Reversal of Breast Cancer Resistance Protein-mediated Drug Resistance by Estrogen Antagonists and Agonists[1]

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Abstract

Breast cancer resistance protein (BCRP), an ATP-binding cassette transporter, confers resistance to a series of anticancer agents such as SN-38, mitoxantrone, and topotecan. In a previous study, we found that estrogens reverse drug resistance of BCRP-expressing cells. In this study, estrogen antagonists, estrogen agonists, and their derivatives were evaluated for BCRP-reversing activity. First, compounds were tested for effects on the cellular accumulation of topotecan in *BCRP*-transduced K562 cells (K562/BCRP). Next, these compounds were examined for their ability to reverse SN-38 and mitoxantrone resistance in K562/BCRP cells. Among commercially available estrogen antagonists and agonists tested, diethylstilbestrol showed the strongest BCRP-reversing activity. Diethylstilbestrol increased the cellular accumulation of topotecan and reversed drug resistance in K562/BCRP cells but showed marginal or no effect in parental K562 cells. The reversal activities of estrone and diethylstilbestrol were more prominent for mitoxantrone than for SN-38. Tamoxifen and toremifene were also found to enhance topotecan uptake in K562/BCRP cells. Next, various tamoxifen derivatives were screened for anti-BCRP activity. In the first cycle of screening with 14 compounds, TAG-11 showed the strongest effect. In the second cycle of screening of 25 TAG-11-related compounds, TAG-139 showed the strongest effect. Reversal of SN-38 and mitoxantrone resistance in K562/BCRP cells by TAG-139 was 5-fold stronger than that by estrone. Dose-dependent characteristics of drug resistance reversal with estrone and TAG-139 were very similar, suggesting that estrone and tamoxifen derivatives interact with the same drug-binding site of BCRP. Derivatives of antiestrogens that exhibit no other biological effects promise to be useful in overcoming BCRP-mediated drug resistance.

Introduction

Tumor cells that acquire resistance to certain chemotherapeutic agents sometimes develop cross-resistance to other structurally unrelated agents (1). This phenomenon is known as multidrug resistance. A family of ABC[3] transporters such as *MDR1* gene product P-glycoprotein (1, 2) and MRP1 (3) is involved in multidrug resistance, which pump out various structurally unrelated antitumor agents in an energy-dependent manner.

BCRP, also called ABCG2, ABCP, or MXR, is a half-molecule ABC transporter with a NH_2-terminal ATP-binding site and a COOH-terminal transmembrane domain (4–8). The *BCRP* gene was first identified as an ABC transporter overexpressed in the placenta (6, 9). We showed that BCRP acts as a homodimer (10). Cells that overexpress BCRP show resistance to SN-38, mitoxantrone, and topotecan. BCRP presumably acts as an efflux pump, resulting in decreased intracellular concentrations of these anticancer agents (4–8).

BCRP is highly expressed in the placenta and digestive tract (9). Our recent study suggests that BCRP may transport estrogens such as estrone and 17β-estradiol from the placenta to mother's body (11). BCRP expression is found in capillary endothelial cells, hematopoietic stem cells, and mother-placenta barrier, suggesting that BCRP may play a protective role against toxic substances and metabolites (12, 13).

Recently, BCRP expression was reported in relapsed or refractory hematological malignancies (14, 15). Some reports showed the association of BCRP expression with poor responses to chemotherapy (15, 16). It is possible that BCRP expression is responsible, at least in part, for clinical drug resistance. If it should be true, overcoming BCRP-mediated drug resistance would contribute to cancer chemotherapy. Reversal of drug resistance was first demonstrated in the inhibition of P-glycoprotein-mediated drug resistance by verapamil (17, 18). Clinical studies of P-glycoprotein inhibitors such as PSC-833 and MS-209 are ongoing (19). After our finding that estrogens reverse BCRP-mediated drug resistance, we examined the effect of synthetic estrogen antagonists and agonists on the cellular uptake of topotecan

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[3] The abbreviations used are: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; K562/BCRP, *BCRP*-transduced K562; ER, estrogen receptor; FACS, fluorescence-activated cell sorting.

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and cytotoxicity of SN-38 and mitoxantrone on BCRP-expressing K562 cells. Among the compounds tested, diethylstilbestrol strongly reversed drug resistance in K562/BCRP cells. Next, various tamoxifen derivatives were screened for anti-BCRP activity, and after two cycles of screening, TAG-139 was identified as the strongest BCRP inhibitor among the tamoxifen derivatives examined. More extensive screening of BCRP antagonists is ongoing in our laboratory.

Materials and Methods

Cells and Cell Culture. K562 human myelogenous leukemia cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. KB-3-1 human carcinoma cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. K562/BCRP cells were established by the transduction of K562 cells with HaMycBCRP retrovirus that carries Myc-tagged human *BCRP* cDNA in Ha retrovirus vector (10). K562/MDR cells were established by the transduction of K562 cells with HaMDR retrovirus that carries human *MDR1* cDNA (20). KB/MRP was made by introducing an expression vector, pJ3Ù-MRP, containing the human *MRP1* cDNA into KB-3-1 cells (21). These stable drug-resistant 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 KLH-conjugated 20-mer peptide corresponding to the amino acid sequence 340–359 of human BCRP protein (10). Anti-P-glycoprotein monoclonal antibody C219 and anti-MRP1 monoclonal antibody MRPm6 were purchased from Centocor (Malvern, PA) and Nichirei (Tokyo, Japan), respectively. Cell lysates were solubilized with 2% SDS, 50 mM Tris-HCl (pH 7.5), 5% 2-mercaptoethanol, and resolved by 4–20% SDS-PAGE (20 μg protein/lane). After electrophoresis, proteins were transferred onto nitrocellulose membranes. Blots were then incubated with anti-BCRP, anti-P-glycoprotein, or anti-MRP1 antibody. After washing, the blots were incubated with the appropriate peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Membrane-bound peroxidase was visualized using ECL Plus chemiluminescence detection kit (Amersham Pharmacia Biotech).

Growth Inhibition Assay. The effects of compounds on the sensitivity of cells to SN-38, mitoxantrone, and topotecan were evaluated by measuring cell growth inhibition after incubation at 37°C for 5 days in the absence or presence of various concentrations of anticancer drugs in combination with the test compounds. Cell numbers were determined with a Coulter counter. IC_{50}s (drug dose causing 50% inhibition of cell growth) were determined from growth inhibition curves.

Intracellular Drug Accumulation. The effect of compounds on cellular accumulation of topotecan was determined by flow cytometry. Cells (5×10^5) were incubated with 20 μM topotecan for 30 min at 37°C in the absence or presence of the test compounds (30 μM), washed in ice-cold PBS, and subjected to fluorescence analysis using a FACS Calibur with 488 nm excitation (Becton-Dickinson, San Jose, CA).

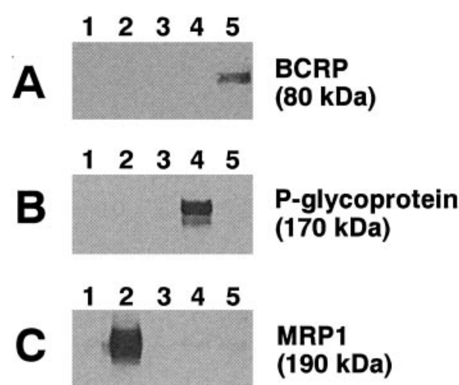

Fig. 1. Expression of BCRP, P-glycoprotein, and MRP1 in the transfectants. Protein samples (20 μg protein/lane) were resolved by 4–20% SDS-PAGE and transferred onto nitrocellulose membranes. *A*, blots were treated with anti-BCRP antibody 3488. *B*, blots were treated with anti-P-glycoprotein antibody C219. *C*, blots were treated with anti-MRP1 antibody MRPm6. The blots were incubated with the appropriate peroxidase-conjugated secondary antibodies. Membrane-bound peroxidase was visualized using ECL Plus chemiluminescence detection kit. *Lane 1:* KB-3-1; *Lane 2:* KB/MRP; *Lane 3:* K562; *Lane 4:* K562/MDR; and *Lane 5:* K562/BCRP.

Antiestrogen Activity. Antiestrogen activity of TAG-compounds was estimated by the inhibition of estradiol binding to ER-α and ER-β using ligand screening system for ERs α and β (Toyobo, Osaka, Japan) under protocols recommended by the supplier. Briefly, to examine anti-ER-α activity, test sample, 17β-estradiol, and recombinant human ER-α were incubated for 60 min at 4°C in a plate coated with anti-ER-α. Free 17β-estradiol that did not bind to ER-α was quantified in the subsequent enzyme immunoassay. The supernatant containing free 17β-estradiol was transferred to an anti-17β-estradiol-coated plate together with horseradish peroxidase-conjugated 17β-estradiol. After the reaction for 60 min at 4°C, horseradish peroxidase bound to the plate was visualized by the addition of a color substrate. Anti-ER-β activity was also measured with a similar method using recombinant human ER-β. The degree of binding inhibition was calculated as the ratio of decrease in estradiol binding in the presence of 37.5 nM test compound divided by that in the presence of 300 nM diethylstilbestrol.

Results

Immunoblot Analysis of BCRP, P-glycoprotein, and MRP1. K562/BCRP cells were established by the transduction of K562 cells with HaMycBCRP retrovirus and the subsequent selection of transduced cells with 20 ng/ml SN-38 for 5 days. K562/MDR cells were established by the transduction of K562 cells with HaMDR retrovirus and the subsequent selection of transduced cells with 4 ng/ml vincristine for 7 days. KB/MRP was established by the transfection of KB-3-1 cells with an *MRP1*-expression plasmid, pJ3Ù-MRP and the subsequent selection of transfected cells with increasing concentrations of doxorubicin. The expressions of BCRP, P-glycoprotein, and MRP1 in these cell lines were confirmed by Western blot analysis (Fig. 1). BCRP expression was detected in K562/BCRP (Fig. 1*A*). The BCRP

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Table 1 Drug resistance of K562/BCRP cells[a]

Drug	IC_{50} (ng/ml)		Degree of resistance
	K562	K562/BCRP	
SN-38	0.42 ± 0.02	10 ± 0.3	24
Mitoxantrone	0.33 ± 0.02	3.6 ± 0.2	11
Topotecan	3.1 ± 0.1	30 ± 2	10

[a] K562 or K562/BCRP cells were cultured for 5 days with increasing concentrations of SN-38, mitoxantrone, or topotecan. Cell numbers were counted with a Coulter counter, and IC_{50} was determined. Degree of resistance is the ratio of IC_{50} for K562/BCRP cells divided by that for K562 cells. Data are represented as mean values ± SD from triplicate determinations.

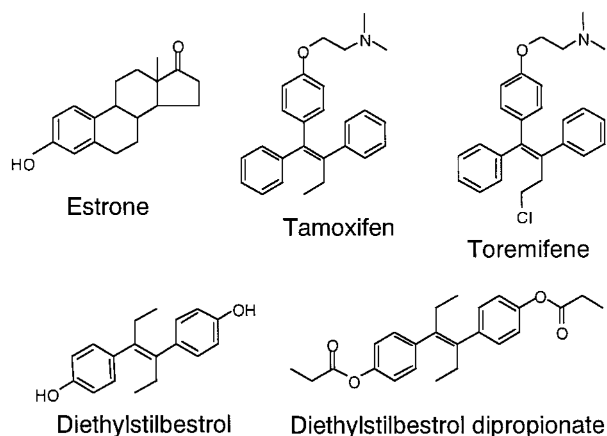

Fig. 2. Chemical structures of estrone, estrogen antagonists, and estrogen agonists.

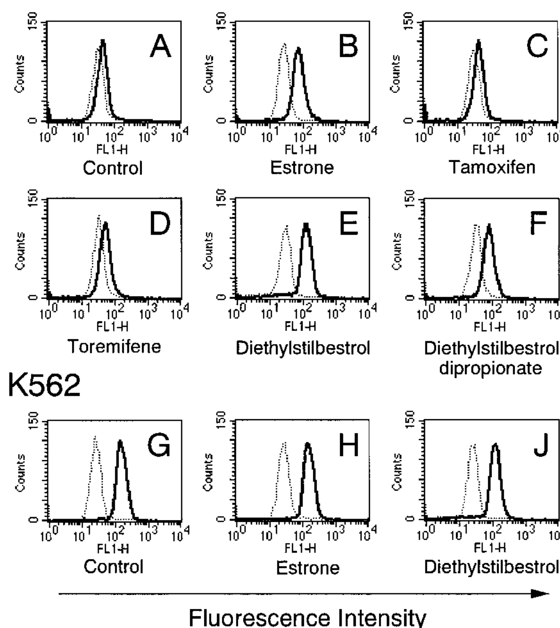

Fig. 3. Effect of estrone, estrogen antagonists, and estrogen agonists on the intracellular accumulation of topotecan in K562 and K562/BCRP cells. Cells were incubated with or without 20 μM topotecan in the presence or absence of 30 μM test compound. Cellular content of topotecan was measured by FACS. *Bold lines*, with topotecan; *dotted lines*, without topotecan. In K562/BCRP cells with topotecan (*bold line*), a fluorescence peak shift to the right indicates cellular uptake of topotecan in the presence of estrone or other test compounds, whereas a slight shift occurred in the absence of test compounds. In contrast, fluorescence peak shifts to the right were observed in K562 cells, irrespective of the type of test compounds.

expression level in K562/BCRP was ~3-fold higher than the level of human leukemia RPMI8226 that expresses the highest amount of BCRP among 59 cell lines in the National Cancer Institute anticancer drug screening panel (22). KB-3-1, KB/MRP, K562, and K562/MDR cells did not express detectable amounts of BCRP. K562/BCRP cells were propagated in drug-free medium. No significant decrease in BCRP expression was observed for 3 months. K562/BCRP cells showed 10–24-fold higher resistances to SN-38, mitoxantrone, and topotecan than parental K562 cells (Table 1). P-glycoprotein expression was detected only in K562/MDR cells (Fig. 1*B*). KB/MRP cells highly expressed MRP1 protein (Fig. 1*C*). Low-level expression of MRP1 was also detected in the other cell lines.

BCRP-reversing Activity of Estrogen Antagonists and Agonists. For the screening of BCRP-reversing agents among commonly used estrogen antagonists and agonists, effect of agents on the cellular accumulation of topotecan was evaluated by flow cytometric analysis. Structures of estrone, a representative steroid with BCRP-reversing activity, and commercially available estrogen antagonists and agonists used in this study are shown in Fig. 2, and results of FACS analysis are shown in Fig. 3. After incubating cells with 20 μM topotecan, cellular fluorescence of K562/BCRP increased only marginally (Fig. 3*A*), whereas significant increase in fluorescence was observed in K562 cells (Fig. 3*G*), suggesting that BCRP expressed in K562/BCRP actively pumps out topotecan from the cells. Intracellular accumulation of topotecan increased in the presence of estrone in K562/BCRP cells (Fig. 3*B*), whereas level was not altered in K562 cells (Fig. 3*H*). Tamoxifen and toremifene slightly increased the topotecan accumulation in K562/BCRP cells (Fig. 3, *C* and *D*). Diethylstilbestrol showed stronger activity in increasing topotecan accumulation in K562/BCRP cells than estrone (Fig. 3*E*) and showed no effect on K562 cells (Fig. 3*J*). Diethylstilbestrol dipropionate also showed BCRP-reversing activity similar to that of estrone (Fig. 3*F*).

Effects of estrone, estrogen antagonists, and agonists on cellular drug resistance were evaluated by growth inhibition assay using fixed doses of test compounds in combination with increasing concentrations of the antitumor agents (Fig. 4). Concentrations of reversing agents used in this experiment were determined based on their toxicity without SN-38 or mitoxantrone. The highest dose was first set as 10 μM, and if an agent showed significant growth inhibition at 10 μM, the concentration was lowered so that the reversing agent would not cause >30% growth inhibition when it was added to the culture alone. Estrone enhanced the cytotoxicities of SN-38 and mitoxantrone on K562/BCRP cells in a dose-dependent manner, whereas it did not affect the cytotoxicity of these drugs on K562 cells (Fig. 4*A*). The reversal activity of estrone

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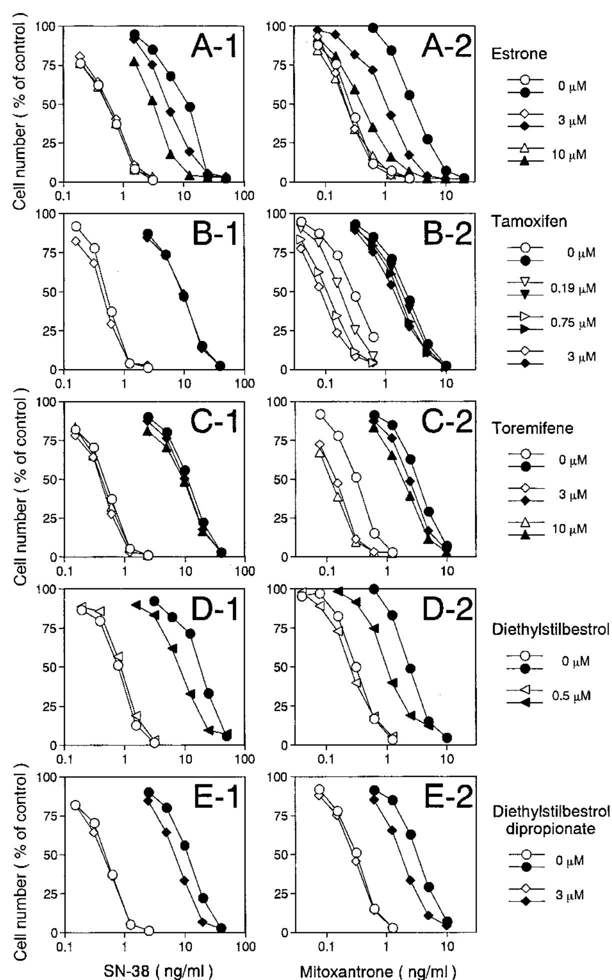

Fig. 4. Reversal of BCRP-mediated drug resistance by estrone, estrogen antagonists, and agonists. K562 (*open symbols*) and K562/BCRP (*closed symbols*) cells were cultured for 5 days with increasing concentrations of SN-38 or mitoxantrone in the absence or presence of fixed doses of estrone, estrogen antagonists, or agonists. Cell numbers were counted with a Coulter counter. Each point is an average of triplicate determinations. SDs are <10% of mean values. *A*, effect of estrone on the sensitivity to SN-38 (*A-1*) and mitoxantrone (*A-2*). *B*, effect of tamoxifen on the sensitivity to SN-38 (*B-1*) and mitoxantrone (*B-2*). *C*, effect of toremifene on the sensitivity to SN-38 (*C-1*) and mitoxantrone (*C-2*). *D*, effect of diethylstilbestrol on the sensitivity to SN-38 (*D-1*) and mitoxantrone (*D-2*). *E*, effect of diethylstilbestrol dipropionate on the sensitivity to SN-38 (*E-1*) and mitoxantrone (*E-2*).

was more prominent for mitoxantrone than for SN-38. Tamoxifen showed no effect on the SN-38 sensitivity of K562/BCRP cells (Fig. 4, *B-1*). Interestingly, mitoxantrone cytotoxicity on parental K562 cells was enhanced in the presence of tamoxifen at low doses in a dose-dependent manner (Fig. 4, *B-2*). Tamoxifen also potentiated the mitoxantrone cytotoxicity on K562/BCRP cells (Fig. 4, *B-2*). Toremifene, an analogue of tamoxifen, also showed a potentiation of mitoxantrone cytotoxicity on K562 and K562/BCRP cells with different dose-dependent characteristics (Fig. 4, *C-2*). Diethylstilbestrol showed strong reversing activity. Diethylstilbestrol at 0.5 μM and estrone at 3 μM showed similar potentiation of SN-38 and mitoxantrone cytotoxicity on K562/BCRP cells

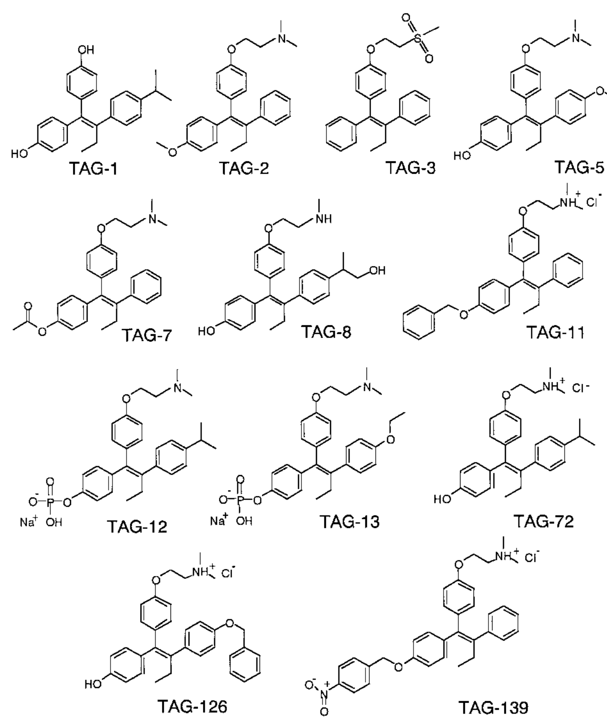

Fig. 5. Chemical structures of TAG-1–TAG-139 used in the screening of anti-BCRP activity.

(Fig. 4*D*). Diethylstilbestrol dipropionate showed somewhat lower reversing activity than diethylstilbestrol (Fig. 4*E*).

Screening of BCRP Antagonists. On the basis of the current finding that antiestrogens reverse BCRP-mediated drug resistance, we undertook to screen BCRP antagonists from a chemical library of tamoxifen derivatives. The first cycle of screening was carried out using 14 compounds (TAG-1–TAG-14) with relatively diverse structures and identified TAG-11 as a first-generation lead compound. The second cycle of screening was carried out using 25 TAG-11-related compounds (TAG-22–TAG-152). Structures of the representative 12 compounds are shown in Fig. 5. The effect of these 12 compounds on the cellular accumulation of topotecan was evaluated by flow cytometric analysis (Fig. 6). Tamoxifen at 30 μM slightly increased topotecan uptake in K562/BCRP cells (Fig. 6*B*). Among 12 compounds, TAG-5, TAG-8, TAG-11, TAG-72, TAG-126, and TAG-139 showed stronger activity in increasing topotecan accumulation in K562/BCRP cells than the other compounds, suggesting that triphenylethylene derivatives with *N*,*N*-dimethylaminoethoxy group have anti-BCRP activity (Fig. 6). TAG-compounds themselves are not fluorescent because cellular fluorescence without topotecan unchanged in the presence of 30 μM TAG-compounds. TAG-compounds did not affect the topotecan fluorescence of K562 cells, suggesting that increases in topotecan fluorescence of K562/BCRP in the presence of TAG-compounds are BCRP specific (data not shown).

Next, the effects of 12 TAG-compounds on SN-38 cytotoxicity in K562/BCRP were evaluated with fixed doses of reversing agents at 1 μM and/or 3 μM (Table 2). Mitoxantrone

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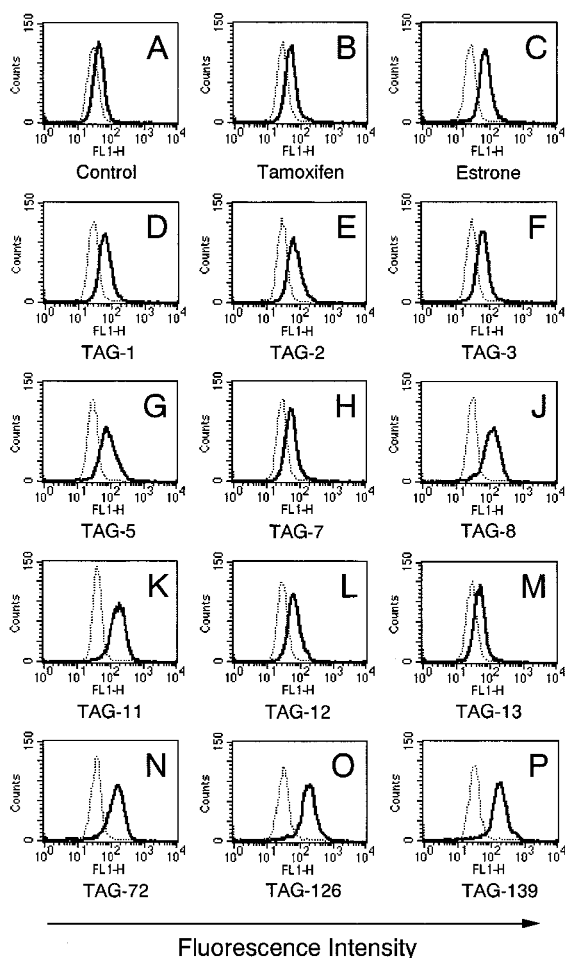

Fig. 6. Effects of TAG-1–TAG-139 on the intracellular accumulation of topotecan in K562/BCRP cells. Cells were incubated with or without 20 μM topotecan in the presence or absence of 30 μM TAG-compounds. Cellular content of topotecan was measured by FACS. *Bold lines*, with topotecan; *dotted lines*, without topotecan. With topotecan (*bold line*), a fluorescence peak shift to the right indicates cellular uptake of topotecan in the presence of TAG-compounds, although a slight shift occurred in the absence of compounds. In K562 cells, TAG-compounds did not affect topotecan uptake (data not shown).

was not used in this experiment because of the synergistic effect of mitoxantrone and tamoxifen derivatives on K562 cells. IC_{50}s of these TAG-compounds, except TAG-72 and TAG-126 in K562 and K562/BCRP cells, were >3 μM. TAG-72 and TAG-126 showed strong growth inhibitory effect at 3 μM, therefore reversal effect of TAG-72 and TAG-126 were tested only at 1 μM. As shown in Table 2, TAG-8, TAG-11, and TAG-139 at 3 μM strongly potentiated SN-38 cytotoxicity on K562/BCRP cells. TAG-139 showed the strongest reversal effect. TAG-126 at 1 μM also showed reversing effect similar to TAG-11 at 1 μM. This result showed good correlation with the FACS results (Fig. 6). TAG-11 and TAG-139 enhanced the cytotoxicity of SN-38 and mitoxantrone on K562/BCRP cells in a dose-dependent manner (Fig. 7). TAG-11 showed similar cytotoxicity on K562 and K562/BCRP cells with the IC_{50} of 4.2 and 4.1 μM, respectively. TAG-139 also showed similar cytotoxicity on K562 and

Table 2 Reversal of BCRP-mediated SN-38 resistance by TAG-compounds[a]

Compound	Reversal index	
	1 μM	3 μM
Tamoxifen	NT	1.02 ± 0.02
TAG-1	NT	1.11 ± 0.06
TAG-2	NT	1.26 ± 0.02
TAG-3	NT	1.08 ± 0.08
TAG-5	NT	1.17 ± 0.11
TAG-7	NT	1.15 ± 0.14
TAG-8	NT	1.67 ± 0.02
TAG-11	1.36 ± 0.04	1.91 ± 0.02
TAG-12	NT	1.07 ± 0.04
TAG-13	NT	1.09 ± 0.01
TAG-72	1.12 ± 0.12	NT
TAG-126	1.31 ± 0.14	NT
TAG-139	2.32 ± 0.08	4.02 ± 0.12

[a] K562/BCRP cells were cultured for 5 days with increasing concentrations of SN-38 in the absence or presence of reversing agents (1 or 3 μM, as indicated). Cell numbers were counted with a Coulter counter, and concentration of drug required for IC_{50} was determined. Reversal index is the ratio of IC_{50} in the absence of reversing agent divided by that in the presence of reversing agent. Data are represented as mean values ± SD from triplicate determinations. *NT*, not tested.

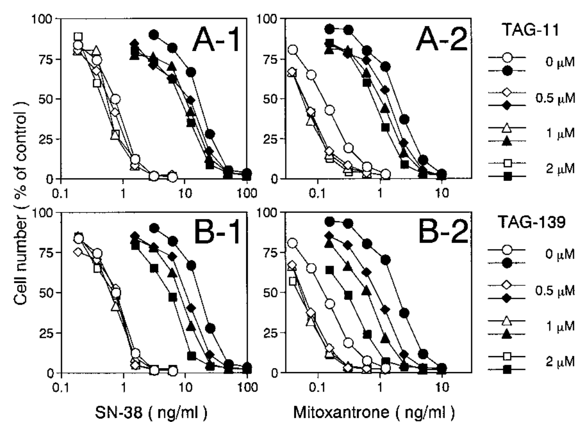

Fig. 7. Reversal of BCRP-mediated drug resistance by TAG-11 and TAG-139. K562 (*open symbols*) and K562/BCRP (*closed symbols*) cells were cultured for 5 days with increasing concentrations of SN-38 or mitoxantrone in the absence or presence of TAG-11 (0.5, 1, or 2 μM). Cell numbers were counted with a Coulter counter. Each point is an average of triplicate determinations. SDs are <10% of mean values. *A*, effect of TAG-11 on the sensitivity to SN-38 (*A-1*) and mitoxantrone (*A-2*). *B*, effect of TAG-139 on the sensitivity to SN-38 (*B-1*) and mitoxantrone (*B-2*).

K562/BCRP cells with the IC_{50} of 3.8 μM. TAG-139 showed stronger reversal effect than TAG-11. The reversal activities of TAG-11 and TAG-139 were more prominent for mitoxantrone than for SN-38. Like tamoxifen, TAG-11 and TAG-139 potentiated the cytotoxicity of mitoxantrone on K562 cells (Fig. 7).

Antiestrogen Activity. To examine whether BCRP-reversing activities of these compounds are associated with antiestrogen activity, effects of compounds on the binding of estradiol to ER-α and ER-β were evaluated. As shown in Fig. 8*A*, tamoxifen and 4-OH-tamoxifen strongly inhibited the binding of estradiol to ER-α. TAG-11 showed weak inhibition of estradiol binding to ER-α. TAG-72 and TAG-126, which

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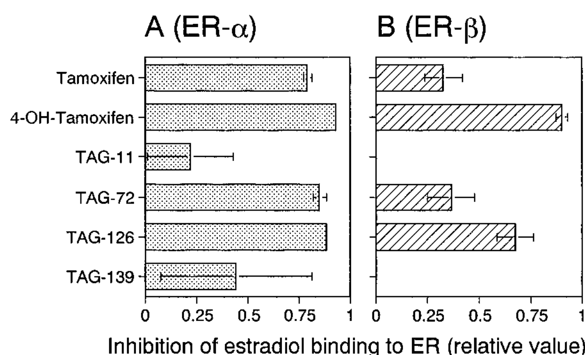

Fig. 8. Antiestrogen activity of TAG-compounds. Antiestrogen activity of TAG-compounds was estimated by the inhibition of estradiol binding to ER-α and ER-β using enzyme immunoassay. Degree of binding inhibition was calculated as the ratio of decrease in estradiol binding in the presence of 37.5 nM test compound divided by that in the presence of 300 nM diethylstilbestrol. Data are represented as mean values ± SD. *A*, inhibition of estradiol binding to ER-α. *B*, inhibition of estradiol binding to ER-β.

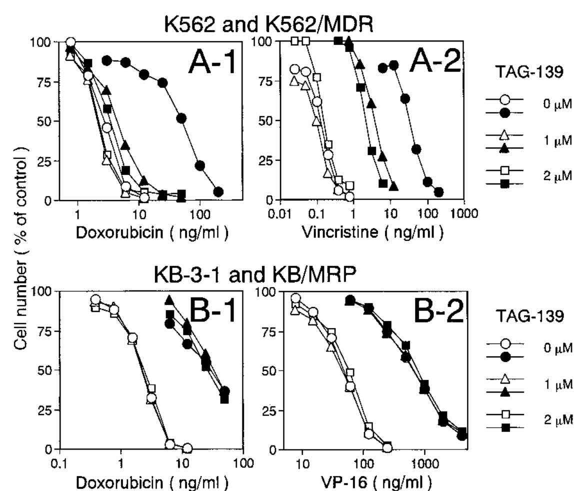

Fig. 9. Effect of TAG-139 on P-glycoprotein- and MRP1-mediated drug resistance. Cells were cultured for 5 days with increasing concentrations of anticancer agents in the absence or presence of TAG-139 (1 or 2 μM). Cell numbers were counted with a Coulter counter. Each point is an average of triplicate determinations. SDs are <10% of mean values. *A*, effect of TAG-139 on the doxorubicin sensitivity (*A-1*) and vincristine sensitivity (*A-2*) of K562 (*open symbols*) and K562/MDR (*closed symbols*) cells. *B*, effect of TAG-139 on the doxorubicin sensitivity (*B-1*) and VP-16 sensitivity (*B-2*) of KB-3–1 (*open symbols*) and KB/MDR (*closed symbols*) cells.

showed modest BCRP-reversing activity, strongly inhibited estradiol binding to ER-α. TAG-139, the strongest BCRP inhibitor among TAG-compounds examined, showed weak interaction with ER-α. Similar results were obtained with ER-β (Fig. 8*B*). These results suggest that BCRP-reversing activity and antiestrogen activity may be disassociated. Therefore, it should be possible to develop BCRP-reversing agents exhibiting no other biological effects including antiestrogen activity.

Effect of TAG-139 on *MDR1*-transduced Cells and *MRP1*-transfected Cells. To examine the transporter specificity of TAG-139, possible reversing effect of TAG-139 on *MDR1*- and *MRP1*-expressing cells were examined. As shown in Fig. 9, TAG-139 at 1 or 2 μM strongly potentiated the cytotoxicity of doxorubicin and vincristine on K562/MDR cells. The reversal activity of TAG-139 was more prominent for doxorubicin than for vincristine (Fig. 9*A*). TAG-139 showed no effect on MRP1-mediated doxorubicin resistance and VP-16 resistance (Fig. 9*B*).

Discussion

In this study, estrogen antagonists and agonists increased the cellular accumulation of topotecan in BCRP-expressing cells and reversed BCRP-mediated drug resistance. Estrone and diethylstilbestrol enhanced the cytotoxicity of SN-38 and mitoxantrone on K562/BCRP cells, whereas they did not affect the cytotoxicity of either of these drugs on K562 cells. Diethylstilbestrol showed stronger BCRP-reversing activity than estrone. Estrone at 3 μM and diethylstilbestrol at 0.5 μM showed similar reversing effects of SN-38 and mitoxantrone resistance in K562/BCRP cells. Therefore, diethylstilbestrol may be a practical parental compound for the screening of BCRP antagonists. However, diethylstilbestrol showed a strong growth inhibitory effect even at 1 μM. Diethylstilbestrol dipropionate showed weaker BCRP antagonist activity and growth inhibitory effect than diethylstilbestrol.

We chose a chemical library of tamoxifen derivatives to screen for BCRP antagonists. This rationale was based on: (*a*) many compounds are already made and available; (*b*) synthesis of new compounds is relatively easy; and (*c*) toxicity, pharmacokinetics, and pharmacodynamics have been well studied. Before starting a screening of TAG-compounds, it was not clear whether tamoxifen and/or toremifene actually reverse BCRP-mediated drug resistance. From a series of experiments using tamoxifen derivatives, however, many TAG-compounds inhibited the function of BCRP. Therefore, the parental compounds, tamoxifen and toremifene, seem to weakly interact with BCRP. TAG-139 was identified as the strongest BCRP antagonist examined. Estrone at 3 μM and TAG-139 at 0.5 μM showed similar reversing effects on SN-38 and mitoxantrone resistance in K562/BCRP cells. Estrone at 10 μM and TAG-139 at 2 μM showed similar reversing effects. TAG-139 at 2 μM showed marginal growth inhibitory effect on either K562 or K562/BCRP cells. Consequently, TAG-139 was 5-fold more active as a BCRP antagonist than estrone. The reversal activities of estrone, diethylstilbestrol, and TAG-139 were more prominent for mitoxantrone than for SN-38. Reversal of SN-38 and mitoxantrone resistances in K562/BCRP cells by TAG-139 was stronger than that by estrone, however, the dose-dependent characteristics of drug resistance reversal by TAG-139 (Fig. 7*B*) and estrone (Fig. 4*A*) were very similar. This suggests that tamoxifen derivatives and estrone interact with the same drug-binding site of BCRP.

We showed that there was no correlation between BCRP-reversing activity and antiestrogen activity (Fig. 8). Therefore it should be possible to develop anti-BCRP compound without antiestrogen activity. As shown in Fig. 8, TAG-139, the best compound found in this study, showed significant antiestrogen activity. The anti-BCRP activity of TAG-139 was

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weaker than that of a reported anti-BCRP agent fumitremorgin C (23). Therefore, TAG-139 should be treated as a lead for the development of the third generation compounds. TAG-139 showed reversing activity on P-glycoprotein-mediated drug resistance but not on MRP1-mediated drug resistance (Fig. 9). Subsequently, it should be practical to develop stronger BCRP-reversing agents that have no antiestrogen or any other biological activity and show low cytotoxicity. In our preliminary study, some BCRP antagonists were effective against BCRP-transduced P388 cells both *in vitro* and *in vivo*. The effect of BCRP-reversing agents against BCRP-expressing tumor cells as well as the specificity of inhibition, bioavailability, pharmacokinetics, and toxicity of drugs should be evaluated more extensively.

Interestingly, mitoxantrone cytotoxicity on parental K562 cells was enhanced in the presence of tamoxifen, toremifene, or TAG-compounds, but SN-38 cytotoxicity was not (Figs. 4 and 7). This synergistic effect also occurred in murine lymphoma P388. We have previously reported a similar synergistic effect with mitoxantrone and progesterone (11). The profiles of synergistic effects of mitoxantrone/progesterone and mitoxantrone/tamoxifen derivatives on K562 cells look very similar. Maximum increase in mitoxantrone sensitivity was ~2-fold, and a synergistic effect occurred with <1 μM of progesterone or tamoxifen derivatives. This synergistic effect does not seem to be related to anti-BCRP activity of tamoxifen derivatives. However, it is not clear at present whether this synergistic effect is related to cytotoxicity or antiestrogen activity of the compounds. This synergistic effect of mitoxantrone and tamoxifen derivatives may have caused some confusion to evaluate anti-BCRP activity of tamoxifen derivatives, but these two potentiation effects are independent of each other.

Recent reports showed the association of BCRP expression with poor responses to chemotherapy (15, 16). It is possible that BCRP expression is responsible, at least in part, for clinical drug resistance. If it should be true, overcoming BCRP-mediated drug resistance would contribute to cancer chemotherapy. Additionally, BCRP is overexpressed in some cancer cell lines other than those of the genital organs (22) and may underlie the natural resistance to antitumor agents. GF120918 and fumitremorgin C have been found to reverse BCRP-mediated drug resistance (23, 24). We previously identified steroid hormones of placental origin as the first endogenous compounds with BCRP-reversing activity (11). To the best of our knowledge, this is the first report that estrogen agonists and antiestrogens reverse BCRP-mediated drug resistance.

Another significance of the current finding is the possible effect of clinically used antiestrogens on the bioavailability of BCRP-interacting anticancer agents. BCRP inhibitors, like P-glycoprotein inhibitors, not only potentiate the cytotoxicity on BCRP-expressing cells but also alter the bioavailability and pharmacokinetics of drugs distributed by BCRP (19, 25). A P-glycoprotein inhibitor GF120918 increased oral bioavailability of topotecan through the inhibition of BCRP function (25). The camptothecins are good BCRP substrates and are increasingly used in chemotherapy. Some topoisomerase I inhibitors also appear to be excellent substrates. Modulation of BCRP activity by inhibitors should alter the pharmacokinetics of such drugs in a number of contexts. These effects might be used to advantage in improving several aspects of chemotherapy such as reduction of the variability in exposure to p.o. administered topotecan and potentiation of the cytotoxic activity of irinotecan. In addition to that, unintentional side effects may be caused because of the modulation of bioavailability by the inhibition of transporters. Recently, methotrexate was identified as a substrate of BCRP (26). Tamoxifen and methotrexate have been used widely for the treatment of breast cancer. Therefore, more attention should be paid to the coadministration of these drugs.

In summary, some estrogen agonists and antiestrogens were found to restore drug sensitivity in K562/BCRP cells by increasing the cellular accumulation of anticancer drugs. These findings should serve for the development of more practical therapies and the design of more effective and safe reagents to circumvent drug resistance. These findings should also contribute to design new types of transporter-directed combination chemotherapy and to avoid unintentional potentiation of anticancer drugs because of the inhibition of BCRP.

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