 0.74 and 0.59, respectively. These results demonstrate that BCRP greatly affects the permeability of SN-38 and sulfasalazine across Caco-2 monolayers.

DISCUSSION

In the present study, we investigated the BCRP-mediated efflux of nine compounds with various physicochemical properties in Caco-2 cells to determine the contribution of BCRP to the intestinal absorption of drugs.

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Using this Caco-2 cell monolayer, we showed that the permeability of daidzein in the absorptive direction was quite high (P_{AB} = 20.3×10^{-6} cm/sec) and it was slightly increased in the presence of WK-X-34 ($P_{AB,+WK-X-34}$ = 29.6×10^{-6} cm/sec) (Fig. 5A, Table 1). Kobayashi et al. (2013) reported that the AP-to-BL transport of daidzein across Caco-2 cells was increased by approximately 1.7-fold in the presence of ES3 as a BCRP substrate. This result is consistent with the present observation. These findings suggest a minimal contribution of BCRP to the transport of daidzein in Caco-2 cells. The relatively low AQ_{BCRP} and *ER* values of daidzein (0.36 and 1.55) also support our findings. However, Enokizono et al. (2007) demonstrated that the area under the curve (AUC) value of daidzein after its oral administration was 3.7-fold higher in Bcrp(-/-) mice compared with wild-type mice. Similar results were reported by Álvarez et al. (2011) and Ge et al. (2017). These reports indicated that BCRP is involved in the intestinal absorption of daidzein in mice. However, the AUC of daidzein after intravenous administration was also increased in Bcrp(-/-) mice. Taking these studies into consideration, we suggest that BCRP is responsible for the elimination of daidzein from the systemic circulation rather than its intestinal absorption.

Our results suggested that the transport of E3S and ciprofloxacin also involved efflux transporters because their *ER* values were above 2.0 (Figs. 4B and 5B, Table 1). The AQ_{BCRP} value of E3S was 0.21, indicating that BCRP slightly contributes to its permeability across

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Caco-2 cells. Gram et al. (2009) showed that the P_{AB} value of E3S in Caco-2 cells increased by 1.4-fold in the presence of fumitremorgin C, a BCRP inhibitor. This finding is similar to our present result. Sai et al. (2006) demonstrated that the uptake of E3S into Caco-2 cells was mediated by organic anion transporting polypeptide (OATP) 2B1 expressed in the apical membrane. It has also been reported that E3S transported into cells by OATP2B1 is exported to the outside of cells by BCRP (Grube et al., 2007). In the present study, since the permeability of E3S in the absorptive direction was relatively high (P_{AB} = 9.88×10^{-6} cm/sec) and its permeability in the absorptive direction was superior to that in the secretory direction in the presence of WK-X-34 ($P_{AB,+WK\text{-}X\text{-}34} = 11.3$, $P_{BA,+WK\text{-}X\text{-}34} = 7.31\times10^{-6}$ cm/sec), it is assumed that OATP2B1 and BCRP functionally interact during the transport of E3S in Caco-2 cells. The AQ_{BCRP} value of ciprofloxacin was 0.03, suggesting that BCRP is not a major transporter of ciprofloxacin in Caco-2 cells. Merinoa et al. (2006) demonstrated that Ko143, a potent and specific BCRP inhibitor, did not affect the AP-to-BL transport of ciprofloxacin in BCRP-expressing MDCKII cells. This observation supports our present result. However, they have also shown that Ko143 decreased the BL-to-AP transport of ciprofloxacin. Since we observed that the permeability of ciprofloxacin in the secretory direction in the presence of WK-X-34 was lower than that in the presence of LY335979 ($P_{BA,+WK\text{-}X\text{-}34} = 4.44\times10^{-6}$ cm/sec, $P_{BA,+LY335979}$ = 5.80×10^{-6} cm/sec), the present results are consistent with the observations reported by

Merinoa et al. In addition, our findings are also in line with Haslam's report (2011), which showed that Ko143 reduced the secretory net flux of ciprofloxacin in high-passage Caco-2 cells (115-120 passages), but had no significant impact in low-passage Caco-2 cells (34-40 passages). In addition, ciprofloxacin has also been reported to be a substrate for influx transporters, including OATP1A2 (Dautrey et al., 1999; Maeda et al., 2007; Lu et al., 2018). Moreover, its fraction absorbed in human intestines was estimated to be 70%-100% (Drusano et al., 1986). In addition, the plasma concentrations of ciprofloxacin after oral and intravenous injection in wild-type mice were 2-fold and 1.5-fold higher than that in Bcrp(-/-) mice, respectively. Taking these findings into consideration, the contribution of BCRP would be higher in the elimination process of ciprofloxacin compared with its intestinal absorption.

Nitrofurantoin showed P_{AB} and *ER* values of 1.8×10^{-6} cm/sec and 7.6, respectively, suggesting the involvement of efflux transporters (Fig. 6A, Table 1). However, its AQ_{BCRP} was relatively low (0.37). Moreover, the *ER* value of nitrofurantoin was above 3, even in the presence of WK-X-34. It has been reported that the transepithelial secretion of nitrofurantoin in Caco-2 cells was inhibited by 92% in the presence of Ko143 due to a decrease in BL-to-AP flux and an increase in AP-to-BL flux (Wright et al., 2011). Similar results were obtained in BCRP-expressing MDCKII cells (Merino et al., 2005). Therefore, we suggest that WK-X-34 has a low affinity toward nitrofurantoin. In summary, it remains unclear whether BCRP acts as

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a barrier for nitrofurantoin permeability in Caco-2 cells.

The permeability of topotecan, SN-38, and sulfasalazine in the absorptive direction was lower than other compounds (P_{AB} = 0.81, 1.31, and 0.19×10^{-6} cm/sec, respectively) (Figs. 4A, 6B,C, Table 1). Moreover, each AQ_{BCRP} value was above 0.6. These results demonstrate that BCRP significantly contributes to the transport of these drugs in Caco-2 cell monolayers. Matsuda et al. (2013) demonstrated that the intestinal availability of topotecan and sulfasalazine after oral administration in rats was increased by approximately 4-fold following the pre-administration of Ko143. Moreover, Dahan et al. (2009) showed that the BCRP inhibitors fumitremorgin C and pantoprazole significantly increased the AP-to-BL transport of sulfasalazine. Our present results are in agreement with these findings. In addition, they also demonstrated that BCRP inhibitors decrease the BL-to-AP transport of sulfasalazine in Caco-2 cells. We observed that the permeability of sulfasalazine in the secretory direction in the presence of WK-X-34 was much lower than that in the presence of LY335979 ($P_{BA,+WK\text{-}X\text{-}34}$ = 6.45, $P_{BA,+LY335979}$ = 15.4×10^{-6} cm/sec), and therefore, our results are consistent with Dahan's previous observation. With respect to sulfasalazine, it has been reported that Caco-2 cells express OATP2B1 and that sulfasalazine is a substrate of OATP2B1 (Sai et al., 2006; Kusuhara et al., 2012). Therefore, sulfasalazine would be taken up from the AP side of the Caco-2 cell monolayer by OATP2B1, although the membrane permeability of sulfasalazine is low because

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of its hydrophilicity. In addition, we also confirmed that the intracellular amount of sulfasalazine when it was added to the BL side of the Caco-2 cell monolayer was much higher than when it was added to the AP side (14.0 nmol/mg protein vs 6.62 nmol/mg protein). This result suggests that the BL uptake of sulfasalazine may also involve transporters.

In contrast, the $AQ_{\text{P-gp}}$ values of topotecan, SN-38, and sulfasalazine were less than 0.1. This result suggests that P-gp is not a major contributor to their permeability in Caco-2 cell monolayers. However, according to Eq.7, the $AQ_{\text{P-gp}}$ was actually determined by the following equation when the transport studies were performed in the presence of a BCRP selective inhibitor, such as Ko143:

$$AQ_{P-gp} = \frac{P_{P-gp}}{P_{AP,eff}+P_{BL,eff}+P_{P-gp}} \quad (8)$$

Since the $AQ_{\text{P-gp}}$ determined by Eq.6 included P_{BCRP} in the denominator, the $AQ_{\text{P-gp}}$ values calculated in this study were likely underestimated. Although the drugs used in this study were BCRP-specific substrates, and therefore, the underestimation of the $AQ_{\text{P-gp}}$ values would not be highly significant, it is necessary to consider this when determining the $AQ_{\text{P-gp}}$ of drugs that are substrates for both P-gp and BCRP.

To date, several BCRP selective inhibitors have been developed and used for investigating the influence of BCRP on drug permeation across intestinal epithelial cells *in vitro*. Particularly, Ko143 is a widely used and valuable inhibitor for determining the contribution of BCRP to drug

transport in Caco-2 cells. However, our method, together with the use of BCRP selective inhibitors, can also be used to accurately evaluate the contribution of BCRP to *in vitro* drug permeability. In addition, the present method is capable of determining the role of not only BCRP but also P-gp in drug transport. Therefore, we consider that our present method using *AQ* provides a useful *in vitro* approach to predict the contribution of BCRP and P-gp to drug absorption.

In conclusion, we summarized the relationship between P_{AB} and AQ_{BCRP} of the compounds used in the present study (Fig. 7). The compounds, which are known substrates for BCRP, showed various P_{AB} and AQ_{BCRP} values. In particular, the higher contribution of BCRP to the permeability of compounds was observed when the P_{AB} value was less than 1.0×10^{-6} cm/sec. Regarding the transport of E3S and ciprofloxacin, which have relatively high P_{AB} values, we suggest that influx transporters, such as OATPs, cooperatively function with BCRP. For these substrates whose transport involves more than two transporters, it is difficult to determine the extent of the contribution of each transporter in an overexpression study. In contrast, Caco-2 cell monolayers would enable the accurate estimation of the contribution of respective transporters in intestinal permeability. In addition, with respect to drug-drug interactions (DDI), the Food and Drug Administration released a draft guidance for the industry in 2017, which describes the importance of determining whether the investigated drug is a substrate of P-gp

and BCRP to evaluate its DDI potential. Thus, evaluating the P-gp and BCRP-mediated drug interactions is a key process for drug development, and our present approach using Caco-2 cell monolayers may also be useful to predict DDI.

Acknowledgments

We would like to thank Dr. Kenji Kusumoto, Mr. Ikuo Nomura, Mr. Kohei Shinozaki and Mr. Naoki Shiota (Kyoto Pharmaceutical University) for their technical assistance. This study was supported by a Grant from the Strategic Research Foundation at Private Universities and Grant-in-Aids for Scientific Research (C) [17K08430] from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Ritsumeikan Global Innovation Research Organization (R-GIRO) Project at Ritsumeikan University. We thank Melissa Crawford, PhD, from Edanz Group (https://en-author-services.edanzgroup.com/) for editing a draft of this manuscript.

Authorship Contributions

Participated in research design: Kawahara, Yamamoto, Fujita.

Conducted experiments: Kawahara, Nishikawa, Fujita.

Contributed reagents or analytic tools: Nishikawa, Kono, Fujita.

Performed data analysis: Kawahara, Nishikawa, Fujita.

Wrote or contributed to the writing of the manuscript: Kawahara, Kono, Fujita.

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Figure Legends

Figure 1. Schematic diagrams for calculating *AQ* using WK-X-34 and LY335979. Models for AP-to-BL permeability considering the intrinsic influx and efflux clearance. (A) Without inhibitors, (B) with WK-X-34, and (C) with LY335979.

Figure 2. Inhibitory effects of LY335979, WK-X-34, and GF120918 on P-gp and BCRP activity in Caco-2 cell monolayers. Concentration-dependent inhibitory effects of LY335979, WK-X-34, or GF120918 on the BL-to-AP transport of paclitaxel (A) and mitoxantrone (B) in Caco-2 cell monolayers.

Figure 3. Bidirectional transport of caffeine (A) and mannitol (B) across Caco-2 cell monolayers. The AP-to-BL and BL-to-AP transport of caffeine (5 μM) and mannitol (5 μM) in the absence or presence of WK-X-34 or LY335979 in Caco-2 cell monolayers. Data are represented as means ± S.D. of 3 experiments.

Figure 4. Bidirectional transport of topotecan (A) and estrone-3-sulfate (B) across Caco-2 cell monolayers. The AP-to-BL and BL-to-AP transport of topotecan (5 μM) and estrone-3-sulfate (5 μM) in the absence or presence of WK-X-34 or LY335979 in Caco-2 cell monolayers.

Data are represented as means ± S.D. for 3 experiments.

Figure 5. Bidirectional transport of daidzein (A) and ciprofloxacin (B) across Caco-2 cell monolayers. The AP-to-BL and BL-to-AP transport of daidzein (5 μM) and ciprofloxacin (5 μM) in the absence or presence of WK-X-34 or LY335979 in Caco-2 cell monolayers. Data are represented as means ± S.D. for 3 experiments.

Figure 6. Bidirectional transport of nitrofurantoin (A), SN-38 (B), and sulfasalazine (C) across Caco-2 cell monolayers. The AP-to-BL and BL-to-AP transport of nitrofurantoin (5 μM), SN-38 (5 μM), and sulfasalazine (5 μM) in the absence or presence of WK-X-34 or LY335979 in Caco-2 cell monolayers. Data are represented as means ± S.D. for 3 experiments.

Figure 7. The relationship between P_{AB} and AQ_{BCRP} of selected compounds. *Key:* 1, caffeine; 2, mannitol; 3, daidzein; 4, estrone-3-sulfate; 5, ciprofloxacin; 6, nitrofurantoin; 7, topotecan; 8, SN-38; 9, sulfasalazine; 10, mitoxantrone.

TABLE 1

Apparent permeability coefficient, efflux ratio, and absorptive quotient values for the absorptive and secretory transport of model compounds across Caco-2 cell monolayers

compound	P_{AB}			AQ_{efflux}	AQ_{BCRP}	$AQ_{P\text{-}gp}$	P_{BA}			ER
	control	+WK-X-34	+LY335979				control	+WK-X-34	+LY335979	
	(x 10^{-6} cm/s)						(x 10^{-6} cm/s)			
1. caffeine	33.2 ± 4.66	32.4 ± 0.90	28.2 ± 0.43	0.00	0.13	0.00	36.4 ± 3.98	42.6 ± 1.80	41.3 ± 0.31	1.10
2. mannitol	0.46 ± 0.09	0.61 ± 0.18	0.49 ± 0.13	0.24	0.20	0.04	0.41 ± 0.10	0.44 ± 0.11	0.46 ± 0.17	0.89
3. daidzein	20.1 ± 1.43	29.6 ± 3.30	18.9 ± 1.30	0.36	0.36	0.00	25.1 ± 1.26	36.2 ± 1.90	26.2 ± 0.45	1.55
4. estrone-3-sulfate	9.88 ± 0.38	11.3 ± 2.86	8.97 ± 0.34	0.21	0.21	0.00	20.3 ± 3.09	7.31 ± 1.94*	17.8 ± 0.03	2.06
5. ciprofloxacin	2.80 ± 0.58	3.31 ± 0.52	3.22 ± 0.40	0.15	0.03	0.13	6.57 ± 1.39	4.44 ± 0.35*	5.80 ± 0.41	2.35
6. nitrofurantoin	1.81 ±0.18	3.36 ± 0.00**	2.11 ± 0.68	0.46	0.37	0.09	13.8 ± 1.01	10.6 ± 1.95	14.2 ± 0.25	7.60
7. topotecan	0.81 ± 0.06	3.23 ± 0.56**	1.25 ± 0.04	0.75	0.61	0.14	10.3 ± 1.53	3.41 ± 0.52**	8.33 ± 0.83	12.7
8. SN-38	1.31 ± 0.50	4.96 ± 0.31*	1.29 ± 0.06	0.74	0.74	0.00	8.83 ± 3.80	4.43 ± 0.16	5.90 ± 0.29	6.73
9. sulfasalazine	0.19 ± 0.04	0.61 ± 0.13**	0.25 ± 0.01	0.70	0.59	0.10	13.3 ± 0.95	6.45 ± 1.34**	15.4 ± 6.47	71.6

Results are expressed as the mean ± SD of 3-6 experiments. *$p < 0.05$; **$p < 0.01$, compared with the corresponding control group.

Figures

Figure 1

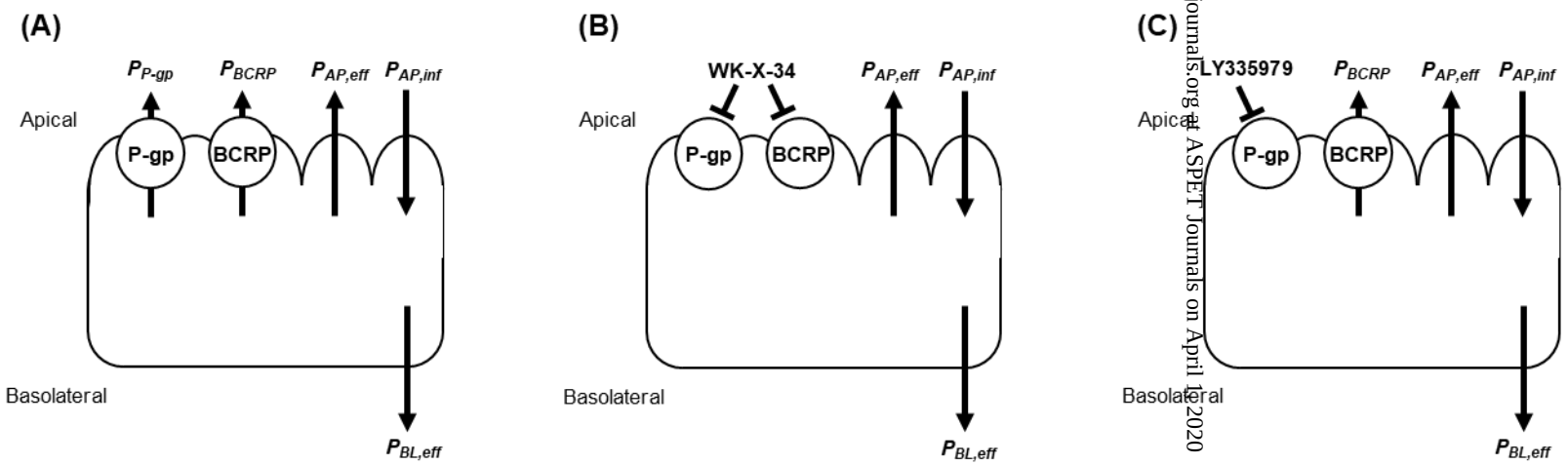

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Figure 2

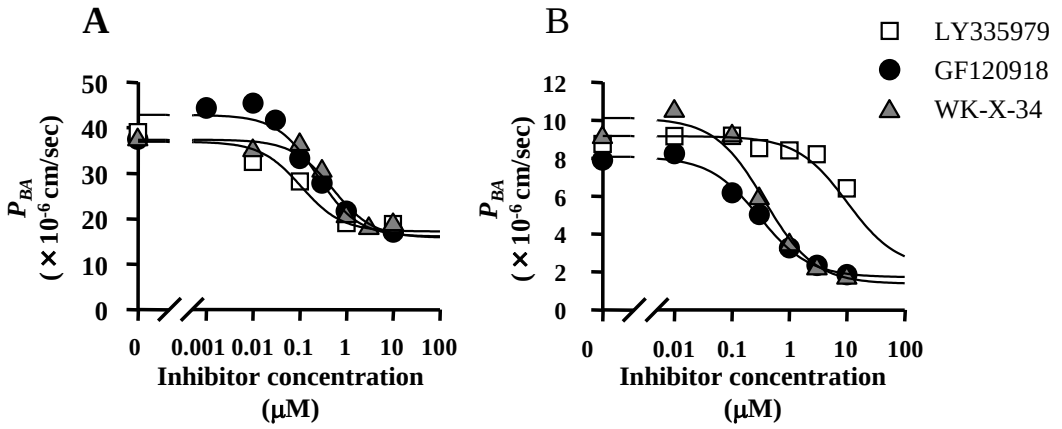

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Figure 3

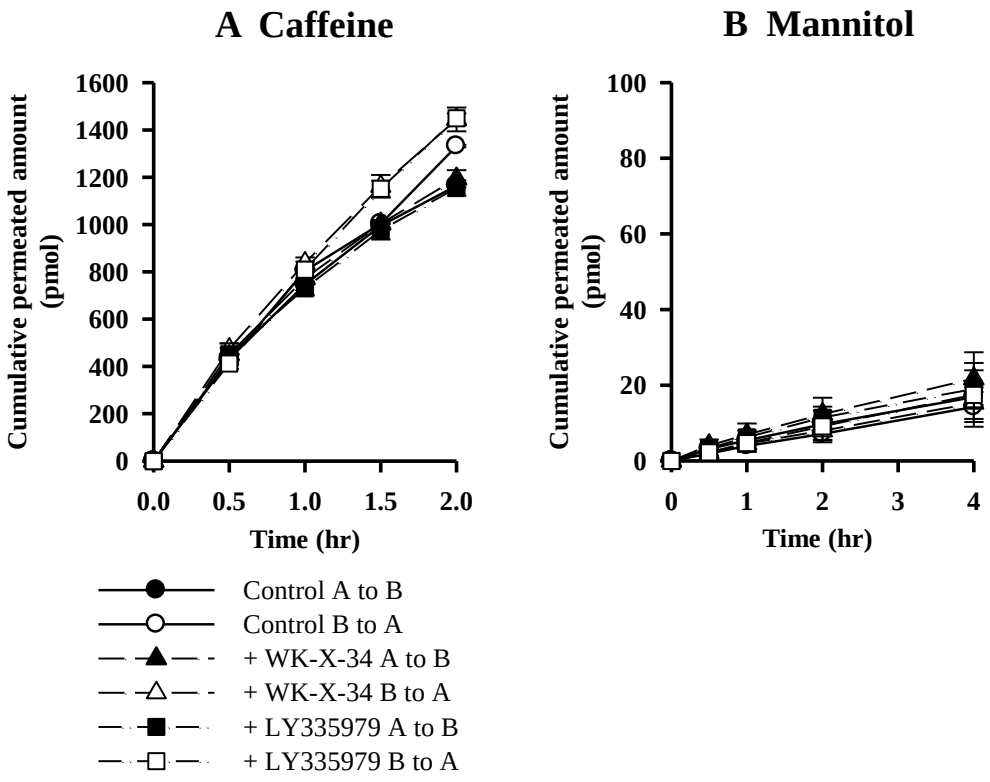

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Figure 4

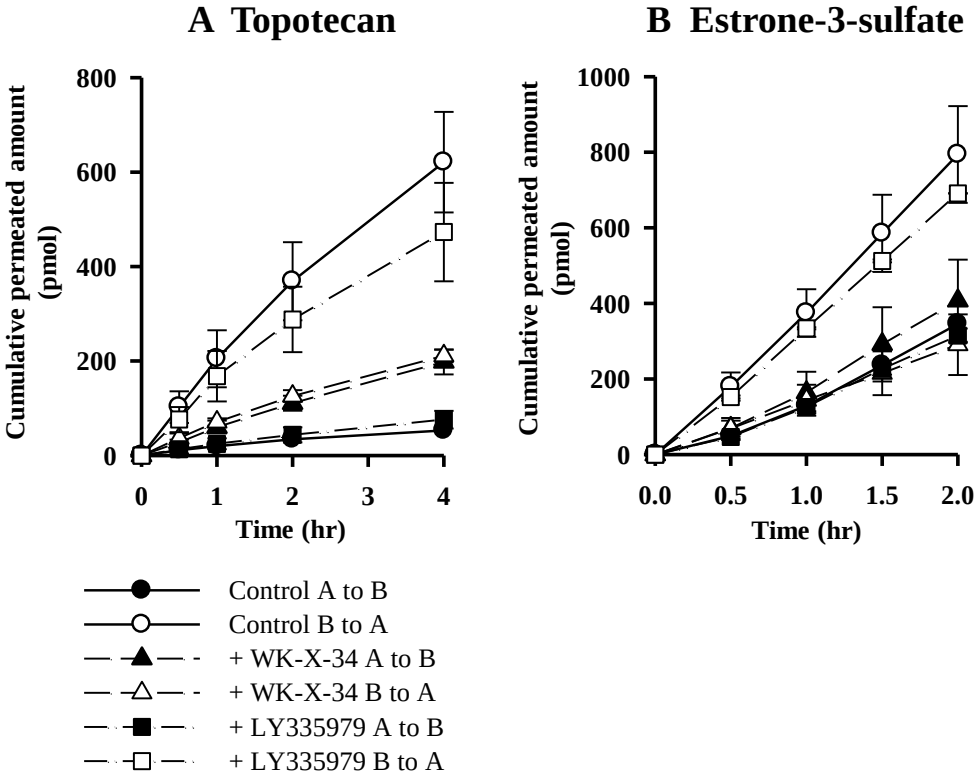

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Figure 5

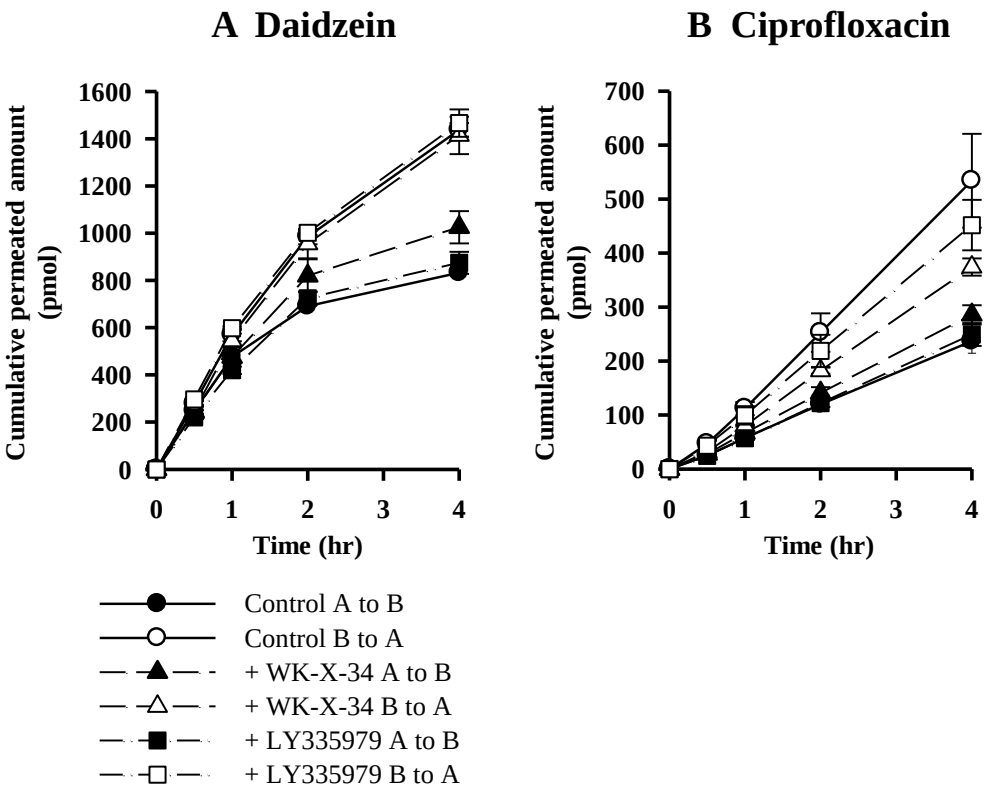

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Figure 6

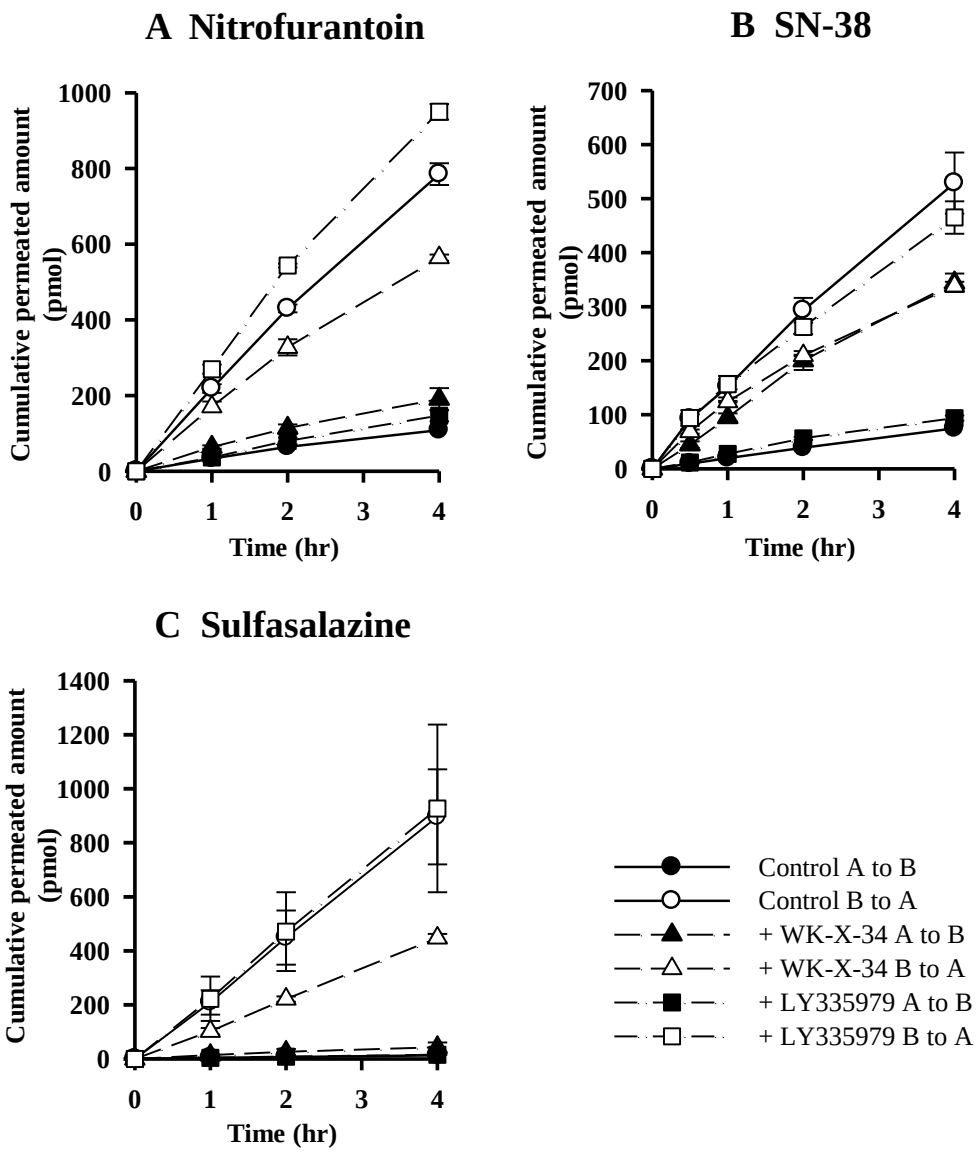

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Figure 7