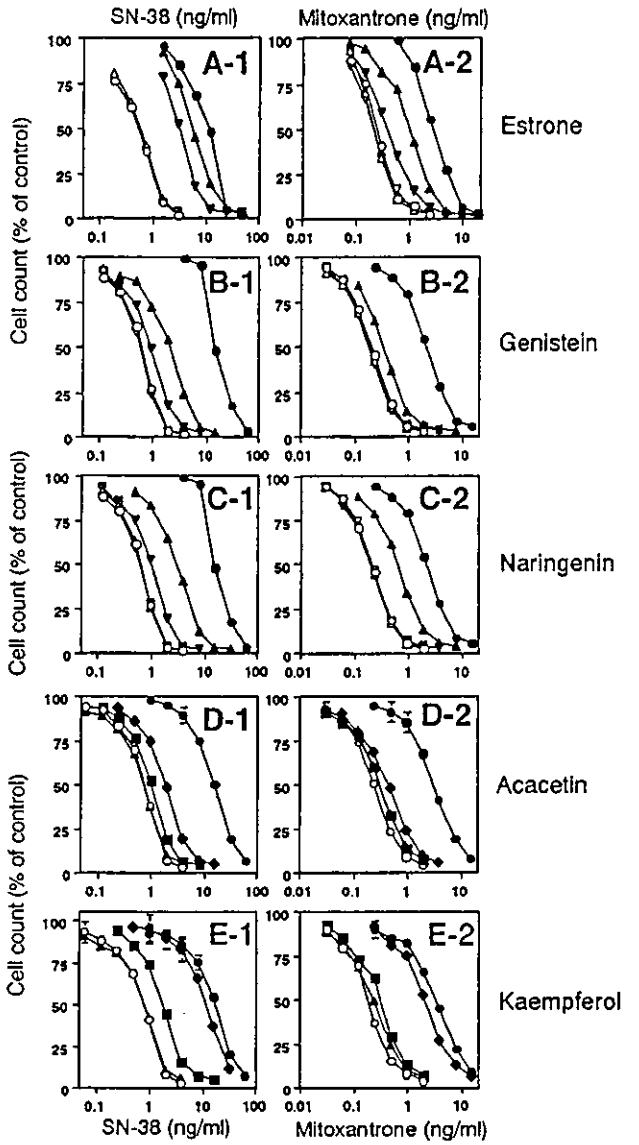

Fig. 3. Reversal effects of estrone and phytoestrogens/flavonoids on breast cancer resistance protein (BCRP)-mediated antitumor drug resistance. K562 (*open symbols*) and K562/BCRP (*closed symbols*) cells were cultured for 5 days in the absence (*circle*) or presence of 0.3 μM (lozenge), 1 μM (*square*), 3 μM (*triangle*), and 10 μM (*inverted triangle*) of the specific compounds indicated under increasing concentrations of antitumor drugs. *A*, estrone. *B*, genistein. *C*, naringenin. *D*, acacetin. *E*, kaempferol. Antitumor agents are SN-38 (N-1) and mitoxantrone (N-2; N, *A–E*). Data points are measurements of the average ± SD from triplicate determinations. Cell numbers were determined with a cell counter.

mitoxantrone were 9.96 ± 0.38 (21.5 ± 0.22) and 10.6 ± 0.99 (14.2 ± 0.95), respectively. However, the flavonoids did not show growth-inhibitory effects on K562 cells under these experimental conditions. K562/BCRP cells treated with estrone or flavonoids, such as genistein, naringenin, and acacetin, for 5 days expressed similar amounts of BCRP as compared with control K562/BCRP cells (Fig. 5). This result suggested that flavonoids sensitized K562/BCRP cells to SN-38 and mitoxantrone not by reducing BCRP expression but by inhibiting BCRP function. We then examined the effects of glycosylated flavonoids on the drug-resistance properties of K562/BCRP cells. Although most glycosylated flavonoids had little effect on BCRP-mediated drug resistance, some glycosides, such as naringenin-7-glucoside and luteolin-4′-*O*-glucoside, displayed moderate reversal activity (Fig. 6). Reversal indexes of 3 μM (10 μM) naringenin-7-glucoside for SN-38 and mitoxantrone were 5.70 ± 0.16 (14.7 ± 0.53) and 5.17 ± 0.23 (9.44 ± 0.42), respectively.

Additional studies showed that the reversal of MDR by genistein and naringenin was specific to BCRP because they did not show any reversal effects on either P-glycoprotein-mediated vincristine resistance or MRP1-mediated VP-16 resistance (Fig. 7).

Intracellular Topotecan Uptake and Cellular [^{3}H]Genistein Accumulation in K562/BCRP Cells. To address whether reversal of BCRP-mediated drug resistance by flavonoids might be associated with the inhibition of BCRP-mediated drug efflux, the cellular accumulation of topotecan was evaluated in the absence or presence of specific flavonoids by flow cytometric analysis. Intracellular accumulation of topotecan increased in the presence of genistein or naringenin in a dose-dependent manner in K562/BCRP cells (Fig. 8), whereas these levels were not altered in K562 cells (data not shown). The results indicate that these flavonoids reverse anticancer drug

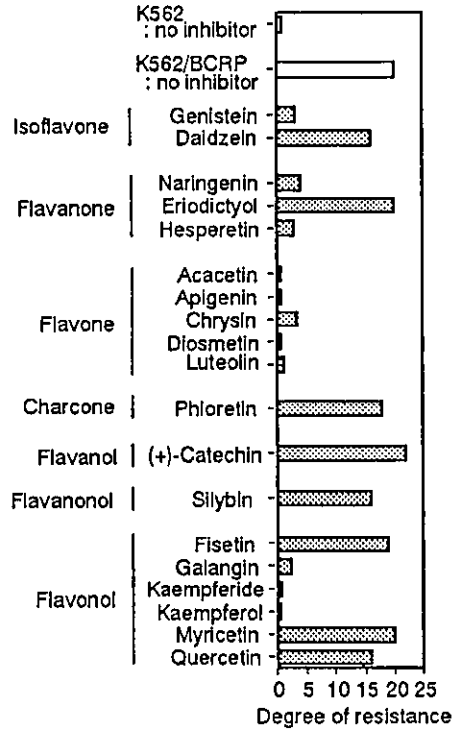

Fig. 4. Inhibitory effects of phytoestrogens/flavonoids on breast cancer resistance protein (BCRP)-mediated SN-38 resistance. K562 and K562/BCRP cells were cultured for 5 days in the absence or presence of 3 μM compound with increasing concentrations of SN-38. Cell numbers were determined using a cell counter, and IC_{50} values then were measured. Open bar, no inhibitor. Dotted bar, treatment with flavonoids. The degree of resistance is the ratio of IC_{50} values of the cells to that of K562 cells under the indicated experimental conditions.

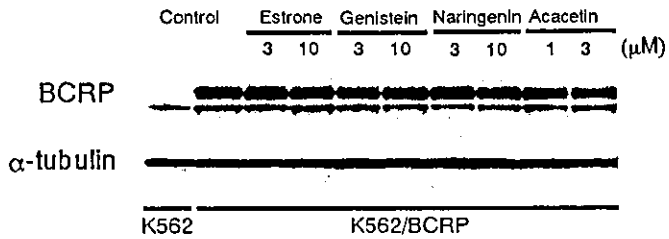

Fig. 5. Western blot analyses of breast cancer resistance protein (BCRP) expression in K562/BCRP cells treated with estrone or flavonoids for 5 days. K562 and K562/BCRP cells were incubated for 5 days in the absence or presence of indicated concentrations of compounds. Cell lysates (20 μg/lane) were used for quantitative analyses of BCRP expression. Expression of α-tubulin was presented as an internal control.

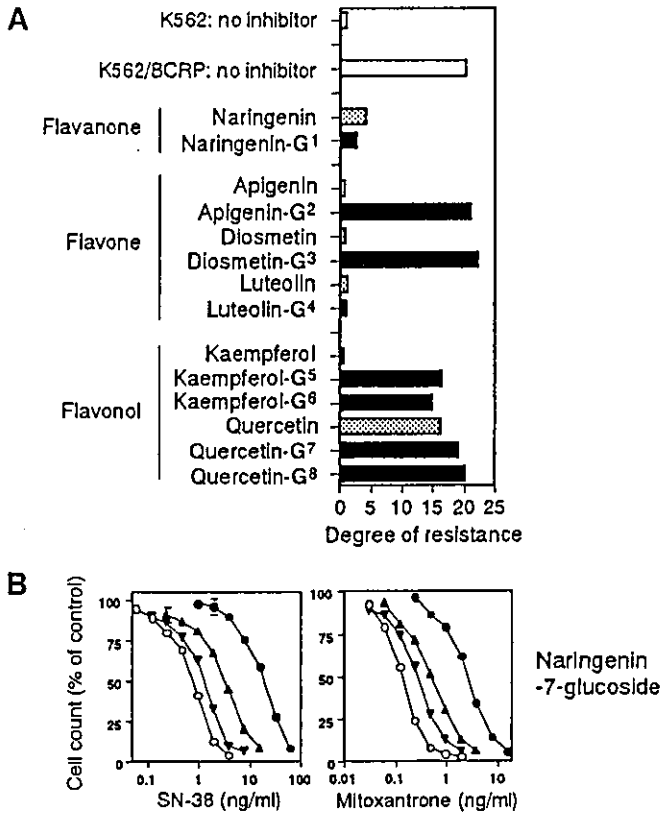

Fig. 6. Inhibitory effects of glycosylated flavonoids on breast cancer resistance protein (BCRP)-mediated drug resistance. *A*, inhibitory effects of glycosylated flavonoids on BCRP-mediated SN-38 resistance. K562 and K562/BCRP cells were cultured for 5 days in the absence or presence of 3 μM flavonoid compounds with increasing concentrations of SN-38. Cell numbers were measured with a cell counter, and IC_{50} values then were determined. The degree of resistance is the ratio of IC_{50} values of the cells to that of K562 cells under the indicated experimental conditions. Open bar, no inhibitor. Dotted bar, treatment with flavonoids. Solid bar, treatment with glycosylated flavonoids. *Naringenin-G*[1], naringenin-7-glucoside; *Apigenin-G*[2], apigenin-7-neohesperidoside (rhoifolin); *Diosmetin-G*[3], diosmetin-7-β-rutinoside (Diosmin); *Luteolin-G*[4], luteolin-4′-*O*-glucoside; *Kaempferol-G*[5], kaempferol-3-glucoside; *Kaempferol-G*[6], kaempferol-7-*O*-neohesperidoside; *Quercetin-G*[7], quercetin-3-arabinoglucoside (peltatoside); and *Quercetin-G*[8], quercetin-3-rutinoside (Rutin). *B*, reversal effects of naringenin-7-glucoside on BCRP-mediated drug resistance. K562 (*open symbol*) and K562/BCRP (*closed symbol*) cells were cultured for 5 days in the absence (*circle*) or presence of 3 μM (*triangle*) and 10 μM (*inverted triangle*) naringenin-7-glucoside with increasing concentrations of antitumor drugs. Data points are the average ± SD from triplicate determinations. Cell numbers were determined by a cell counter.

resistance by increasing the cellular levels of anticancer drugs in BCRP-expressing cells.

To examine whether flavonoids themselves are transported by BCRP, the intracellular accumulation of [^{3}H]genistein in K562 and K562/BCRP cells also was examined. K562/BCRP cells accumulated a significantly smaller amount of [^{3}H]genistein than K562 cells, suggesting that there is BCRP-mediated efflux of genistein out of the cells (Fig. 9).

Transcellular Transport of [^{3}H]Genistein. BCRP-mediated transport of genistein was examined by transcellular transport assays using LLC/BCRP cells, which express BCRP in the apical membrane (8). The paracellular fluxes monitored by [^{14}C]inulin appearance in the other side of the growth chambers were <1% of the total radioactivity/h. Basal-to-apical transport (secretion) of [^{3}H]mitoxantrone, a BCRP substrate, was greater in LLC/BCRP cells than that in LLC-PK1 cells (8). In the present study, secretion of [^{3}H]genistein in LLC/BCRP cells also proved to be greater than that in LLC-PK1 cells, whereas apical-to-basal transport (reabsorption) of [^{3}H]genistein in LLC/BCRP cells was reduced compared with LLC-PK1 cells (Fig. 10). However, in the presence of 3 μM fumitremorgin C, secretion and reabsorption of [^{3}H]genistein were at similar levels between LLC-PK1 and LLC/BCRP cells.

Our previous study demonstrated that [^{3}H]estrone was converted to [^{3}H]estrone sulfate in LLC-PK1 cells and that the latter was exported by BCRP (8). Therefore, transported radioactivity over the apical membrane of the cells was analyzed by silica gel TLC. [^{3}H]genistein

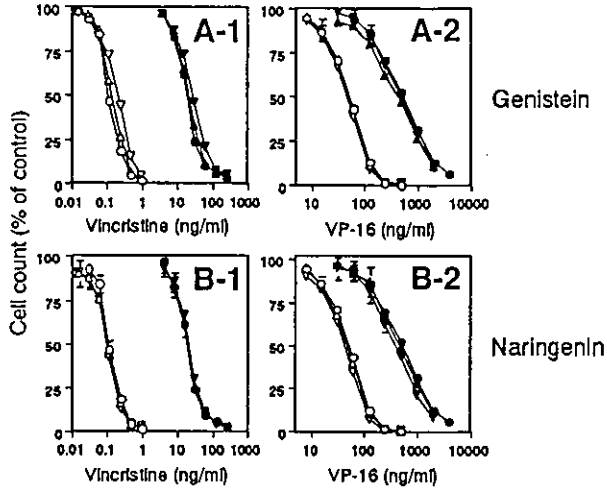

Fig. 7. Reversal effects of genistein and naringenin on either P-glycoprotein- or MRP1-mediated antitumor drug resistance. Parental cells (*open symbol*) and transfected cells (*closed symbol*) were cultured for 5 days in the absence (*circle*) or presence of 3 μM (*triangle*) or 10 μM (*inverted triangle*) compounds with increasing concentrations of the antitumor agents. Cell numbers were determined using a cell counter. *A-1*, effect of genistein on the vincristine sensitivity of K562 and K562/MDR cells. *A-2*, effect of genistein on the VP-16 sensitivity of KB-3–1 and KB/MRP cells. *B-1*, effect of naringenin on the vincristine sensitivity of K562 and K562/MDR cells. *B-2*, effect of naringenin on the VP-16 sensitivity of KB-3–1 and KB/MRP cells. Data points are the average ± SD from triplicate determinations.

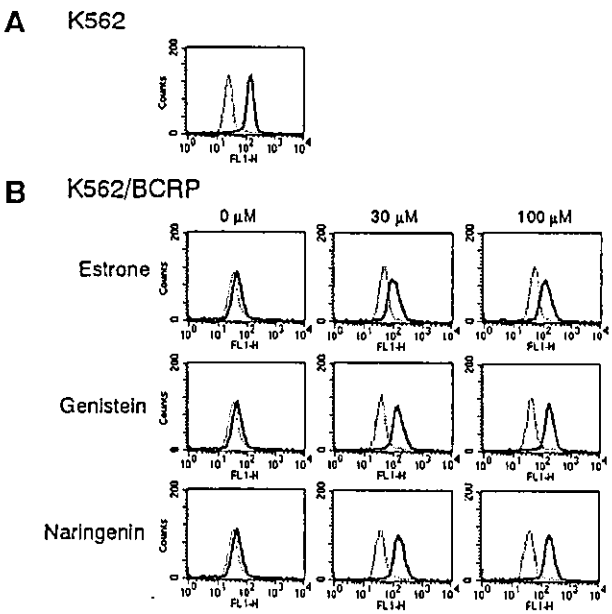

Fig. 8. Effects of estrone and flavonoids on the cellular uptake of topotecan in K562/BCRP cells. Cells were incubated with (*bold line*) or without (*dotted line*) 20 μM topotecan in the absence or presence of the indicated compound. Uptake of topotecan was measured using fluorescence-activated cell sorter. A fluorescence peak shift to the right was observed in K562 cells. In K562/BCRP cells, a fluorescence peak shift was not observed in the absence of estrone or flavonoids, but a fluorescence peak shift to the right indicating cellular uptake of topotecan occurred in a dose-dependent manner of the compounds.

migrated at Rf = 0.8 (segment 11). TLC of transported radioactivity after 4-h incubation showed that almost all of the [^{3}H]genistein was transported in its native form and migrated at Rf = 0.8 (segment 11) under these experimental conditions. Secretion of [^{3}H]genistein was significantly greater in LLC/BCRP cells than that in LLC-PK1 cells (Fig. 11), indicating that genistein itself is directly transported by BCRP. In a separate experiment, when [^{3}H]genistein was incubated with LLC-PK1 cells for 24 h, >80% of the radioactivity shifted to Rf = 0.12–0.21 (segment 3–4) and approximately 10% shifted to Rf = 0.38–0.47 (segment 6–7), in which metabolites of genistein were expected to exist (data not shown). However, only small amounts of such metabolites were found in the 4-h transport assay (Fig. 11).

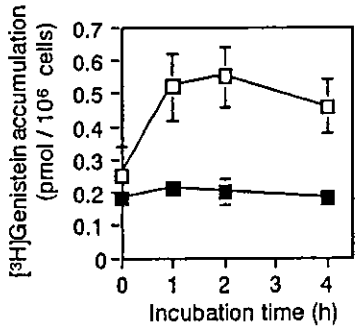

Fig. 9. Intracellular accumulation of [^{3}H]genistein in K562/BCRP cells. Cells (2×10^6) were incubated with 30 nM [^{3}H]genistein for 0, 1, 2, or 4 h at 37°C. After washing, the cells were dissolved in 100 μl PBS and 400 μl Soluene-350 and mixed with 5 ml ACS II scintillation mixture. Radioactivity was measured using a scintillation counter. The data are mean ± SD from triplicate determinations.

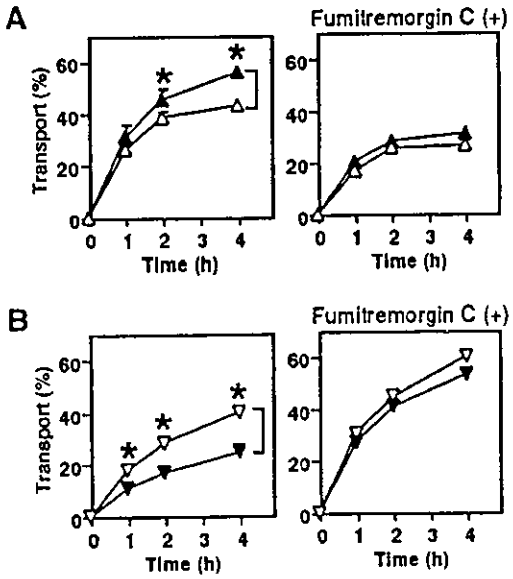

Fig. 10. Transcellular transport of [^{3}H]genistein by breast cancer resistance protein (BCRP). Cells (2.4×10^6/well) were plated on 3-μm pore filters and cultured for 3 days. The apical and the basal sides of the medium were replaced with 2 ml serum-free medium 1.5 h before beginning the experiment. When required, 3 μM fumitremorgin C was added to the apical and basal side medium at this time. [^{3}H]genistein (30 nM) was added to either the apical or basal side medium. After 1, 2, and 4 h, the percentage of radioactivity that appeared in the opposite side was measured. *A*, basal-to-apical transport of [^{3}H]genistein. *B*, apical-to-basal transport of [^{3}H]genistein. Open triangles, basal-to-apical transport in LLC-PK1, *closed triangles*, basal-to-apical transport in LLC/BCRP; *inverted open triangles*, apical-to-basal transport in LLC-PK1; and *inverted closed triangles*, apical-to-basal transport in LLC/BCRP. The data, expressed as a percent fraction of the total radioactivity, are mean ± SD of triplicate determinations from three different cultures. When a vertical bar is not shown, the SD is within the symbol. *$P < 0.05$. The data are representative of two independent experiments.

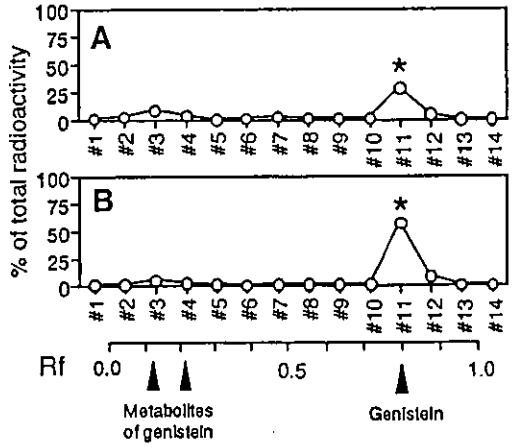

Fig. 11. Silica gel TLC of secreted [^{3}H]genistein and its corresponding metabolites. The experimental procedure was the same as that of the transepithelial transport assay until sample collection. Fifty μl of medium in the apical side after 4-h incubation were mixed with 100 μl of methanol, spotted, and run on silica gel plates in chloroform/methanol/acetic acid (8:3:1). Separated zones then were excised, and their radioactivities were measured. The radioactivity measurements are expressed as a percentage fraction of the total radioactivity before incubation. Data points are mean ± SD of triplicate determinations from three different cultures. *A*, TLC analysis of secreted ^{3}H-labeled compounds for 4 h in LLC-PK1 cells. *B*, TLC analysis of secreted ^{3}H-labeled compounds for 4 h in LLC/BCRP cells. Numbers 1–14 indicate segments in the silica gel TLC plate. [^{3}H]genistein is fractionated in segment 11, and metabolites of [^{3}H]genistein are found in segments 3 and 4. The data are means of triplicate determinations from three different cultures. The SD is within the symbol. *$P < 0.05$ between LLC-PK1 and LLC/BCRP cells.

DISCUSSION

Estrone and 17β-estradiol are the first endogenous compounds that were shown to exert strong BCRP-reversing activity (7). Synthesized estrogen agonists and antagonists also showed strong reversing activity of BCRP-mediated drug resistance (9). Therefore, we extended our studies to natural estrogenic compounds in the search for BCRP inhibitors.

Isoflavones derived from soybean, such as genistein and daidzein, constitute a subset of flavonoids that have been reported to have weak estrogenic activity (10). In addition, chemical structures of isoflavones resemble those of estrone and 17β-estradiol (Fig. 2). Naringenin, a flavanone contained in grapefruit juice, also resembles 17β-estradiol in chemical structure, albeit to a lesser extent than isoflavone (Fig. 2). Flavonoids are remarkably safe nutrients, being the most abundant polyphenolic compounds present in the human diet in fruits, vegetables, and plant-derived beverages such as tea and red wine (15). Some flavonoids also have been reported to interact with ABC transporters, such as P-glycoprotein, MRP1, MRP2, and cystic fibrosis transmembrane conductance regulator (16–21).

Genistein and naringenin displayed stronger interaction with BCRP than estrone (Fig. 3, *A–C*), and many flavones, such as acacetin, apigenin, chrysin, diosmetin, and luteolin, and some flavonols, such as kaempferide and kaempferol, demonstrated strong reversing activity of BCRP-mediated drug resistances (Fig. 3, *D* and *E*, and Fig. 4). Flavanol, flavanonol, and one-half of flavonols tested did not show BCRP-reversing activity (Fig. 4). Two glycosylated flavonoids, naringenin-7-glucoside and luteolin-4′-*O*-glucoside, did show anti-BCRP activity, whereas six other glycosylated flavonoids did not (Fig. 6). From these results, we speculated that the 3-hydroxyl group of the C ring might be important for BCRP-inhibitory activity, although some exceptions do exist. The flavonoids did not show growth-inhibitory effects in K562 and KB-3–1 cells at the highest concentrations used in our experiments, suggesting that they might be safely used for circumventing BCRP-mediated drug resistance in clinical practice. Glycosylated flavonoids with anti-BCRP activity

also may be useful because of their water solubility. In our preliminary animal experiments, some flavonoid aglycones were insoluble to either water or hydrophilic solvents and therefore would be difficult to administer i.v. Therefore, use of either glycosylated flavonoids or water-soluble derivatives of flavonoids would be an alternative way to develop BCRP inhibitors.

GF120918 was first developed as a P-glycoprotein inhibitor but also was shown to be a BCRP inhibitor (22, 23). TAG-139, which we identified as a tamoxifen-derived BCRP inhibitor, also, like tamoxifen, inhibited P-glycoprotein (9). Genistein was reported to be a substrate/inhibitor of MRP1 because genistein inhibited daunorubicin transport out of cells overexpressing MRP1 at a concentration of 50 μM (20). In the present study, genistein and naringenin effectively inhibited BCRP at a concentration of 3 μM (Fig. 3) but showed little effect on vincristine resistance in K562/MDR cells or on VP-16 resistance in KB/MRP cells even at a concentration of 10 μM (Fig. 7). Therefore, these flavonoids could be specific inhibitors of BCRP.

In previous studies, we showed that estrone inhibits BCRP function but was not transported by BCRP in its native form (7, 8). In contrast, progesterone is known to inhibit the function of P-glycoprotein but is not transported by P-glycoprotein (24). In the case of genistein, K562/BCRP cells accumulated smaller amounts of [^{3}H]genistein than parental K562 cells (Fig. 9). Secretion of [^{3}H]genistein from LLC/BCRP cells was greater than from LLC-PK1 cells in transcellular transport assays (Fig. 10). TLC analysis of transported [^{3}H]genistein suggested that there was increased transport of genistein aglycone in LLC/BCRP cells compared with LLC-PK1 cells (Fig. 11). Intracellular accumulation of [^{3}H]genistein also was decreased in LLC/BCRP cells compared with levels in parental cells (data not shown). These results suggest that inhibition of BCRP-mediated drug resistance by genistein is caused by the competitive transport of genistein by BCRP. Unlike estrone and 17β-estradiol, genistein would be transported in its native form but not in either sulfated or glucuronated forms (25, 26).

Another possible mechanism of BCRP inhibition by flavonoids is the interaction with the nucleotide-binding domain of BCRP because some flavonoids, including genistein, have been shown previously to interact with nucleotide-binding domain of P-glycoprotein, which was predicted to suppress ATP-hydrolysis and energy-dependent drug transport (16–18). In the case of BCRP, modulation of ATPase activity by the flavonoids should be investigated further to clarify this possibility.

The data presented here might have clinically important implications because some flavonoids effectively inhibited BCRP-mediated drug resistance at relatively low concentrations. For instance, we showed that 3 μM of genistein effectively circumvented BCRP-mediated drug resistance. Soybean (100 g) contains 100–200 mg isoflavones consisting of genistein, daidzein, glycitein, and their corresponding glycosides. Several groups have investigated the pharmacokinetics of soy isoflavone. A single bolus ingestion of 50 mg genistein in healthy premenopausal women was shown to result in a peak plasma concentration of 1.26 $\pm$ 0.27 μM at 9.33 $\pm$ 1.33 h (25). The rate of unconjugated genistein was only approximately 2–4%. However, in another report of 6-consecutive-day feeding study of 25 g soymilk powder in young adult women, the percentage of plasma aglycone genistein sampled on days 5 and 6 was 26 $\pm$ 7% of total genistein (26). In addition, glucuronide was the main metabolite in that study. Therefore, the concentration of active genistein in cancer tissues may reach sufficient level for BCRP inhibition via oral ingestion because β-glucuronidase activity is elevated in cancer (27). Parenteral administration may validate genistein as BCRP inhibitor. Subcutaneous injection of genistein was shown previously to enable high concentration of active genistein in mice (28).

The findings that glycosylated flavonoids naringenin-7-glucoside and luteolin-4′-*O*-glucoside effectively inhibited BCRP-mediated drug resistance might prove to be of great importance. These glycosylated flavonoids are well soluble in water. Because deglycosidation by intestinal enzymes precedes glucuronidation via oral routes, the i.v. injection of glycosylated flavonoids would bypass deglycosidation and retain their native structures, which are effective as BCRP inhibitors. In fact, i.v. naringenin-7-glucoside was excreted in urine mostly as its native glucoside form in a rat model (29).

Despite the promising results using flavonoids in the reversal of BCRP-mediated drug resistance, we must bear in mind that that coadministration or intake of flavonoids with BCRP-substrate antitumor agents may result in the alteration of their pharmacokinetics and may increase the toxicity of the antitumor drugs in the recipient patients. In this regard, orally administered GF120918 has been reported to increase oral bioavailability of topotecan (30).

In summary, phytoestrogens/flavonoids reverse BCRP-mediated drug resistance effectively, and these findings may bring direct and immediate clinical benefits via more effective and safer cancer chemotherapy treatments.

ACKNOWLEDGMENTS

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Gefitinib reverses breast cancer resistance protein–mediated drug resistance

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Abstract

Breast cancer resistance protein (BCRP) is an ATP binding cassette transporter that confers resistance to a series of anticancer agents such as 7-ethyl-10-hydroxycamptothecin (SN-38), topotecan, and mitoxantrone. In this study, we evaluated the possible interaction of gefitinib, a selective epidermal growth factor receptor tyrosine kinase inhibitor, with BCRP. *BCRP*-transduced human epidermoid carcinoma A431 (A431/BCRP) cells acquired cellular resistance to gefitinib, suggesting that BCRP could be one of the determinants of gefitinib sensitivity in a certain sort of cells. Next, the effect of gefitinib on BCRP-mediated drug resistance was examined. Gefitinib reversed SN-38 resistance in *BCRP*-transduced human myelogenous leukemia K562 (K562/BCRP) or *BCRP*-transduced murine lymphocytic leukemia P388 (P388/BCRP) cells but not in these parental cells. In addition, gefitinib sensitized human colon cancer HT-29 cells, which endogenously express BCRP, to SN-38. Gefitinib increased intracellular accumulation of topotecan in K562/BCRP cells and suppressed ATP-dependent transport of estrone 3-sulfate, a substrate of BCRP, in membrane vesicles from K562/BCRP cells. These results suggest that gefitinib may overcome BCRP-mediated drug resistance by inhibiting the pump function of BCRP. Furthermore, P388/BCRP-transplanted mice treated with combination of irinotecan and gefitinib survived significantly longer than those treated with irinotecan alone or gefitinib alone. In conclusion, gefitinib is shown to interact with BCRP. BCRP expression in a certain sort of cells is supposed to be one of the determinants of gefitinib sensitivity. Gefitinib inhibits the transporter function of BCRP and reverses BCRP-mediated drug resistance both *in vitro* and *in vivo*. [Mol Cancer Ther 2004;3(9):1119–25]

Introduction

ATP binding cassette (ABC) transporters, such as P-glycoprotein (1, 2) and MRP1 (3), are involved in multidrug resistance. They pump out various structurally unrelated anticancer agents in an energy-dependent manner. Breast cancer resistance protein (BCRP, also known as ABCG2) is a half-molecule ABC transporter with an NH_2-terminal ATP binding site and a COOH-terminal transmembrane domain (4–8) and acts as a homodimer (9). BCRP is widely expressed in normal human tissues such as placenta, liver, prostate, small intestine, ovary, colon, and capillary endothelial cells and hematopoietic stem cells (5, 10–12). BCRP is presumed to play a protective role against toxic substrates and metabolites. In addition, overexpression of BCRP confers resistance to anticancer agents such as 7-ethyl-10-hydroxycamptothecin (SN-38), topotecan, 9-aminocamptothecin, and mitoxantrone (7, 13–15). The role of BCRP in clinical drug resistance has not been established, but some reports have shown an association between BCRP expression and poor responses to chemotherapy (16, 17). BCRP has been also shown to be an important determinant of oral bioavailability of BCRP substrate drugs (18).

Gefitinib (ZD1839, Iressa) is an orally active, selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that blocks signal transduction pathways implicated in the proliferation and survival of tumor cells (19, 20). Gefitinib markedly inhibits the autophosphorylation of epidermal growth factor–stimulated EGFR in a broad range of EGFR-expressing human cancer cell lines and xenograft models and has also shown a marked antitumor activity either on its own or in combination in various human tumor xenograft models, especially non-small cell lung cancers (19–22). Gefitinib also showed superior antitumor activity against lung cancers that were refractory to chemotherapy in clinical studies and is now an approved cancer drug in several countries including Japan and the United States (23–27). Further clinical studies of gefitinib in other tumor types, such as breast, head and neck, prostate, breast, gastric, and colorectal tumors, are also ongoing worldwide.

In this study, we evaluated the possible interaction of gefitinib with BCRP. *BCRP*-transduced A431 cells showed resistance to gefitinib. Gefitinib inhibited the transporter function of BCRP and reversed BCRP-mediated drug resistance both *in vitro* and *in vivo*.

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Materials and Methods

Cells and Cell Culture

A431 human epidermoid carcinoma cells, KB3-1 human epidermoid carcinoma cells, and HT-29 human colon cancer cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. K562 human myelogenous leukemia cells and P388 murine lymphocytic leukemia cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. A431/BCRP, K562/BCRP, and P388/BCRP cells were established by the transduction of A431, K562, and P388 cells, respectively, with a HaBCRP retrovirus that carries Myc-tagged human *BCRP* cDNA in the Ha retrovirus vector (9). A431/MDR and K562/MDR cells were established by the transduction of A431 and K562 cells, respectively, with a HaMDR retrovirus that carries human *MDR1* cDNA in the same vector (28). The KB/MRP cells were made by introducing the expression vector, pJ3Ω-MRP, containing human *MRP1* cDNA into KB3-1 cells (29). The stably transfected cell lines were maintained in drug-free medium for up to 3 months.

Western Blot Analysis

The anti-BCRP polyclonal antibody 3488 was raised by immunizing rabbits with a keyhole limpet hemocyanin–conjugated 20-mer peptide corresponding to amino acid sequence 340 to 359 of human BCRP protein (9). The anti–P-glycoprotein antibody, C219, the anti-MRP1 antibody, MRPm6, and anti–α-tubulin antibody, B-5-1-2, were obtained from Centocor (Malvern, PA), Nichirei (Tokyo, Japan), and Sigma-Aldrich Chemical (St. Louis, MO), respectively. Western blot assays were done as reported previously (9). In brief, cell lysates were solubilized with 2% SDS, 50 mmol/L Tris-HCl (pH 7.5), and 5% 2-mercaptoethanol and resolved by 5% to 20% SDS-PAGE (20 μg protein per lane). After electrophoresis, proteins were transferred onto nitrocellulose membranes. Blots were incubated with anti-BCRP (8 μg/mL), anti–P-glycoprotein (2 μg/mL), anti-MRP1 (2 μg/mL), or anti–α-tubulin (1 μg/mL) antibody. After washing, the blots were incubated in 1:500 dilution of the appropriate peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Membrane-bound peroxidase was visualized using Enhanced Chemiluminescence Plus detection kit (Amersham Pharmacia Biotech). Each blot was exposed for 1 minute (BCRP, P-glycoprotein, and MRP) or 5 seconds (α-tubulin).

Growth Inhibition Assay

Gefitinib was supplied by AstraZeneca UK Ltd. (London, United Kingdom). Cells (2×10^5–3×10^5 cells/well) were plated in 12-well dishes and treated with increasing doses of anticancer agents. After 5 days of treatment, cell number was counted with a Coulter counter and drug dose causing 50% inhibition of cell growth (IC_{50}) was determined.

Intracellular Drug Accumulation

The effect of gefitinib on the cellular accumulation of topotecan was determined by flow cytometry. K562 and K562/BCRP cells (5×10^5 cells each) were incubated with 20 μmol/L topotecan for 30 minutes at 37°C in the absence or presence of gefitinib (10, 30, and 100 μmol/L), washed in ice-cold PBS, and subjected to fluorescence analysis using a FACSCalibur (Becton Dickinson, San Jose, CA) with 488 nm excitation.

Intravesicular Transport Assay

Membrane vesicles of K562/BCRP were prepared according to the method described previously (30). The vesicular transport assay was done by a rapid centrifugation technique using ^{3}H-labeled estrone 3-sulfate (E1S), a substrate of BCRP (31). [^{3}H]E1S was purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA). First, the transport reaction mixture [50 μL; 50 mmol/L Tris-HCl (pH 7.4), 10 mmol/L $MgCl_2$, 250 mmol/L sucrose, 10 mmol/L phosphocreatine, 100 μg/mL creatine phosphokinase, with or without 5 mmol/L ATP, 50 nmol/L [^{3}H]E1S, and membrane vesicles (50 μg protein)] was kept on ice for 5 minutes and incubated at 37°C for an appropriate time. The reaction was terminated by the addition of ice-cold stop solution [1 mL; 10 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, 250 mmol/L sucrose]. The membrane vesicles were centrifuged at 18,000 × *g* for 10 minutes at 4°C. The pellet was solubilized with 0.1 mol/L NaOH (100 μL) and neutralized by the addition of 0.1 mol/L HCl. The radioactivity levels were measured by a liquid scintillation counter.

Animal Studies

Six-week-old female CDF1 mice were supplied by Charles River Japan, Inc. (Kanagawa, Japan) and maintained under specific pathogen-free conditions and provided with sterile food and water *ad libitum*. The mice were transplanted with P388 or P388/BCRP cells (10^6 cells per mouse) i.p. from the left flank. For the evaluation of the drug sensitivity of P388/BCRP *in vivo*, irinotecan (30 mg/kg, Yakult Honsha, Tokyo, Japan) and/or gefitinib (150 mg/kg) were given i.p. to the transplanted mice four times with 3-day intervals. The effects of these anticancer agents were evaluated by their effects on survival (the mean survival ratio of treated mice to control mice). Statistical evaluation of a difference between two sets of data was done by a two-tailed Student's *t* test. $P < 0.05$ was considered significant.

Results

Immunoblot Analysis of ABC Transporters

A431/BCRP cells were established by the transduction of A431 cells with the HaBCRP retrovirus and subsequent selection with 51 nmol/L SN-38 for 5 days. K562/BCRP and P388/BCRP cells were also established by a similar SN-38 selection. A431/MDR and K562/MDR were established by the transduction of A431 and K562 cells, respectively, with HaMDR retrovirus and subsequent selection with 4 nmol/L vincristine (VCR) for 7 days. KB/MRP was established by the transfection of KB3-1 cells with pJ3Ω-MRP and subsequent selection with increasing concentrations of doxorubicin. The expressions of BCRP, P-glycoprotein, and MRP1 in these variants were confirmed by Western blot analysis (Fig. 1). BCRP expression

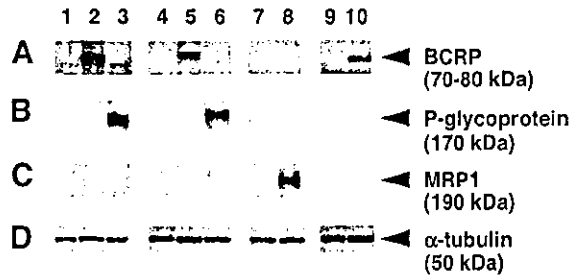

Figure 1. Expression of BCRP, P-glycoprotein, and MRP1 in the transfectants. A, BCRP expression. B, P-glycoprotein expression. C, MRP1 expression. D, α-tubulin expression. *Lane 1*, A431; *lane 2*, A431/BCRP; *lane 3*, A431/MDR; *lane 4*, K562; *lane 5*, K562/BCRP; *lane 6*, K562/MDR; *lane 7*, KB3-1; *lane 8*, KB/MRP; *lane 9*, P388; *lane 10*, P388/BCRP.

was detected in A431/BCRP, K562/BCRP, and P388/BCRP cells (Fig. 1A). A431/MDR and K562/MDR cells expressed P-glycoprotein (Fig. 1B) and MRP1 expression was detected in KB/MRP cells (Fig. 1C).

Drug Resistance of ABC Transporter–Expressing Cells

The sensitivity of ABC transporter–expressing cells to anticancer agents was examined using a growth inhibition assay (Fig. 2; Table 1). A431/BCRP and K562/BCRP cells acquired resistance to SN-38, and A431/MDR and K562/MDR cells acquired resistance to VCR as compared with respective parental cells (Fig. 2A and C; Table 1). P388/BCRP cells acquired resistance to SN-38 (Table 1). KB/MRP cells also acquired ~10-fold higher resistance to etoposide (VP-16) than the parental cells (Table 1). As shown in Fig. 2B, A431/BCRP cells showed cross-resistance to gefitinib. The IC_{50} value of gefitinib in A431/BCRP cells was 75.8 nmol/L, which was 9.07-fold higher than that in the parental A431 cells (8.43 nmol/L; Fig. 2B; Table 1). A431/MDR cells also showed a 2.36-fold higher resistance to gefitinib than A431 cells (Table 1). These results suggest that BCRP and P-glycoprotein are involved in the cellular resistance to gefitinib in A431 cells. In contrast, K562/BCRP, K562/MDR, and P388/BCRP cells did not show gefitinib resistance (Fig. 2D; Table 1).

Reversal of BCRP-Mediated Drug Resistance by Gefitinib

The effects of gefitinib on BCRP-mediated drug resistance were examined. As shown in Fig. 3A, ≤1 μmol/L gefitinib strongly enhanced the cytotoxicity of SN-38 in K562/BCRP cells but not in K562 cells. Gefitinib alone at 1 μmol/L showed little growth inhibitory effects on K562 or K562/BCRP cells (Fig. 2D). Gefitinib also potentiated the cytotoxicity of SN-38 in P388/BCRP cells (Fig. 3B). Next, effects of gefitinib on drug resistance in either P-glycoprotein–expressing or MRP1-expressing cells were examined to evaluate transporter specificity. Gefitinib enhanced VCR cytotoxicity in K562/MDR cells in a dose-dependent manner but showed no effect on MRP1-mediated etoposide (VP-16) resistance in KB/MRP cells (Fig. 3C and D). Possible effect of gefitinib on cells that endogenously express BCRP was examined using human colon cancer cells HT-29 (Fig. 4). As shown in Fig. 4B, gefitinib at 0.3 and 1 μmol/L increased the SN-38 cytotoxicity of HT-29 cells. Gefitinib alone showed no effect on the growth of HT-29 cells at 1 μmol/L (data not shown).

Effect of Gefitinib on BCRP-Mediated Transport

The effects of gefitinib on the intracellular accumulation of topotecan in BCRP-expressing cells were examined by flow cytometric analysis. After incubating cells with 20 μmol/L topotecan, significant increases in fluorescence intensity occurred in K562 cells (Fig. 5A). However, the cellular fluorescence of K562/BCRP cells increased only marginally, suggesting that BCRP exports topotecan out of the cells (Fig. 5B). Gefitinib treatment enhanced the intracellular accumulation of topotecan in K562/BCRP cells in a dose-dependent manner (Fig. 5D, F, and H) but showed no effect on that in parental K562 cells (Fig. 5C, E, and G). Similar effects of gefitinib were observed in P388/BCRP cells (data not shown). Next, the effects of gefitinib on the BCRP-mediated transport of E1S were examined using membrane vesicles from K562/BCRP cells. As shown in Fig. 6, ATP-dependent uptake of [^{3}H]E1S was inhibited by gefitinib in a dose-dependent manner. The IC_{50} value of gefitinib to inhibit the pump function of BCRP was 1.01 ± 0.30 μmol/L. No ATP-dependent E1S uptake was observed in K562 membrane vesicles (data not shown). Taken together, these results suggest that gefitinib inhibits BCRP-mediated transport.

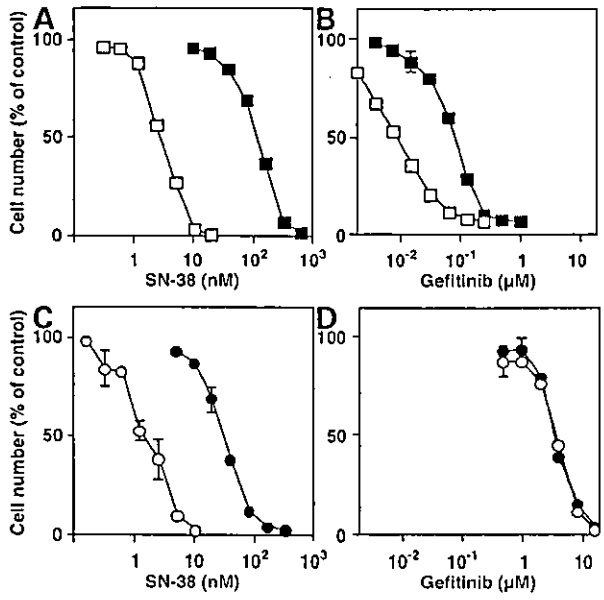

Figure 2. A, SN-38 sensitivity of A431 and A431/BCRP cells. B, gefitinib sensitivity of A431 and A431/BCRP cells. C, SN-38 sensitivity of K562 and K562/BCRP cells. D, gefitinib sensitivity of K562 and K562/BCRP cells. Cells were cultured for 5 days with increasing concentrations of SN-38 (A and C) or gefitinib (B and D). Cell numbers were determined with a Coulter counter. *Points*, mean of triplicate determinations; *bars*, SD. □, A431; ■, A431/BCRP; ○, K562; ●, K562/BCRP.

Table 1. Drug resistance of ABC transporter–expressing cells

Cell	IC_{50} (nmol/L)				Degree of Resistance			
	SN-38	VCR	Gefitinib	VP-16	SN-38	VCR	Gefitinib	VP-16
A431	2.91 ± 0.08	0.15 ± 0.02	8.43 ± 1.15					
A431/BCRP	120 ± 1.15	ND	75.8 ± 5.57		41.4	—	9.07	
A431/MDR	ND	57.6 ± 1.83	19.7 ± 0.38		—	380	2.36	
K562	1.68 ± 0.56	0.07 ± 0.005	3,480 ± 52.9					
K562/BCRP	30.6 ± 0.82	ND	3,250 ± 108		18.2	—	0.93	
K562/MDR	ND	23.8 ± 3.65	4,140 ± 137		—	367	1.19	
P388	15.2 ± 1.71	ND	2,420 ± 191					
P388/BCRP	194 ± 9.07	ND	4,130 ± 214		12.8	—	1.71	
KB3-1				111 ± 2.67				
KB/MRP				1,200 ± 151				10.8

NOTE: Cells were cultured for 5 days in the absence or presence of increasing concentrations of the indicated anticancer agents. Cell numbers were determined with a Coulter counter and IC_{50} values were calculated. The degree of resistance is the ratio of the IC_{50} values for *BCRP*-transduced, *MDR1*-transduced, or *MRP*-transduced cells divided by those for the parental cells. Data are means ± SD from triplicate determinations. ND, not determined; VP-16, etoposide.

Reversal of BCRP-Mediated Drug Resistance by Gefitinib *In vivo*

To evaluate the circumvention of drug resistance by gefitinib *in vivo*, mice (six per group) were transplanted i.p. with either P388 or P388/BCRP cells (10^6 cells per mouse) and subsequently treated with irinotecan (30 mg/kg) and/or gefitinib (150 mg/kg) four times on days 1, 4, 7, and 10 successively. The survival time of P388-transplanted mice without treatment was 9.3 ± 0.5 days. The survival time of the P388-inoculated mice, treated with irinotecan alone, was 26.7 ± 2.9 days, with a treated versus control of 287%. Gefitinib was not effective against P388 cells *in vivo* (Table 2). Treatment of neither irinotecan alone nor gefitinib alone was effective in P388/BCRP-transplanted mice. These results suggest that BCRP is responsible for irinotecan resistance *in vivo*. However, coadministration of irinotecan with gefitinib extended the survival of P388/BCRP-inoculated mice, with a treated versus control of 140% (P = 0.017; Table 2). These results indicate that gefitinib reverses BCRP-mediated irinotecan resistance *in vivo*.

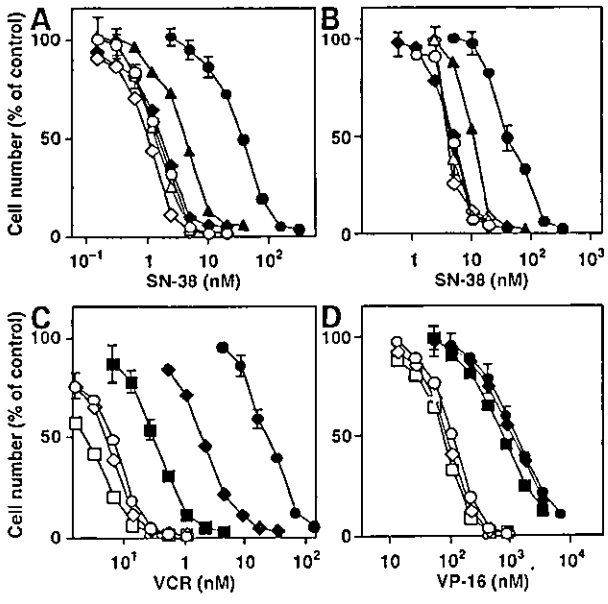

Figure 3. Effects of gefitinib on ABC transporter–mediated drug resistance. Cells were cultured for 5 days with increasing concentrations of anticancer agents in the absence or presence of gefitinib. Cell numbers were determined with a Coulter counter. *Points*, mean of triplicate determinations; *bars*, SD. A, effect of gefitinib on the SN-38 sensitivity of K562 (○, △, and ◇) and K562/BCRP (●, ▲, and ◆) cells. B, effect of gefitinib on the SN-38 sensitivity of P388 (○, △, and ◇) and P388/BCRP (●, ▲, and ◆) cells. C, effect of gefitinib on the VCR sensitivity of K562 (○, △, and ◇) and K562/MDR (●, ▲, and ◆) cells. D, effect of gefitinib on the etoposide (*VP-16*) sensitivity of KB3-1 (○, △, and ◇) and KB/MRP (●, ▲, and ◆) cells. ○ and ●, without gefitinib; △ and ▲, 0.3 μmol/L gefitinib; ◇ and ◆, 1 μmol/L gefitinib; □ and ■, 3 μmol/L gefitinib.

Discussion

In this study, we describe the interaction of gefitinib with BCRP. A431/BCRP cells showed higher resistance to gefitinib than the parental cells (Fig. 2B; Table 1), suggesting

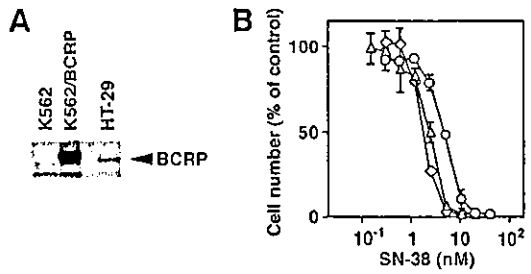

Figure 4. Effect of gefitinib on cells that express endogenous BCRP. HT-29 cells were cultured for 5 days with increasing concentrations of SN-38 in the absence or presence of gefitinib. A, BCRP expression. B, effect of gefitinib on the SN-38 sensitivity of HT-29 cells. ○, without gefitinib; △, 0.3 μmol/L gefitinib; ◇, 1 μmol/L gefitinib.

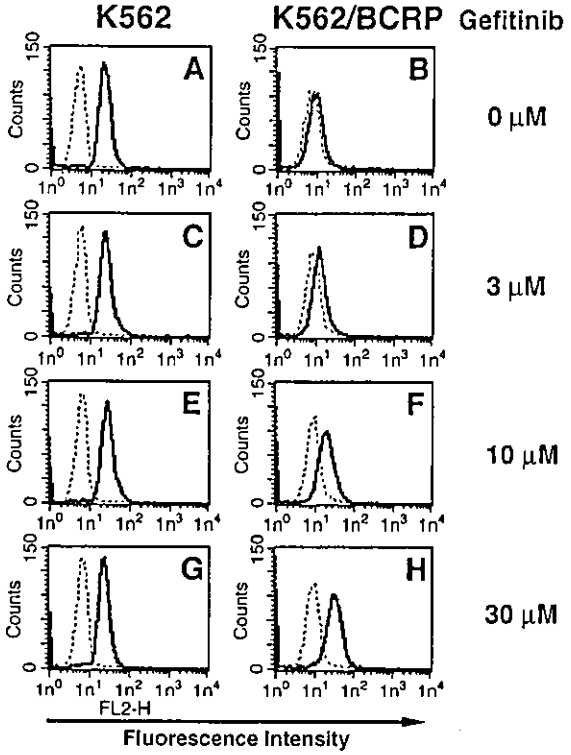

Figure 5. Effect of gefitinib on the intracellular accumulation of topotecan in K562 (**A**, **C**, **E**, and **G**) and K562/BCRP (**B**, **D**, **F**, and **H**) cells. Cells were incubated in the absence (*dotted lines*) or presence (*bold lines*) of 20 μmol/L topotecan in combination with gefitinib at doses of 0 μmol/L (**A** and **B**), 3 μmol/L (**C** and **D**), 10 μmol/L (**E** and **F**), or 30 μmol/L (**G** and **H**). Cellular topotecan content was measured by fluorescence-activated cell sorting.

that BCRP may be one of the determinants of gefitinib resistance in a certain sort of cells. Reversal of BCRP-mediated drug resistance by gefitinib was also observed in both K562/BCRP and P388/BCRP cells (Fig. 3A and B). Gefitinib increased the cellular accumulation of topotecan in K562/BCRP (Fig. 5) and suppressed the ATP-dependent transport of E1S in membrane vesicles from K562/BCRP cells (Fig. 6). These findings suggest that gefitinib inhibits the transporter function of BCRP and increase the cytotoxicity of BCRP substrate anticancer agents.

As described above, A431/BCRP cells acquired gefitinib resistance, whereas K562/BCRP cells did not. We do not know the reason why A431/BCRP is resistant to gefitinib but K562/BCRP is not. K562/BCRP and A431/BCRP cells expressed similar levels of BCRP and showed similar levels of SN-38 resistance. Therefore, difference in the BCRP expression levels is not the reason for this discrepancy. In our preliminary observation, *BCRP*-transduced human non-small cell lung cancer PC-9 (PC-9/BCRP) cells also acquired gefitinib resistance. PC-9 and A431 are highly sensitive to gefitinib (32). PC-9 and A431 cells express EGFR and their growth is supposed to be EGFR dependent. Gefitinib seems to show growth inhibitory effect against PC-9 and A431 through, at least in part, EGFR-dependent signaling pathway. In contrast, K562/BCRP and P388/BCRP cells showed similar levels of gefitinib sensitivity to their parental cells (Table 1). K562 and P388 cells do not express EGFR and are relatively resistant to gefitinib. Mechanism of gefitinib cytotoxicity against K562 and P388 is not clear because EGFR-dependent pathway does not function in these cells. Growth inhibitory effects of gefitinib in such EGFR-negative cells should be independent of epidermal growth factor signaling and EGFR phosphorylation; therefore, some other low-affinity molecular targets of gefitinib may exist. Anyway, we could hypothesize from the current study that BCRP expression confers gefitinib resistance in cells that express EGFR and grow EGFR dependently. BCRP could be an important determinant for anticancer activity of gefitinib in clinical situations.

There are several possible mechanisms in the enhancement of SN-38 cytotoxicity in A431/BCRP by gefitinib. One is the competitive inhibition of the BCRP-mediated SN-38 efflux by gefitinib. To clarify this mechanism, direct transport experiments such as membrane vesicle transport assays or drug accumulation/efflux studies are currently ongoing in our laboratory. Whether gefitinib is a member of the BCRP substrate group of anticancer agents is a very important subject. Other tyrosine kinase inhibitors, CI1033 and imatinib mesylate, have been shown to inhibit the function of BCRP (33, 34). The second possible mechanism is the inhibition of BCRP function through the inhibition of protein phosphorylation. At present, no report exists on the role of possible BCRP phosphorylation on the transporter function. In addition, other unknown gefitinib-sensitive protein phosphorylation pathway may exist and regulate the drug efflux function of BCRP. In this study, we have also shown that A431/MDR cells acquired low gefitinib resistance, but K562/MDR cells did not. These results suggest that gefitinib resistance mediated by ABC transporters depends, at least in part, on the EGFR-dependent growth of tumor cells or cellular gefitinib sensitivity. The third

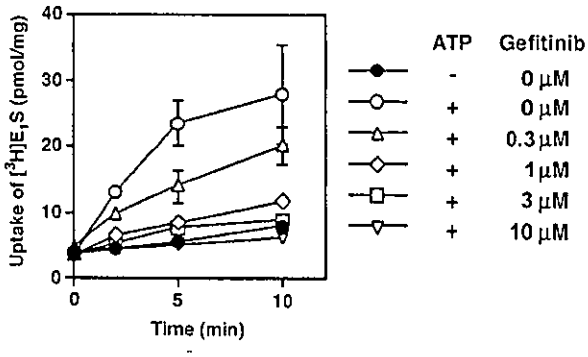

Figure 6. Effect of gefitinib on the intravesicular uptake of E1S. Membrane vesicles from K562/BCRP were incubated at 37°C with 50 nmol/L [^{3}H]E1S and increasing concentrations of gefitinib in the absence (●) or presence (○, △, ◇, □, and ▽) of 5 mmol/L ATP. *Points*, mean of triplicate determinations; *bars*, SD.

Table 2. Circumvention of BCRP-mediated drug resistance *in vivo* by gefitinib

Tumor	Irinotecan (mg/kg)	Gefitinib (mg/kg)	Survival (d)*	T/C (%)†	T/I (%)‡
P388	0	0	9.3 ± 0.5	100	
	30	0	26.7 ± 2.9	287§	100
	0	150	9.3 ± 0.8	100	
	30	150	27.8 ± 3.1	299§	104
P388/BCRP	0	0	11.3 ± 1.0	100	
	30	0	11.5 ± 0.5	102	100
	0	150	10.2 ± 1.2	90	
	30	150	15.8 ± 1.8	140‖	137

*Data are means ± SD.
† The ratio of survival time to that of mice without treatment.
‡ The ratio of survival time to that of irinotecan-treated mice.
§ $P < 0.01$ compared with control.
‖ $P = 0.017$ compared with control.

explanation would be that gefitinib and other kinase inhibitors may inhibit energy production needed for pump function by competing with ATP for binding to BCRP. This possibility will also be examined in the future.

On the other hand, the reversal activity of BCRP-mediated drug resistance by gefitinib in K562/BCRP and P388/BCRP cells suggests that gefitinib can interact with BCRP in these cell types, too. The BCRP inhibitory effect of gefitinib may be also explained by competitive inhibition of BCRP-mediated drug export. We examined the effect of gefitinib on SN-38 resistance of K562/BCRP and P388/BCRP cells at gefitinib concentrations of 0.3 and 1 μmol/L (Fig. 3A and B). Gefitinib at 0.3 and 1 μmol/L showed good reversal activity of the SN-38 resistance of K562/BCRP and P388/BCRP cells (Fig. 3A and B). K562/BCRP and P388/BCRP did not show any SN-38 resistance in the presence of 1 μmol/L gefitinib. Reportedly, the maximum plasma concentrations resulting from clinically relevant gefitinib doses were 0.5 to 1 μmol/L (27). This suggests that the blood levels of gefitinib would be sufficient for the reversal of BCRP-mediated drug resistance *in vivo*. In general, doses of anticancer agents used in mice are much higher than those in human. Kunimoto et al. (35) have examined the antitumor activities of irinotecan in mice at doses of 100 or 200 mg/kg. In this study, irinotecan was given to mice at similar dosage (30 mg/kg/d for 4 days, 120 mg/kg in total). Gefitinib has shown the antitumor activities in xenografts of A549, Dul45, A431, CR10, HCT15, HT-29, Lovo, KB, or HX62 cells at doses of 12.5 to 200 mg/kg/d (36). In this study, gefitinib was given to mice at 150 mg/kg. Gefitinib dosage may be relatively high in our study because inhibitor of transporter (gefitinib) is used generally at higher dosage than the substrate of transporter (irinotecan). Evaluation of clinical significance of coadministration of irinotecan and gefitinib must await further clinical investigations.

Although gefitinib seems to be effective in reversal of BCRP-mediated resistance to irinotecan *in vivo*, the reversal is not complete (~50% of the control). BCRP transfectants used in this study have been established after the selection of the transfected cells with appropriate anticancer agents for only several days. Therefore, other drug resistance mechanism such as P-glycoprotein or DNA repair pathway would not exist. Reason for the incomplete reversal of the drug resistance *in vivo* may be attributable to the bioavailability and pharmacokinetics/pharmacodynamics of the drugs.

As both irinotecan and gefitinib are effective against non-small cell lung cancer, they may be used in combination in clinical practice. The pharmacologic interaction of these agents, through the inhibition of BCRP, should be considered if such regimens are employed. Previously, interaction between irinotecan and gefitinib was reported in human colorectal cancer cell lines (37). In our *in vivo* study, no weight loss was observed in irinotecan-alone–treated or gefitinib-alone–treated mice. However, combined treatment of irinotecan and gefitinib showed toxicity in mice; especially, P388-transplanted mice lost 4 g on day 15 and regained on day 18. This may be due to the inhibition of endogenous BCRP by gefitinib. Gefitinib may also increase the cytotoxicity of irinotecan in BCRP-expressing normal tissues.

In summary, we have shown that gefitinib interacts with BCRP. BCRP expression is supposed to be one of the determinants of gefitinib sensitivity in a certain sort of cells. Gefitinib inhibits the transporter function of BCRP and reverses BCRP-mediated drug resistance both *in vitro* and *in vivo*.

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Induction of cellular resistance to nucleoside reverse transcriptase inhibitors by the wild-type breast cancer resistance protein

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Abstract

Breast cancer resistance protein (BCRP/ABCG2) is a novel member of ATP-binding cassette transporters, which induce multidrug resistance in cancer cells. We previously reported that a high level of BCRP expression in $CD4^+$ T cells conferred cellular resistance to nucleoside reverse transcriptase inhibitors (NRTIs) of human immunodeficiency virus type 1 (HIV-1). However, this BCRP was found to have a mutation of Arg to Met at position 482 ($BCRP_{R482M}$). The present study demonstrated that the wild-type BCRP ($BCRP_{WT}$) also conferred cellular resistance to NRTIs. MT-4 cells (a $CD4^+$ T-cell line) highly expressing $BCRP_{WT}$ (MT-4/BCRP) were generated and the expression of $BCRP_{WT}$ was confirmed by genotypic and phenotypic analyses. Compared to the parental MT-4 cells, MT-4/BCRP cells displayed resistance to zidovudine (AZT) in terms of antiviral activity as well as drug cytotoxicity. In addition, other NRTIs were also less inhibitory to HIV-1 replication in MT-4/BCRP cells than in MT-4 cells. Significant reduction of intracellular AZT accumulation was observed in MT-4/BCRP cells. An analysis for intracellular metabolism of AZT suggested that the resistance was attributed to the increased efflux of AZT and its metabolites in MT-4/BCRP cells. Furthermore, the BCRP-specific inhibitor fumitremorgin C completely restored the reduction of AZT in MT-4/BCRP cells. These results indicate that, like $BCRP_{R482M}$, $BCRP_{WT}$ also plays an important role in cellular resistance to NRTIs.

Keywords: BCRP/ABCG2; Wild type; T-cell line; HIV-1; NRTI; Resistance

1. Introduction

Significant advances in the treatment of human immunodeficiency virus type 1 (HIV-1) infection have been achieved with highly active antiretroviral therapy (HAART), which is conducted by combination with drugs that block different steps in the viral replication cycle, such as reverse transcription and protein processing. However, the decrease of efficacy with increasing a treatment period becomes a major concern associated with long-term therapy with antiretroviral agents. In fact, HAART often results in treatment failure due to the emergence of drug-resistant HIV-1 mutants [1]. On the other hand, some patients display a sign of drug resistance without emergence of resistant mutants [2], suggesting that certain cellular factors in part account for the failure of antiretroviral therapy.

Abbreviations: HIV-1, human immunodeficiency virus type 1; HAART, highly active antiretroviral therapy; ABC, ATP-binding cassette; P-gp, P-glycoprotein; PI, protease inhibitor; CNS, central nervous system; AZT, zidovudine; MRP, multidrug resistance protein; NRTI, nucleoside reverse transcriptase inhibitor; PMEA, phosphornyl-methoxyethyladenine; BCRP, breast cancer resistance protein; DOX, doxorubicin; 3TC, lamivudine; d4T, stavudine; ddI, didanosine; ddC, zalcitabine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; APMSF, *p*-amindinophenylmethanesulfonylfluoride; mAb, monoclonal antibody; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; IC_{50}, 50% inhibitory concentration; EC_{50}, 50% effective concentration; AZTMP, AZT 5′-monophosphate; AZTDP, AZT 5′-diphosphate; AZTTP, AZT 5′-triphosphate; NNRTI, non-nucleoside reverse transcriptase inhibitor; BBB, blood–brain barrier

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A common mechanism of resistance to anticancer agents is drug-induced expression of the ATP-binding cassette (ABC) transporters, which act as an ionic pump and prevent intracellular accumulation of anticancer agents in tumor cells. P-glycoprotein (P-gp/ABCB1), one of the ABC transporters, has proved to be an important determinant for the oral bioavailability of HIV-1 protease inhibitors (PIs) and their entrance into central nervous system (CNS). P-gp also appears to affect drug penetration into other tissues serving as sanctuaries for HIV-1 [3]. It was reported that overexpression of P-gp was associated with reduced antiviral activity of zidovudine (AZT) against HIV-1 [4]. In addition, overexpression of multidrug resistance protein (MRP/ABCC) 4 was found to severely impair the anti-HIV-1 activity of AZT and other nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs), such as phosphonyl-methoxyethyladenine (PMEA) [5].

Recently, breast cancer resistance protein (BCRP/ABCG2), a new member of the ABC transporter superfamily, was identified in the atypical multidrug-resistant human breast cancer cell line MCF-7, which was selected in the presence of doxorubicin (DOX) and verapamil [6]. BCRP is the second member of the G (white) subfamily of ABC transporters and is also known as the mitoxantrone-resistance protein MXR [7] or the placental ABC transporter ABCP [8]. This glycosylated plasma membrane protein is a half-size transporter evolutionarily distinct from other full-size ABC transporters [9]. Cells highly expressing BCRP showed resistance to mitoxantrone, and to a lesser extent, to DOX, daunorubicin, and topotecan. We have recently established a DOX-resistant $CD4^+$ T-cell line MT-4/DOX_{500} and found that the cells are expressing a high level of BCRP but not other multidrug resistance proteins [10]. This BCRP was found to have a mutation of Arg to Met at position 482 ($BCRP_{R482M}$). Using this cell line, it was demonstrated that high level expression of $BCRP_{R482M}$ in $CD4^+$ T cells brought about reduced anti-HIV-1 activity of AZT and lamivudine (3TC). However, it still remains to be elucidated whether the wild-type BCRP ($BCRP_{WT}$) similarly interacts with NRTIs and affects their antiviral activity and cytotoxicity.

In the present study, we have generated MT-4 cells highly expressing $BCRP_{WT}$ (MT-4/BCRP) and examined NRTIs for their cytotoxicity and anti-HIV-1 activity in MT-4/BCRP cells. The results clearly show that, like $BCRP_{R482M}$, $BCRP_{WT}$ also confers cellular resistance to NRTIs through increasing drug efflux from the cells.

2. Materials and methods

2.1. Compounds

DOX, paclitaxel, mitoxantrone, and rhodamine 123 were purchased from Sigma. 7-Ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11) and 7-ethyl-10-hydroxycamptothecin (SN-38) were obtained from Daiichi Pharmaceuticals. AZT, stavudine (d4T), didanosine (ddI), and zalcitabine (ddC) were also purchased from Sigma. 3TC was kindly provided by Mitsubishi Chemical Corporation. The BCRP-specific inhibitor fumitremorgin C [11,12] was a generous gift from Dr. Rabindran, Wyeth-Ayerst Research.

2.2. Cells

The human $CD4^+$ T-cell line MT-4 [13] was grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin (culture medium). MT-4 cells were transduced with an HaBCRP retrovirus supernatant, and the cells were treated and maintained with 4 ng/ml mitoxantrone [14]. The obtained mitoxantrone-resitant cells, MT-4/BCRP, were used in this study. MT-4/DOX_{500} cells [10] were maintained in the presence of 500 ng/ml DOX. Prior to cytotoxicity and antiviral assays, MT-4/BCRP and MT-4/DOX_{500} cells were cultured in the absence of any compounds at least for 7 days.

2.3. Cytotoxicity assay

The cells (1×10^5 cells/ml) were cultured in the presence of various concentrations of test compounds. After a 4-day incubation at 37 °C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [15].

2.4. Drug accumulation and retention assay

Intracellular drug accumulation and retention in MT-4, MT-4/BCRP, and MT-4/DOX_{500} cells were determined by a slight modification of the flow cytometric method described previously [16]. Briefly, the cells were exposed to either 10 μg/ml DOX, 10 μg/ml mitoxantrone, or 100 ng/ml rhodamine 123 for 30 min (accumulation phase) and washed with ice-cold phosphate buffered saline (PBS). The cells were resuspended in culture medium in the absence of compounds, further incubated for 60 min at 37 °C (retention phase), and analyzed for intracellular drug concentrations by flow cytometry (FACScan™, Becton Dickinson). To determine the effect of fumitremorgin C, the cells were exposed to 10 μg/ml mitoxantrone in the absence or presence of 5 μM fumitremorgin C for 20 min at 37 °C, and its intracellular concentration was evaluated by flow cytometry.

2.5. Preparation of crude membrane fractions

Crude membrane fractions were prepared from MT-4 and MT-4/BCRP cells, according to the method described previously [17,18]. To prepare the crude membrane fractions, the cells were washed with 1% aprotinin-containing

PBS and treated with lysis buffer [10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.4), 1 mM ethylene diaminetetraacetic acid (EDTA), 1 mM *p*-amindinophenylmethanesulfonylfluoride (APMSF), and 2 μg/ml aprotinin]. After 10 min on ice, the cells were homogenized with approximately 80 strokes of Dounce homogenizer. The intact cells and nuclei in the homogenate were removed by centrifugation (1500 × *g*) for 10 min. To prepare membrane-enriched fractions, the supernatants were ultracentrifuged (100,000 × *g*) for 30 min at 4 °C, and the pellets were resuspended in dilution buffer [10 mM Tris–HCl (pH 7.4), 0.25 M sucrose, and 1 mM APMSF]. Protein concentrations were determined by Bradford's method [19], and each protein was kept at −80 °C until use.

2.6. Western blot analysis

The crude membranes were subjected to the analysis for BCRP expression. An anti-BCRP antibody was prepared, according to the procedures described previously [14]. The antibody was generated by immunizing rabbits with a peptide that corresponds to amino acids 340–359 of the human BCRP protein. For Western blot analysis, the extracted proteins (100 μg) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyldenedifluoride membrane [10]. The transferred proteins were treated with the anti-BCRP antibody and a horseradish peroxidase-conjugated goat anti-rabbit IgG monoclonal antibody (Amersham Pharmacia Biotech). Antibody binding was visualized with an enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech).

2.7. Anti-HIV-1 assay

The activity of the compounds against HIV-1 replication was based on the inhibition of virus-induced cytopathicity in MT-4 and MT-4/BCRP cells, as previously described [20]. Briefly, the cells (1×10^5 cells/ml) were infected with HIV-1 (III_B strain) at a multiplicity of infection of 0.3 and cultured in the presence of various concentrations of the test compounds. After a 4-day incubation at 37 °C, the number of viable cells was determined by the MTT method.

2.8. Determination of intracellular concentration of AZT and its metabolites

MT-4 and MT-4/BCRP cells were treated with [methyl-^{3}H]AZT (15.4 Ci/mmol) for different periods of time, rapidly washed with ice-cold buffer, and extracted with 60% (v/v) methanol. The methanol extracts were heated at 95 °C for 1.5 min, and their radioactivity was determined. The intracellular concentrations of AZT and its metabolites was also determined in MT-4 and MT-4/BCRP cells exposed to [methyl-^{3}H]AZT at various concentrations ranging from 62.5 to 1000 nM.

2.9. Effect of fumitremorgin C on the efflux of AZT and its metabolites

The effect of fumitremorgin C on the efflux of intracellular AZT and its metabolites from MT-4 and MT-4/BCRP were analyzed by a slight modification based on high performance liquid chromatography (HPLC), as previously reported [21]. Five million cells were incubated with 1 μM [methyl-^{3}H]AZT in the absence or presence of fumitremorgin C (5 μM). After a 2-h incubation, the cells were washed three times with ice-cold medium and immediately frozen in dry ice. The cells were then extracted with 60% (v/v) methanol, and the methanol extracts were heated at 95 °C for 1.5 min. The extracts were clarified by centrifugation (12,000 × *g*) for 6 min. Separation and detection of AZT and its metabolites were performed with a 25-cm Whatman Partisil-10 SAX column (Gilson) by HPLC. After injection of the samples (25 μl), a buffer gradient was applied, starting at zero time with 5 mM potassium phosphate and increasing linearly to 750 mM potassium phosphate over 55 min at 1 ml/min. Then, 750 mM potassium phos-phate was further pumped for 10 min. The elution was fractionated at 1-min intervals (1 ml) and analyzed for radioactivity.

3. Results

3.1. Establishment of MT-4/BCRP cells

To determine whether $BCRP_{WT}$ affects the anti-HIV-1 activity and cytotoxicity of NRTIs, we have established MT-4/BCRP cells expressing a high level of $BCRP_{WT}$. $BCRP_{WT}$ in the crude membrane fractions of the cells was detected with the anti-BCRP polyclonal antibody 3488. As show in Fig. 1, a limited level of BCRP protein was expressed in the parental MT-4 cells, while a high level of BCRP protein expression was observed in MT-4/BCRP cells. Sequence analysis for the full-length BCRP cDNA obtained from MT-4 and MT-4/BCRP cells revealed that only $BCRP_{WT}$ mRNA was expressed in these cell lines (data not shown).

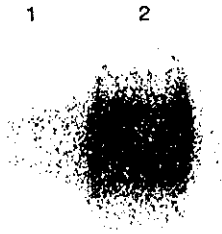

Fig. 1. BCRP expression in MT-4 and MT-4/BCRP cells. Crude membranes (100 μg) from MT-4 and MT-4/BCRP cells were separated by SDS-PAGE and transferred to a polyvinyldenedifluoride membrane. The transferred proteins were immunoblotted with a mAb against human BCRP. Lanes 1 and 2 are the proteins obtained from MT-4 and MT-4/BCRP cells, respectively.

Table 1
Cytotoxicity of anticancer agents and AZT in MT-4, MT-4/BCRP, and MT-4/DOX500 cells[a]

Compound	IC_{50}[b]		
	MT-4	MT-4/BCRP	MT-4/DOX_{500}
Doxorubicin (ng/ml)	4.5 ± 1.0	7.2 ± 2.1	653 ± 25
Mitoxantrone (ng/ml)	0.10 ± 0.07	22 ± 6.2	678 ± 172
SN-38 (μM)	4.1 ± 0.1	44 ± 8.5	133 ± 32
CPT-11 (μM)	2.3 ± 0.2	14 ± 3.7	78 ± 21
Paclitaxel (ng/ml)	0.89 ± 0.16	1.03 ± 0.24	0.77 ± 0.23
AZT (μM)	94 ± 16	154 ± 12	240 ± 12

[a] The cells were cultured for 4 days in the presence of the compounds. The number of viable cells was determined by the MTT method, as described in Section 2. All data represent means ± standard deviations for three separate experiments.

[b] Fifty percent inhibitory concentration, required to reduce cell proliferation and viability by 50%.

3.2. Resistance profile of MT-4/BCRP cells

Several anti-cancer agents were examined for their cytotoxicity in MT-4, MT-4/BCRP, and MT-4/DOX_{500} cells. The level of BCRP expression was approximately 10-fold lower in MT-4/BCRP cells than that in MT-4/DOX_{500} cells (data not shown), although the BCRPs expressing in the former and the latter were $BCRP_{WT}$ and $BCRP_{R482M}$, respectively. MT-4/DOX_{500} cells were found to be resistant to DOX, mitoxantrone, CPT-11, and SN-38 but remained sensitive to paclitaxel (Table 1), which was in agreement with our previous results [10]. MT-4/BCRP cells proved to be resistant to mitoxantrone, CPT-11 and SN-38 but not resistant to paclitaxel. Furthermore, MT-4/BCRP cells showed little, if any, resistance to DOX compared with the parental MT-4 cells (Table 1).

To further confirm the difference in substrate specificity between $BCRP_{WT}$ and $BCRP_{R482M}$, the intracellular accumulation and retention of rhodamine 123, DOX, and mitoxantrone were examined in MT-4/BCRP and MT-4/DOX_{500} cells by flow cytometry. The intracellular accumulation and retention of rhodamine 123 and DOX were not apparent in MT-4/BCRP cells (Fig. 2). However, significant reduction of intracellular mitoxantrone was observed in MT-4/BCRP cells after removal of the compound from culture medium. Furthermore, much lower levels of rhodamine 123, DOX, and mitoxantrone were identified in MT-4/DOX_{500} cells compared with MT-4 and MT-4/BCRP cells (Fig. 2). The BCRP-specific inhibitor fumitremorgin C was found to increase the mitoxantrone accumulation up to a similar level in all of the three cell lines (Fig. 3). These results were in agreement with those reported in several previous studies on the substrate specificity of $BCRP_{WT}$ and mutant BCRPs.

3.3. Cytotoxicity and anti-HIV-1 activity of NRTIs in MT-4/BCRP cells

When the cytotoxicity of AZT was evaluated in MT-4, MT-4/BCRP, and MT-4/DOX_{500} cells, its 50% inhibitory concentrations (IC_{50}s) were 94, 154, and 240 μM, respectively (Table 1). The IC_{50} of other NRTIs could not be determined because of their low cytotoxicity in these cell lines (data not shown). The anti-HIV-1 assays were also conducted for AZT and other NRTIs to determine whether

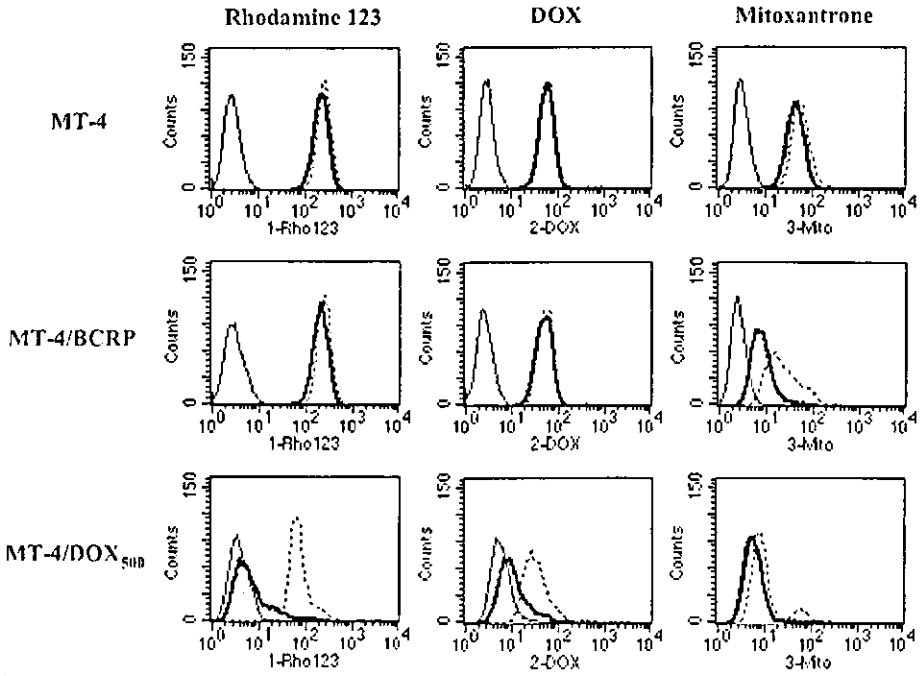

Fig. 2. Accumulation and retention of DOX, mitoxantrone, or rhodamine 123 in MT-4 and MT-4/BCRP cells. The cells were incubated for 30 min in media alone (—), or incubated with 10 μg/ml DOX, 10 μg/ml mitoxantrone, or 100 ng/ml rhodamine 123 (- - -); washed, and further incubated for 60 min in media alone (—). The intracellular DOX, mitoxantrone, or rhodamine 123 concentration was determined by flow cytometry.

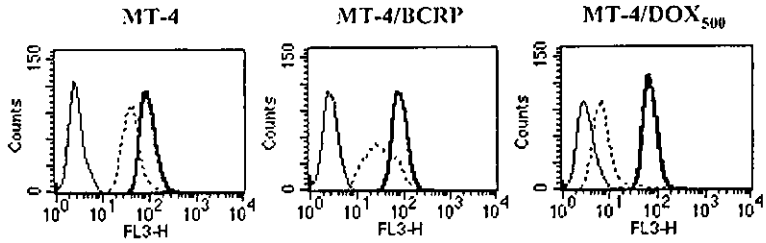

Fig. 3. Accumulation of mitoxantrone in MT-4, MT-4/BCRP, and MT-4/DOX_{500} in the absence or presence of fumitremorgin C. The cells were incubated for 60 min with 10 μg/ml mitoxantrone in the absence (- - -) or presence of 5 μM fumitremorgin C (—). The intracellular concentrations of mitoxantrone were determined by flow cytometry.

Table 2
Anti-HIV-1 activity of NRTIs in MT-4 and MT-4/BCRP cells[a]

Compound	EC_{50}[b] (μM)		Fold resistance to MT-4[c]	
	MT-4	MT-4/BCRP	MT-4/BCRP	MT-4/DOX_{500}[d]
AZT	0.013 ± 0.004	0.032 ± 0.020 *	2.5	7.5
3TC	0.88 ± 0.34	1.58 ± 0.41*	1.8	>77
ddI	11.6 ± 1.6	62.7 ± 28.5*	5.4	2.7
ddC	1.3 ± 0.6	2.4 ± 0.4*	1.9	n.d.[e]
D4T	0.29 ± 0.11	0.36 ± 0.16	1.2	1.6

[a] The infected cells were cultured for 4 days in the presence of the compounds. The number of viable cells was determined by the MTT method, as described in Section 2. All data represent means ± standard deviations for five separate experiments. The statistical significance between the EC_{50} of MT-4 cells and EC_{50} of MT-4/BCRP cells was determined by the *t*-test.

[b] Fifty percent effective concentration, required to reduce cell HIV-1-induced cytopathicity by 50%.

[c] Fold resistance, the ratio of EC_{50} in MT-4 cells to EC_{50} in MT-4/BCRP cells or the ratio of EC_{50} in MT-4 cells to EC_{50} in MT-4/DOX_{500} cells.

[d] Data are taken from [10].

[e] Not determined.

* $P < 0.05$.

their activities were also affected by BCRP. AZT proved 2.5-fold less inhibitory to HIV-1 replication in MT-4/BCRP cells than in MT-4 cells (Table 2). The 50% effective concentrations (EC_{50}) of AZT were 0.013 and 0.032 μM in MT-4 and MT-4/BCRP cells, respectively. Furthermore, the activities of 3TC, ddI, and ddC were also impaired to some extent (1.8–5.4-fold) in MT-4/BCRP cells (Table 2). These results indicate that, like $BCRP_{R482M}$, the expression of $BCRP_{WT}$ is associated with cellular resistance to NRTIs.

3.4. AZT accumulation in MT-4/BCRP cells

It was assumed that the resistance of MT-4/BCRP cells to NRTIs could be attributed to the reduction of intracellular drug concentration. A steady-state level of AZT and its metabolites was already achieved after 10 min of drug exposure (Fig. 4). No significant difference was observed between MT-4 and MT-4/BCRP cells at this time point. When the cells were further incubated in the presence of AZT, the intracellular drug concentration retained at the same level in MT-4 cells until 2 h and gradually decreased thereafter. On the other hand, the intracellular drug concentration started to decrease at 30 min in MT-4/BCRP cells and further decreased over an incubation period. The intracellular drug concentrations in MT-4/BCRP cells were always less than half of those in MT-4 cells after 1 h (Fig. 4). Furthermore, similar results were also obtained in MT-4/BCRP cells after a 2 h-exposure to various concentrations of AZT (Fig. 5).

3.5. The effect of the BCRP-specific inhibitor fumitremorgin C

To gain insight into the mechanism of increased efflux of AZT and its metabolites in MT-4/BCRP cells, the intracellular metabolism of AZT was investigated in MT-4 and MT-4/BCRP cells. An HPLC analysis revealed that the intracellular levels of AZT and its metabolites, especially

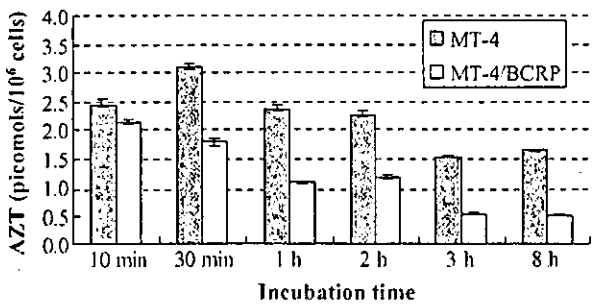

Fig. 4. Intracellular uptake of [methyl-^{3}H]AZT in MT-4 and MT-4/BCRP cells. MT-4 (closed bars) and MT-4/BCRP (open bars) cells were incubated in the presence of 0.5 μM [methyl-^{3}H]AZT. At the indicated times, the cells were extensively washed with ice-cold PBS. Fractions extracted with methanol were determined for their radioactivity. The results represent the means ± standard derivations in three independent samples.

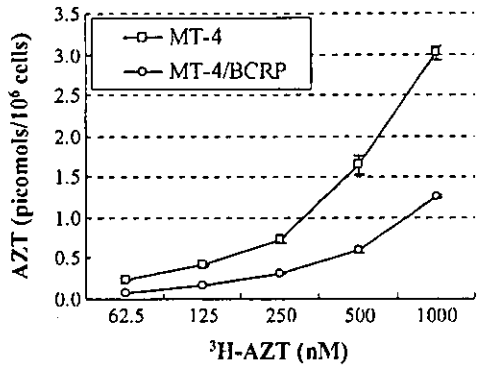

Fig. 5. Concentration-dependent intracelluar uptake of [methyl-^{3}H]AZT in MT-4 and MT-4/BCRP cells. MT-4 (open square) and MT-4/BCRP (open circle) cells were incubated in the presence of various concentrations of [methyl-^{3}H]AZT. After 2 h, the cells were extensively washed with ice-cold PBS. Fractions extracted with methanol were determined for their radioactivity. The points and error bars represent the means ± standard derivations in three independent samples.

Table 3
Effects of fumitremorgin C on intracellular concentrations of AZT and its metabolites in MT-4 and MT-4/BCRP cells[a]

Cells	Fumitremorgin C	Concentration (pmol/10^6 cells)			
		AZT	AZTMP	AZTDP	AZTTP
MT-4	(−)	0.135	1.461	0.101	0.076
MT-4	(+)	0.160	1.596	0.112	0.080
MT-4/BCRP	(−)	0.071	0.410	0.046	0.066
MT-4/BCRP	(+)	0.180	1.599	0.098	0.084

[a] The cells were incubated with [methyl-^{3}H]AZT in the absence or presence of fumitremorgin C (5 μM). After a 2-h incubation, the cells were subjected to HPLC analysis, as described in Section 2. Intracellular concentrations of AZT and its metabolites are expressed as pmol/10^6 cells.

AZT 5′-monophosphate (AZTMP), were highly diminished in MT-4/BCRP cells (Table 3). Only 28% of the AZTMP level in MT-4 cells was identified in MT-4/BCRP cells. In addition, the levels of AZT, AZT 5′-diphosphate (AZTDP), and AZT 5′-triphosphate (AZTTP) were also 52.8, 45, and 86.6% of those in MT-4 cells, respectively (Table 3). The BCRP-specific inhibitor fumitremorgin C could completely restore the decreased levels of AZT and its metabolites in MT-4/BCRP cells at a concentration of 5 μM (Table 3). Fumitremorgin C did not affect the viability and proliferation of MT-4 and MT-4/BCRP cells at this concentration (data not shown). These results suggest that the reduced anti-HIV-1 activity of AZT in MT-4/BCRP cells is due to the increased efflux of AZT and its metabolites by $BCRP_{WT}$ and subsequent decrease of their intracellular concentrations.

4. Discussion

Host cellular factors are assumed to be in part involved in the resistance to anti-retroviral agents [2,22]. One of such factors is the ABC transporter family, which acts as an ionic pump and prevents intracellular accumulation of various drugs. Among the ABC transporters, P-gp was found to be an important determinant for oral bioavailability and CNS penetration of PIs [3]. In addition, it was reported that the overexpression of MRP4 in $CD4^+$ T cells increased the efflux of some NRTIs from the cells and reduced their anti-HIV-1 activity [5]. However, the role of other ABC transporters in NRTI-resistance is still unknown and remains to be elucidated.

We have recently demonstrated that high level of $BCRP_{R482M}$ expression in $CD4^+$ T cells brings about reduced anti-HIV-1 activity of NRTIs but not non-nucleoside reverse transcriptase inhibitors (NNRTIs) or PIs [10]. The arginine at position 482 (R482) is considered to play a crucial role in BCRP function; point mutation of this amino acid residue may significantly change the substrate specificity of BCRP and the phenotype of drug resistance [23]. In fact, this study has shown clear difference in cytotoxicity and efflux of drugs between $BCRP_{WT}$ and $BCRP_{R482M}$ (Table 1 and Figs. 2 and 3). Therefore, it would be possible that the NRTI-resistance could be conferred only by $BCRP_{R482M}$ but not by $BCRP_{WT}$. To exclude this possibility, we have generated the $CD4^+$ T-cell line (MT-4/BCRP) expressing a high level of $BCRP_{WT}$ and examined whether it also affects the anti-HIV-1 activity and cytotoxicity to NRTIs. The present study has clearly demonstrated that, like $BCRP_{R482M}$, $BCRP_{WT}$ also diminishes the anti-HIV-1 activity of NRTIs. Although the reduction rate (fold resistance) seems to be considerably smaller in MT-4/BCRP cells than in MT-4/DOX_{500} cells [10], this may be in part attributed to the 10-fold higher expression of BCRP in MT-4/DOX_{500} cells than in MT-4/BCRP cells. In fact, apart from DOX (a substrate of $BCRP_{R482M}$ but not $BCRP_{WT}$), MT-4/DOX_{500} was found to be much more resistant to mitoxantrone, SN-38, and CPT-11 (Table 1). Furthermore, experiments using MT-4/DOX_{100} cells [10], which express an almost identical level of $BCRP_{R482M}$ compared to the $BCRP_{WT}$ level in MT-4/BCRP cells, revealed that AZT was 3.3-fold less inhibitory to HIV-1 replication in MT-4/DOX_{100} cells than in MT-4 cells (data not shown). Since the anti-HIV-1 activity of AZT was 2.5-fold lower in MT-4/BCRP cells than in MT-4 cells (Table 2), these results suggest that $BCRP_{WT}$ interacts similarly with nucleoside analogs.

HIV-1 has been found in several tissues in vivo and can infect many different types of human cells in vitro. BCRP has also been detected in some normal tissues, including placenta, liver, breast, and venous and capillary endothelium [24]. More importantly, BCRP mRNA could be detected in bone marrow and peripheral blood mononuclear cells [25]. If HIV-1 could upregulate BCRP expression in the infected cells, the intracellular NRTI concentrations would decrease in these tissues, resulting in insufficient suppression of HIV-1 replication and increasing opportunity for the emergence of drug-resistant mutants.

In the brain, HIV-1 infection is restricted, in most cases, to microglia and brain macrophages. The CNS disorders associated with HIV-1 infection, such as encephalopathy and dementia, occur at the late stage of the disease [26]. The development of HAART has decreased the incidence rates for HIV-1-associated encephalopathy and dementia, nevertheless its impact on the future incidence and course of dementia remains debatable [27,28]. Effective treatment of HIV-1-associated dementia or encephalopathy needs efficient distribution of anti-HIV-1 agents into the CNS. The blood–brain barrier (BBB) is well recognized to play a crucial role in restricting the penetration of many drugs and toxins into the brain from the systemic circulation. In the case of PIs, P-gp expression in the BBB enhances the elimination of PIs from CNS, and specific inhibitors of P-gp are able to dramatically increase their penetration into the CNS [29].

Limited distribution of AZT, ddI, and related nucleoside derivatives in the CNS has been demonstrated after their systemic administration [30–32]. Although several forms of organic anion transporters have substantial activities for NRTI transport into the brain, the role of the ABC transporters in penetration of NRTIs through the BBB has not fully been understood yet [32–35]. It was reported that MRP4 acted as a transporter of some NRTIs, such as AZTMP and PMEA [5]. However, even now, there are no solid evidences of MRP4 expression in the BBB. Recently, the brain multidrug resistance protein was discovered in the porcine BBB, and the protein was shown to be highly homologous to the human and mouse BCRP [36,37]. BCRP was also identified in the BBB of both normal and tumor human brain tissues and mainly located at the luminal surface of microvessel endothelium [38]. Although the extracellular concentrations of AZT and its metabolites were not measured in this study, it is assumed that not only $BCRP_{R482M}$ but also $BCRP_{WT}$ are involved in the efflux of AZT and its metabolites, presumably AZTMP [5]. The effect of $BCRP_{WT}$ on the intracellular concentrations of AZT and AZTMP were completely restored by the presence of the BCRP-specific inhibitor fumitremorgin C, also suggesting this possibility (Table 3). Additional experiments are still required to determine whether the same result can be obtained for other NRTIs in the presence of fumitremorgin C. However, if the expression of $BCRP_{WT}$ in the BBB may commonly restrict the entry of NRTIs into the brain, it seems important to reduce the affinity of anti-HIV-1 agents for the ABC transporters to achieve their sufficient concentrations in the brain. A strategy involving either chemical modification of existing anti-HIV-1 agents or discovery of a selective inhibitor of the ABC transporters is needed for effective treatment of the CNS disorders associated with HIV-1 infection. In this point of view, BCRP inhibitors, such as fumitremorgin C, may be useful to improve the entry of NRTIs into the brain and increase their concentrations in the cerebrospinal fluid [11,12].

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