Impact of Breast Cancer Resistance Protein Expression on the In Vitro Efficacy of Anticancer Drugs in Pancreatic Cancer Cell Lines

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ABSTRACT

Breast cancer resistance protein (BCRP) overexpression confers multidrug resistance to cancer cells, and the efficacy of anticancer drugs has been reported to be significantly affected by BCRP in cell lines transfected with BCRP or selected with drugs. It is unclear whether the in vitro efficacy of anticancer drugs is affected by endogenous BCRP, although cancer cell lines consisting of defined tumor cell lines with endogenous BCRP have been used to screen for anticancer drugs in the pharmaceutical industry. We assessed the impact of BCRP expression on the efficacy of anticancer drugs using pancreatic cancer cell lines expressing varying levels of endogenous BCRP. Pancreatic cancer cell lines were selected from the Cancer Cell Line Encyclopedia (CCLE). The EC50 of 7-ethyl-10-hydroxycamptothecin (SN-38), topotecan, and mitoxantrone decreased in the presence of a BCRP inhibitor in PANC-1 and AsPC-1 cells, which exhibit high BCRP expression. However, no significant alterations in EC50 were observed in HPAF-II, SW 1990, and MIA PaCa-2, which show moderate or low BCRP expression. The shift of EC50 of anticancer drugs with and without a BCRP inhibitor increased with an increase in BCRP mRNA expression levels; however, the shift was obvious only in cells highly expressing BCRP. Thus, the in vitro efficacy of anticancer drugs on cell proliferation may be minimally affected by BCRP in most pancreatic cancer cell lines, considering that 72% of pancreatic cancer cell lines in CCLE show moderate or low BCRP expression. The effect of BCRP should be carefully evaluated in pancreatic cell lines that highly express BCRP.

Introduction

Breast cancer resistance protein (BCRP) was discovered as a transporter overexpressed in a multidrug-resistant human breast cancer cell line (Doyle et al., 1998). BCRP has been reported to recognize many anticancer drugs as substrates, such as the cytotoxic drugs capetibamine (Zhang et al., 2015), methotrexate (Volk et al., 2000), 7-ethyl-10-hydroxycamptothecin (SN-38) (Nakatomi et al., 2001), topotecan (Maliepaard et al., 2001), mitoxantrone (Miyake et al., 1999), etoposide (Allen et al., 2003), and teniposide (Lin et al., 2011), and the molecular-targeted drugs dinaciclib (Chihara et al., 2015), flavopiridol (Nakanishi et al., 2003a), and imatinib (Burger et al., 2011), and pump these drugs out of cells. Thus, BCRP is known to be involved in multidrug resistance in cancer cells (Nakanishi and Ross, 2012). Until now, cell lines that overexpress BCRP, such as BCRP-transfected and drug-selected cells, have been used for in vitro studies to investigate BCRP-mediated drug resistance, and it has been reported that the efficacy of anticancer drugs that are substrates for BCRP can be significantly altered by BCRP. For example, MCF-7/AdrVp cells that were selected by the incremental increase of adriamycin in the presence of a P-glycoprotein (P-gp) inhibitor, verapamil, and MCF-7 cells stably transfected with BCRP cDNA (MCF-7/BCRP) showed 3900-fold and 32-fold higher LC50 of mitoxantrone, respectively, than that in nontransfected MCF-7 cells (Doyle et al., 1998).

However, it is unclear whether the efficacy of anticancer drugs is affected by endogenous BCRP expression, although in pharmaceutical industries, cancer cell line panels consisting of well defined tumor cell lines without enforced or in vitro-induced BCRP expression under artificial conditions have been frequently used to screen for anticancer drugs (Shoemaker, 2006; Niu and Wang, 2015). In drug discovery and development, the current standard procedure often starts with in vitro cell culture-based tests to screen for the efficacy of drugs, but only approximately 10% of the drugs progress successfully through clinical development (Edmondson et al., 2014). To increase the success rate of clinical trials, accurate and efficient in vitro screening of anticancer drugs is required. However, currently it remains unclear whether clinically promising drug candidates may be excluded during screening using cancer cell line panels, due to the effect of native BCRP.

The Cancer Cell Line Encyclopedia (CCLE) provides public access to mRNA expression data of approximately 1000 cancer cell lines (Barretina et al., 2012). When mRNA expression levels of BCRP were examined in cancer cell lines using the CCLE database, it was found that endogenous BCRP expression varies largely among the cell lines (Fig. 1; Supplemental Table 1). In the present study, we assessed the impact of BCRP expression on the efficacy of anticancer drugs using several cell lines transfected with BCRP...
cancer cell lines displaying varied levels of endogenous BCRP expression. In selecting cancer cell lines, BCRP expression levels in cancer cell lines were classified according to the signal intensity (SI) of BCRP: SI > 200, high; 100 < SI < 200, moderate; and SI < 100, low. Based on this classification, all five cancer cell lines were selected from pancreatic cancer–derived cells to avoid an effect caused by the derivation of tissues. PANC-1 and AsPC-1, HPAF-II and SW 1990, and MIA PaCa-2 were selected as representative cancer cell lines that show high, moderate, and low BCRP expressions, respectively (Fig. 1; Supplemental Table 1). Furthermore, Madin-Darby canine kidney (MDCKII) cells with enforced expression of BCRP were used as a positive control for the experiments.

Materials and Methods

Reagents. SN-38, etoposide, flavopiridol hydrochloride hydrate, and methotrexate hydrate were purchased from Sigma-Aldrich (St. Louis, MO). Dinaciclib was from Adooq BioScience (Irvine, CA). Teniposide was from Tokyo Chemical Industry (Tokyo, Japan). Mitoxantrone dihydrochloride was from Abcam Biochemicals (Cambridge, UK). Topotecan hydrochloride was from LKT Laboratories (St. Paul, MN). All other chemicals and reagents were commercial products of reagent grade.

Cell Culture. Human pancreatic cancer cell lines PANC-1, AsPC-1, HPAF-II, SW 1990, and MIA PaCa-2 were obtained from the American Type Culture Collection (Manassas, VA). Parental MDCKII cells were kindly provided by Dr. Piet Borst (Netherlands Cancer Institute, Amsterdam, Netherlands) (Evers et al., 1998). PANC-1, HPAF-II, SW 1990, and MIA PaCa-2 cell lines were grown in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). The parental MDCKII cells and their transduced subline, MDCKII-BCRP, were grown in low-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, and AsPC-1 cells were grown in Roswell Park Memorial Institute 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, in a humidified atmosphere of 5% CO2/air at 37°C.

Transduction of the MDCKII Cell Line with Human BCRP. BCRP cDNA (Matsushima et al., 2005) was subcloned into BamHI and EcoRI sites of the pQCXIN vector (Clontech, Mountain View, CA) to express the target gene along with the neomycin selection marker. Human embryonic kidney 293-T cells were seeded onto collagen-coated culture dishes 1 day before the transfection. BCRP-pQCXIN and
pPAM3psi, which is an amphotropic retrovirus helper plasmid (Miller and Buttimore, 1986), were transfected into human embryonic kidney 293-T cells using FuGENE 6 transfection reagent (Roche Life Science, Penzberg, Germany), following the manufacturer’s instructions. After 2 days, virus particles were harvested and concentrated. One day before infection, parental MDCKII cells were seeded onto collagen-coated culture plates. The next day, virus particles collected from the cells transfected with BCRP-pQCXIN were added to parental MDCKII cells with 8 μg/ml of polybrene. Infected cells were selected with G418 (250–500 μg/ml) for BCRP expression. Established cell lines were screened for BCRP activity on the basis of prazosin transport.

TaqMan Reverse-Transcription Polymerase Chain Reaction Gene Expression Analysis and Data Normalization. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The yield of extracted RNA was measured spectrophotometrically at 260 nm. For first-strand cDNA synthesis,

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**Fig. 3.** Confocal immunofluorescence microscopic analysis of BCRP protein localization in pancreatic cancer cell lines and MDCKII cells. Cells were stained with monoclonal antibody against BCRP (green) and polyclonal antibody against Na+/K+-ATPase (red). Nuclei were stained with DAPI (blue). Images are representative of two independent experiments.
500 ng of total RNA was reverse-transcribed in a final volume of 50 μl following the protocol for the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). We used 1 μl of cDNA for quantitative real-time polymerase chain reaction (PCR) using the CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The assay identification numbers of inventoried TaqMan reverse-transcription PCR analysis of human BCRP, human β-actin, and dog β-actin genes were Hs01053790_m1, Hs09999903_m1, and C03023880_g1, respectively (Applied Biosystems, Carlsbad, CA). Assays were performed as technical triplicates. Gene expression data were normalized to β-actin as an endogenous control quantified in the same sample. Relative expression differences were calculated using the comparative ΔΔCt method (Livak and Schmittgen, 2001).

Western Blot Analysis. For Western blot analysis, crude membrane fractions were prepared from human pancreatic cancer cell lines and MDCKII cells according to the method described in a previous report (Gant et al., 1991). The protein concentrations in the fraction were determined using the method of Lowry et al. (1951), with bovine serum albumin as a standard. The membrane fraction was dissolved in Laemmli sample buffer with 10% 2-mercaptoethanol and loaded onto a 10% Bis-Tris gel. Proteins were transferred electrophoretically to a polyvinylidene difluoride membrane using the Trans-Blot Turbo Transfer System (Bio-Rad) at 1.3 A for 7 minutes. The membrane was reacted with mouse monoclonal anti-BCRP antibody, clone BXP-21 (1:100; KAMIYA Biomedical Company, Seattle, WA), or with rabbit monoclonal anti-β-actin antibody (1:200; Cell Signaling Technology, Danvers, MA) and then reacted with donkey anti-mouse (for BCRP, 1:500) or donkey anti-rabbit (for β-actin, 1:2,000) IgG conjugated with horseradish peroxidase to detect proteins using the iBind Flex Western Device (Thermo Fisher Scientific, Waltham, MA). Bands were detected using the ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, IL).

Confocal Immunofluorescence Microscopy. Cells grown on glass cover slips were fixed in methanol for 10 minutes at −20°C. The cells were permeabilized with 1% Triton X-100 in phosphate-buffered saline (PBS) for 5 minutes at room temperature. Cells washed with PBS were incubated in blocking solution (5% FBS in PBS) for 60 minutes and then with mouse monoclonal anti-BCRP antibody, clone BXP-21 (1:25; KAMIYA Biomedical Company), and goat polyclonal anti-Na+/K+-ATPase antibody, N-15 (1:50; Santa Cruz Biotechnology, Dallas, TX). The immunoreactions were visualized by incubation of the cells with donkey Alexa Fluor 488-anti-mouse IgG (for BCRP, 1:1,000) and donkey Alexa Fluor 647-anti-goat IgG (for Na+/K+-ATPase, 1:1,000). Finally, cover slips containing the immunolabeled cells were mounted with DAPI (Vector Laboratories, Burlingame, CA) and observed using a Zeiss (Oberkochen, Germany) LSM 800. The immunoreactions were visualized by incubation of the cells with donkey Alexa Fluor 488-anti-mouse IgG (for BCRP, 1:1,000) and donkey Alexa Fluor 647-anti-goat IgG (for Na+/K+-ATPase, 1:1,000). Bands were detected using the ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, IL).

mRNA Quantification after Treatment with Anticancer Drugs. Cells were seeded into 24-well plates at a density of 6000 cells/well. The next day, the cells were exposed to anticancer drugs at the indicated concentrations for 72 hours. RNA isolation, reverse transcription, and quantitative real-time PCR were performed as described in the previous section.

Data Analysis. The percentage of control cell growth was calculated using the following equation:

\[ \text{% of control cell growth} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{day 0}}}{\text{OD}_{\text{neg control}} - \text{OD}_{\text{day 0}}} \]

where ODsample and ODneg control are mean ODs of the cells with and without anticancer drugs, respectively, and OD_{day 0} is the mean OD of the cells before anticancer drugs were added.

The concentration that induces 50% of the maximum effect on cell growth inhibition (EC50) of anticancer drugs tested was determined by iterative nonlinear regression analysis using Excel (Microsoft, Redmond, WA) and its add-in program XLfit (IDBS, Guildford, UK). EC50 values were calculated, assuming standard hyperbolic kinetics, in accordance with the following equation:

\[ E = E_{\text{min}} + \frac{E_{\text{max}} - E_{\text{min}}}{1 + \left(\frac{x}{x_{50}}\right)^{h}} \]

where \( x \) is the drug concentration; \( E_{\text{min}} \) and \( E_{\text{max}} \) are the percentages of cell growth when \( x \) is infinity and 0, respectively; and \( H \) is the slope factor.

Results

mRNA and Protein Expression of BCRP in Pancreatic Cancer Cell Lines and MDCKII Cells. BCRP mRNA expression levels in five

### Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>MDCKII-MOCK</th>
<th>MDCKII-BCRP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>EC50 ,−FTC (range)</td>
<td>EC50 ,+FTC</td>
<td>N  RR</td>
</tr>
<tr>
<td></td>
<td>(μM)</td>
<td>(μM)</td>
</tr>
<tr>
<td>Cytotoxic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antimetabolites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capetabine</td>
<td>&gt;200 (−)</td>
<td>&gt;200 (−)</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.035 (−)</td>
<td>0.028 (−)</td>
</tr>
<tr>
<td>Topoisomerase I inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SN-38</td>
<td>0.16 (0.14−0.17)</td>
<td>0.051 (0.044−0.057)</td>
</tr>
<tr>
<td>Topotecan</td>
<td>0.39 (0.31−0.46)</td>
<td>0.26 (0.19−0.33)</td>
</tr>
<tr>
<td>Topoisomerase II inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>0.29 (0.29−0.29)</td>
<td>0.26 (0.26−0.26)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>2.8 (−)</td>
<td>1.8 (−)</td>
</tr>
<tr>
<td>Teniposide</td>
<td>0.57 (−)</td>
<td>0.42 (−)</td>
</tr>
<tr>
<td>Molecular targeted</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclin-dependent kinase inhibitor</td>
<td>0.065 (−)</td>
<td>0.030 (−)</td>
</tr>
<tr>
<td>Dimacilb</td>
<td>0.12 (0.12−0.12)</td>
<td>0.12 (0.11−0.12)</td>
</tr>
<tr>
<td>Flavopiridol</td>
<td></td>
<td></td>
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</tbody>
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NC, not calculated.
pancreatic cancer cell lines and MDCKII cells were determined using TaqMan real-time PCR analysis and normalized to β-actin expression as an endogenous control. The relative mRNA expression level of BCRP was the highest in MDCKII-BCRP cells, followed by PANC-1, AsPC-1, HPAF-II, SW 1990, and MIA PaCa-2 cells. BCRP mRNA was not detected in MDCKII-MOCK cells (Fig. 2A). The experimentally determined BCRP mRNA expression ratio in five pancreatic cancer cell lines was comparable to that from microarray data in CCLE (data not shown). The mRNA expression levels of P-gp, which also confers multidrug resistance, were also quantified in pancreatic cancer cell lines. The mRNA expression levels of P-gp were below the detection limit in AsPC-1, SW 1990, and MIA PaCa-2 cells or more than 6900-fold lower than those of β-actin in PANC-1 and HPAF-II cells, suggesting that P-gp expression in these cell lines was very low (data not shown).

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Subcellular Localization of the BCRP Protein in Pancreatic Cancer Cell Lines and MDCKII Cells. Because the BCRP protein was detected in Western blot analysis in MDCKII-BCRP, PANC-1, and AsPC-1 cells (Fig. 2B), the subcellular localization of the BCRP protein was examined in these cells using confocal immunofluorescence microscopic analysis. The Na+/K+-ATPase protein was costained with BCRP as a marker of the plasma membrane. In MDCKII-BCRP cells, the BCRP protein was expressed not only in the plasma membrane but also throughout the cells, and a stronger signal could be observed in the plasma membrane than inside the cells (Fig. 3). The BCRP protein was not detected in MDCKII-MOCK cells (Fig. 3). In PANC-1 and AsPC-1 cells, BCRP was expressed mainly in the plasma membranes (Fig. 3).

EC_{50} Values of Anticancer Drugs in the Absence and Presence of FTC in MDCKII Cells. Nine anticancer drugs that are substrates of BCRP and have different modes of action were selected (Table 1), and EC_{50} values of these drugs were evaluated in the absence and presence of a BCRP inhibitor, FTC, in MDCKII-MOCK and MDCKII-BCRP cells. FTC was used as a selective BCRP inhibitor (Ahmed-Belkacem et al., 2006) to evaluate the impact of only BCRP inhibition. Resistance ratio (RR) was calculated based on the ratio of EC_{50} for a given drug without FTC over that with FTC in each cell line. Methotrexate, SN-38, topotecan, mitoxantrone, etoposide, teniposide, dinaciclib, and flavopiridol inhibited the growth of MDCKII-BCRP cells up to 200 μM. Although RRs of SN-38 (3.1) and dinaciclib (2.1) were >2, RRs of most anticancer drugs were <2 in MDCKII-MOCK cells in which BCRP was not expressed (Table 1). Therefore, RR > 2 was a significant effect by BCRP inhibition. RR of methotrexate was <2, and RRs of SN-38, topotecan, mitoxantrone, etoposide, teniposide, dinaciclib, and flavopiridol in MDCKII-BCRP cells were >2 (94, 32, 4.0, 2.5, 3.0, 6.7, and 5.5, respectively). By the addition of FTC, EC_{50} values of these drugs in MDCKII-BCRP cells decreased to values comparable to those of MDCKII-MOCK cells, which suggested that FTC completely inhibited BCRP activity in MDCKII-BCRP cells (Table 1). Among nine anticancer drugs investigated, EC_{50} values of SN-38, topotecan, mitoxantrone, dinaciclib, and flavopiridol were further determined in pancreatic cancer cell lines and MDCKII cells were determined using TaqMan real-time PCR analysis and normalized to β-actin expression as an endogenous control. The relative mRNA expression level of BCRP was the highest in MDCKII-BCRP cells, followed by PANC-1, AsPC-1, HPAF-II, SW 1990, and MIA PaCa-2 cells. BCRP mRNA was not detected in MDCKII-MOCK cells (Fig. 2A). The experimentally determined BCRP mRNA expression ratio in five pancreatic cancer cell lines was comparable to that from microarray data in CCLE (data not shown). The mRNA expression levels of P-gp, which also confers multidrug resistance, were also quantified in pancreatic cancer cell lines. The mRNA expression levels of P-gp were below the detection limit in AsPC-1, SW 1990, and MIA PaCa-2 cells or more than 6900-fold lower than those of β-actin in PANC-1 and HPAF-II cells, suggesting that P-gp expression in these cell lines was very low (data not shown).

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the selected pancreatic cancer cell lines because these drugs showed larger RRs in MDCKII-BCRP cells.

**EC₅₀ Values of Anticancer Drugs in the Absence and Presence of FTC in Selected Cancer Cell Lines.** EC₅₀ values of SN-38, topotecan, mitoxantrone, dinaciclib, and flavopiridol in the absence and presence of FTC were evaluated in five pancreatic cancer cell lines, and RRs were calculated. RRs of SN-38, topotecan, and mitoxantrone were 18, 11, and 3.7, respectively, in PANC-1 and 3.5, 3.9, and 3.0, respectively, in AsPC-1 (Table 2). RRs of dinaciclib and flavopiridol in PANC-1 and AsPC-1 cells were <2. In HPAF-II, SW 1990, and MIA PaCa-2 cells, none of these five drugs showed RRs >2, except SN-38 in MIA PaCa-2 cells (Table 2).

**Relationship between RRs of Anticancer Drugs and BCRP Expression in Pancreatic Cancer Cell Lines and MDCKII-BCRP Cells.** The relationship between RRs of SN-38, topotecan, mitoxantrone, dinaciclib, and flavopiridol and mRNA expression levels of BCRP in five pancreatic cancer cell lines and MDCKII-BCRP cells was examined. The results are shown in Fig. 4. RRs of all these drugs increased with an increase in BCRP mRNA expression.

**Effect of Drug Treatment on BCRP mRNA Expression in Pancreatic Cancer Cell Lines.** Effect of anticancer drugs on BCRP mRNA expression was examined in pancreatic cancer cell lines after treatment with SN-38, topotecan, mitoxantrone, dinaciclib, and

![Diagram](https://example.com/diagram.png)

**Fig. 4.** Relationship between RR of SN-38 (A), topotecan (B), mitoxantrone (C), dinaciclib (D), and flavopiridol (E) and BCRP mRNA expression levels of MDCKII-BCRP (closed circle), PANC-1 (closed square), AsPC-1 (closed triangle), HPAF-II (open circle), SW 1990 (open square), and MIA PaCa-2 (open triangle) cells. Dotted lines represent an RR of 2.
flavopiridol at concentrations close to EC_{50} values for 72 hours. In PANC-1, AsPC-1, HPAF-II, and SW 1990 cells, alterations in BCRP mRNA expression after treatment with anticancer drugs were within 2-fold. In Mia PaCa-2 cells, a concentration-dependent, >10-fold increase in BCRP mRNA expression was observed after treatment with SN-38, topotecan, and mitoxantrone, with increases in expression at higher concentrations of 15-, 17-, and 20-fold, respectively (Table 3).

**Discussion**

In CCLE, 28% pancreatic cancer cell lines (12 of 43) express BCRP at high levels, and the rest of them show moderate or low BCRP expression based on the classification of SI (SI > 200, high; 100 < SI < 200, moderate; and SI < 100, low) (Fig. 1). In the present study, we investigated the impact of BCRP expression on the efficacy of anticancer drugs that are BCRP substrates in the presence or absence of a selective BCRP inhibitor, using several pancreatic cancer cell lines with varying levels of BCRP expression. Our results show that the EC_{50} values of some anticancer drugs significantly decrease in the presence of a BCRP inhibitor in PANC-1 and AsPC-1 cells expressing high levels of BCRP. The largest shift of EC_{50} observed in the present study was 18-fold, in the case of SN-38 in PANC-1 cells. EC_{50} values were not affected by the BCRP inhibitor in HPAF-II, SW 1990, and Mia PaCa-2 cells expressing moderate or low levels of BCRP (Table 2). Overall, RRs of anticancer drugs became larger with an increase in BCRP mRNA expression (Fig. 4). Because all cell lines used in this study were derived from pancreatic cancer, further investigations are required to evaluate whether the findings are true in cell lines derived from other cancer types.

Because it is well recognized that mRNA expression is a surrogate of protein expression, and that transporters are functional when expressed in plasma membranes of cells, a relationship between mRNA (Fig. 2A), protein expression (Fig. 2B; Supplemental Fig. 1A), and protein subcellular localization (Fig. 3) was examined. In HPAF-II, SW 1990, and Mia PaCa-2 cells, BCRP protein was not detected despite BCRP mRNA expression being detected in significant levels. This can be attributed to the higher sensitivity of real-time PCR compared with that of Western blot analysis. Considering robust BCRP protein expression in plasma membranes of PANC-1 and AsPC-1 cells, BCRP mRNA expression levels might serve as a good indicator for functional BCRP, at least in PANC-1 and AsPC-1 cells. In Western blot analysis, the protein molecular weight of BCRP in AsPC-1 cells was less than that in MDCKII-BCRP or PANC-1 cells (Fig. 2B; Supplemental Fig. 1A). This observation might be accounted for by the difference in N-linked glycosylation of BCRP between AsPC-1 and MDCKII-BCRP cells or PANC-1 cells. However, the difference may not have a significant impact on BCRP function because the absence of N-linked glycosylation did not affect the plasma membrane localization of BCRP (Mohrmann et al., 2005). In fact, RRs of SN-38, topotecan, and mitoxantrone were >2 in AsPC-1 cells, which suggested that BCRP expressed in AsPC-1 cells was functional.

**BCRP** mRNA expression increased in the Mia PaCa-2 cells treated with anticancer drugs (Table 3); however, the increased expression levels in these cells still remained the lowest in rank order among the five pancreatic cancer cell lines used in this study. Thus, no changes in BCRP mRNA expression occurred after incubation with anticancer drugs, which affected RRs of these drugs and interpretation of the results shown in Fig. 4. Because the number of cell lines used in this study was limited, further investigations are required to identify any cancer cell lines that show increased BCRP expression sufficient to alter RRs after treatment with anticancer drugs.

As shown in Fig. 4, the overall RRs of anticancer drugs could be explained by BCRP mRNA expression levels. However, in the present study, RR of SN-38 was 2.1 in Mia PaCa-2 cells, which was of borderline significance for the BCRP effect despite low BCRP expression (Table 2), and such a high RR value of SN-38 was also observed in MDCKII-MOCK cells (3.1) (Table 1). SN-38 was reported to be a substrate of not only BCRP but also other efflux transporters, such as P-gp, multidrug-resistance protein 1, and multidrug-resistance protein 2 (Kwak et al., 2012). Because the mRNA expression level of P-gp in Mia PaCa-2 cells was low, endogenous transporters other than BCRP and P-gp, whose function can be altered by FTC, may be involved in this resistance.

Transcellular transport experiments of SN-38, topotecan, mitoxantrone, and flavopiridol using MDCKII-MOCK and MDCKII-BCRP cells have been reported (Xiao et al., 2006; Zhou et al., 2009), and the corrected efflux ratio, which is the normalized ratio of basal-to-apical versus apical-to-basal permeability in MDCKII-MOCK cell monolayers with respect to that in MDCKII-BCRP cell monolayers, of these four drugs was comparable (7.6–8.4 in SN-38, 2.6–5.4 in topotecan, 4.5–6.1 in mitoxantrone, and 6.0 in flavopiridol). However, our study showed that RRs of topoisomerase 1 inhibitors (SN-38 and topotecan) were much larger than those of mitoxantrone and flavopiridol in MDCKII-BCRP cells (Table 1). The magnitude of RRs may not be

<table>
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<tr>
<th>Drug</th>
<th>PANC-1</th>
<th>AsPC-1</th>
<th>HPAF-II</th>
<th>SW 1990</th>
<th>MIA PaCa-2</th>
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<tbody>
<tr>
<td></td>
<td>Concentration Mean ± S.D.</td>
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<td>Concentration Mean ± S.D.</td>
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**TABLE 3**

Relative changes in BCRP mRNA expression in pancreatic cancer cell lines after treatment with anticancer drugs compared with control (no treatment)

Analysis of mRNA expression was performed after a 72-hour incubation with each anticancer drug. BCRP mRNA expression levels were normalized to those of β-actin. Data represent the mean ± S.D. (n = 3).
directly explained by the efflux ratio alone, which is an indicator of the importance of efflux transporters in transporting drugs across the cell membrane (Giacomini et al., 2010). Namely, there may be a determinant rather than the active efflux of anticancer drugs by BCRP. For example, the anticancer efficacy of drugs depends on their chemical structure within the tumor microenvironment. SN-38 and topotecan are pharmacologically active in the lactone form, but most drugs are in the inactive carboxylate form at physiologic pH (Oguma, 2001). With reference to the transport of lactone and carboxylate forms of SN-38, we have recently shown that they are similarly transported by another type of transporter, organic-anion-transporting polypeptide 2B1 (Fujita et al., 2016). However, the same result may not be directly applicable to BCRP-mediated transport, and evaluation of the efflux ratio of the lactone forms of these drugs separately from their carboxylate forms will be required.

**BCRP** mRNA expression in pancreatic cancer cell lines used in this study was compared with that in cancer biopsies available from the Expression Project for Oncology (accession number: GSE2109; Gene Expression Omnibus), which provides public access to mRNA expression data of more than 2000 cancer biopsies (Fig. 5; Supplemental Table 2). The comparison showed that mRNA expression levels of **BCRP** in cancer biopsies were lower than those in PANC-1 and AsPC-1 cells, in which significant decreases in EC50 values of several anticancer drugs were observed when BCRP was inhibited. **BCRP** mRNA expression in cancer biopsies was within a comparable range or lower than that in HPAF-II, SW 1990, and MIA PaCa-2 cells, in which expression in cancer biopsies was within a comparable range or lower than pancreas; open square, pancreatic cancer cell lines). SI of **BCRP** to **β-actin**, and derivation of each cancer biopsy is shown in Supplemental Table 2.

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**Fig. 5.** Comparison of relative mRNA expression levels of **BCRP** to **β-actin** between cancer biopsies and cancer cell lines. mRNA expression levels of **BCRP** and **β-actin** in 2157 cancer biopsies and cancer cell lines used in this study, represented as SI of microarray analysis, were obtained from Expression Project for Oncology (expO) and CCLE, respectively, and compared (open circle, pancreatic cancer biopsies; closed circle, cancer biopsies derived from malignant tissues other than pancreas; open square, pancreatic cancer cell lines). SI of **BCRP** and **β-actin**, relative SI of **BCRP** to **β-actin**, and derivation of each cancer biopsy is shown in Supplemental Table 2.

In conclusion, our results suggest that the in vitro efficacy of anticancer drugs on cell proliferation was significantly affected by BCRP in pancreatic cancer cell lines abundantly expressing BCRP, which account for 28% pancreatic cancer cell lines in CCLE. However, the in vitro efficacy of anticancer drugs in the remaining 72% of pancreatic cancer cell lines expressing BCRP at moderate or low levels was not significantly affected. Although we are unable to exclude the possibility that BCRP expression induced by anticancer drug treatment during incubation affected their in vitro efficacy, it is unlikely that clinically promising anticancer drugs for treatment of pancreatic cancer have been excluded during screening because of BCRP, considering that most pancreatic cancer cell lines show moderate or low BCRP expression. However, when pancreatic cancer cell lines with high levels of BCRP expression are used, the effect of BCRP should be carefully evaluated, considering information such as BCRP expression in cancer cell lines and cancer tissues from patients, to avoid errors in the selection of drug candidates.

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**Authorship Contributions**

**Participated in research design:** Washio, Nakanishi, Ishiguro, Yamamura, Tamai.

**Conducted experiments:** Washio.

**Performed data analysis:** Washio, Nakanishi, Ishiguro, Tamai.

**Wrote or contributed to the writing of the manuscript:** Washio, Nakanishi, Ishiguro, Tamai.

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poise oral availability is limited primarily by P-glycoprotein. *Cancer Res* 63:1339–1344.


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