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Estrogen-mediated post transcriptional down-regulation of P-glycoprotein in *MDR1*-transduced human breast cancer cells

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The human multidrug resistance gene 1 (*MDR1*) encodes the plasma membrane P-glycoprotein (P-gp/ABCB1) that functions as an efflux pump for various anticancer agents. We recently reported that estrogens down-regulate the expression of breast cancer resistance protein (BCRP/ABCG2). In our present study we demonstrate that estrogens also down-regulate P-gp expression in the *MDR1*-transduced, estrogen receptor α (ER-α)-positive human breast cancer cells, MCF-7/MDR and T-47D/MDR. The P-gp expression levels in MCF-7/MDR cells treated with 100 pM estradiol were found to be 10–20-fold lower than the levels in these same cells that were cultured without estradiol. In contrast, estradiol did not affect the P-gp expression levels in the ER-α-negative cancer cells, MDA-MB-231/MDR and NCI/ADR-RES. Estrone and diethylstilbestrol were also found to down-regulate P-gp in MCF-7/MDR cells, but progesterone treatment did not produce this effect. Tamoxifen reversed the estradiol-mediated down-regulation of P-gp in MCF-7/MDR cells, suggesting that ER-α activity is necessary for the effects of estradiol upon P-gp. However, estradiol was found not to alter the *MDR1* transcript levels in either MCF-7/MDR and T-47D/MDR cells, suggesting that post-transcriptional mechanisms underlie its effects upon P-gp down-regulation. MCF-7/MDR cells also showed eight-fold higher sensitivity to vincristine when treated with 100 pM estradiol, than when treated with 1 pM estradiol. These results may serve to provide a better understanding of the expression control of ABC transporters, and possibly allow for the establishment of new cancer chemotherapy strategies that would control P-gp expression in breast cancer cells and thereby increase their sensitivity to *MDR1*-related anticancer agents. (*Cancer Sci* 2006; 97: 1198–1204)

P-glycoprotein (P-gp), also known as ABCB1, is a 170–180 kDa transmembrane glycoprotein encoded by the human *multidrug resistance gene 1* (*MDR1*). P-gp is also a member of the ATP-binding cassette (ABC) transporter family and functions as an efflux pump for various structurally unrelated anticancer agents such as the *vinca* alkaloids, anthracyclines and taxans.[1-4] Cancer cells transfected with *MDR1* cDNA have been shown to have significantly higher resistance to vinblastine and doxorubicin than their parental cell controls.[5] Moreover, the *MDR1* gene expression levels in acute myelogenous leukemic cells have been correlated with a poor response to induction chemotherapy.[6] *MDR1* transcript levels have been shown to be generally high in untreated, intrinsically drug-resistant tumors of the colon, kidney, liver and pancreas, and are elevated in some cancers following a relapse after chemotherapy.[7] Hence, P-gp expression is one of the most important determinants of the effectiveness of chemotherapeutic agents in the treatment of cancer.

To circumvent P-gp-mediated drug resistance in human malignancies, various inhibitors that competitively inhibit the P-gp-mediated efflux of anticancer agents have been studied.[8,9] Another possible strategy for circumventing P-gp-mediated multidrug resistance would be the use of compounds that reduce its expression in cancer cells. However such compounds have not yet been reported. P-gp is also widely expressed in normal cells and tissues, such as the liver, kidney, adrenal, intestine, placenta, and the endothelial cells at both blood–brain and blood–testis barriers.[10,11] This suggests that P-gp functions in the protection of these tissues from cytotoxic agents and xenobiotics. Therefore, the expression levels and activity of P-gp in these tissues would be expected to have an impact on the blood and tissue levels of such compounds. Indeed, clinical studies of P-gp inhibitors have now shown that they increase the plasma concentrations of P-gp substrate anticancer agents.[12,13] Hence, the suppression of P-gp may also affect the pharmacokinetics of its chemotherapeutic substrates.

We previously reported that both estrogens[14] and antiestrogens[15] inhibit breast cancer resistance protein (BCRP)-mediated drug resistance and subsequently showed that sulfated estrogens are physiological substrates of BCRP.[16,17] In the course of analyzing the interaction of estrogens with BCRP, we also found that the physiological levels of estradiol (E_2) down-regulate both endogenous and exogenous BCRP expression in estrogen receptor α (ER-α)-positive cells by post-transcriptional mechanisms.[18] Therefore, the two effects of estrogens on BCRP, i.e. the inhibition of drug efflux and the down-regulation of protein expression, occur by different mechanisms.[18,19] In this study, we examined the effect of estrogens in *MDR1*-transduced, ER-α-positive human breast cancer cells and found that they down-regulate P-gp expression in these cells.

Materials and Methods

Antibodies. The anti-P-gp antibody C219,[20] anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody and anti-ER-α antibody (NCL-ER--6F11) were obtained from Cencor (Malvern, PA), Chemicon (Temecula, CA) and Novocastra (Newcastle, UK), respectively. The peroxidase-conjugated sheep antimouse secondary antibody was purchased from Amersham (Buckinghamshire, UK). The biotinylated $F(ab')_2$ fragment of the anti-P-gp antibody, MRK16, was prepared in our laboratory as described previously.[21]

Cell cultures. The estrogen-free basal medium used in this study consists of phenol red free-DMEM (Dulbecco's modified Eagle medium) and 7% charcoal/dextran-treated fetal bovine serum (HyClone, Logan, UT). The human cancer cell lines MCF-7, T-47D, MDA-MB-231 and NCI/ADR-RES were obtained from the 60 cell line panel of the National Cancer Institute (NCI, Bethesda, MD) and maintained in basal medium supplemented with 10 pM E_2 at 37°C in a humidified incubator with 5% CO_2. NCI/ADR-RES was used as a control cell line that overexpresses

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endogenous P-gp in the NCI cell line panel, but lacks the expression of ER-α.[22] The MCF-7, T-47D and MDA-MB-231 cell lines were transduced with HaMDR retrovirus[23–25] and selected in 4 ng/mL vincristine for seven days. The resulting mixed populations of the transduced cells that stably expressed P-gp were designated MCF-7/MDR, T-47D/MDR and MDA-MB-231/MDR, respectively.

Western blot analyzes of P-gp and ER-α. Cells were cultured in basal medium in the absence or presence of various concentrations of E_2 for four days. For the Western blot analysis of P-gp, the cells were harvested and the resulting cell pellets were resuspended in lysis buffer (10 mM Tris-HCl [pH 8.0], 0.1% Triton X-100, 10 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM 4-[2-aminoethyl]-benzenesulfonylfluoride, 1% aprotinin, 1 mM dithiothreitol [DTT]). The cell lysates were obtained after freeze-thawing and brief centrifugation, and then solubilized in sample buffer A (2% SDS, 50 mM Tris-HCl [pH 8.0], 0.2% bromophenol blue, 5% 2-mercaptoethanol). The solubilized proteins were separated by SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were incubated with the anti-P-gp antibody C219 (1 μg/mL) and anti-GAPDH antibody (0.44 μg/mL), followed by a treatment with the peroxidase-conjugated sheep antimouse secondary antibody. Membranes were subsequently developed with an Enhanced Chemiluminescence Plus detection kit (Amersham).

For the Western blot analysis of ER-α, cell pellets were directly solubilized in sample buffer B (2% SDS, 62 mM Tris-HCl, 10% glycerol). The solubilized proteins from 1.5×10^5 cells were separated by SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were incubated with the anti-ER-α antibody NCL-ER--6F11 (1:30 dilution) and anti-GAPDH antibody (0.44 μg/mL). The membranes were then processed as described above.

Fluorescence-activated cell sorting (FACS) analysis of P-gp expression levels. To determine the expression levels of P-gp on the surface of E_2-treated cells, the human-specific monoclonal antibody, MRK16, that reacts with a cell surface epitope of P-gp was employed. Cells were incubated in basal medium in the absence or presence of various concentrations of E_2 for four days. The cells (5×10^5 per test) were then incubated with or without the biotinylated $F(ab')_2$ fragment of MRK16 (100 μg/mL) and then washed and incubated with R-phycoerythrin-conjugated streptavidin (400 μg/mL; BD Biosciences, Franklin Lakes, NJ).[25] The fluorescence staining levels were detected using FACS Calibur (BD Biosciences).

Reverse transcription polymerase chain reaction analysis of *MDR1* mRNA. *MDR1* mRNA levels were examined by reverse transcription polymerase chain reaction (RT-PCR) analysis. Cells were incubated in basal medium in the absence or presence of various concentrations of E_2 for four days. The extraction of total RNA and subsequent RT-PCR reactions were performed using an RNeasy kit (Qiagen, Valencia, CA) and an RNA LA PCR kit (Takara, Ohtsu, Japan), respectively, according to the manufacturer's instructions. First-strand *MDR1* cDNA was synthesized with 0.3 μg of total RNA, and a 702 bp fragment of *MDR1* cDNA was subsequently amplified with the following primers; forward 5′-GATATCAATGATACAGGGTT-3′ and reverse 5′-TGTCC-AATAGAATATTCCCC-3′. As an internal control, amplification of *GAPDH* cDNA (551 bp fragment) was carried out with the following primers; forward 5′-ATCACCATCTTCCAGGAGCGA-3′ and reverse 5′-GCTTCACCACCTTCTTGATGT-3′. The PCR conditions were as follows: 95°C for 5 min; the indicated cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and a final extension step for 7 min at 72°C.

Intracellular accumulation of rhodamine 123 and vincristine. To examine rhodamine 123 accumulation, MCF-7 and MCF-7/MDR cells were treated with either 1 or 100 pM E_2 for four days and then trypsinized, harvested and resuspended in basal medium. These cells (1×10^6 per test) were then incubated with 100 nM rhodamine 123 in basal medium supplemented with the same concentrations of E_2 for 20 min at 37°C, washed twice with ice-cold PBS, and subjected to FACS analysis using FACS Calibur.[26]

To examine vincristine accumulation, MCF-7 and MCF-7/MDR cells were seeded on 12-well plates and cultured in basal medium in the presence of either 1 or 100 pM E_2 for four days. The cells were then washed and incubated with 50 nM [^{3}H]vincristine (5.4 Ci/mmol, Amersham) for 0.5, 10, and 30 min at 37°C in basal medium supplemented with the same concentration of E_2. At specified times the cells were washed three times with ice-cold PBS and then solubilized by the addition of 1 N NaOH. The cell extract was neutralized and the radioactivity levels were determined in a liquid scintillation counter (Aloka, Tokyo, Japan).

Drug sensitivity assay. The sensitivity of MCF-7/MDR cells to anticancer agents was evaluated by cell growth inhibition assays. MCF-7/MDR cells were pretreated with the indicated concentrations of E_2 for two days and then treated with various concentrations of vincristine in the presence of the same concentrations of E_2. After four days of vincristine treatment, the cell numbers were determined with a cell counter (Sysmex, Kobe, Japan). Data are represented as the mean ± SD from triplicate determinations. In instances where a horizontal bar is not shown, the SD is low and is contained within the symbol.

Results

Establishment of *MDR1* transduced cells. In a previous study we demonstrated that estrogens down-regulate both endogenous and exogenous BCRP expression in human breast cancer cells. In this study we initially screened the 60 cell line panel of the NCI for ER-α and P-gp expression. However, most of these established human breast cancer cells are estrogen-independent and do not express ER-α. Two estrogen-dependent human breast cancer cell lines, MCF-7 and T-47D cells, expressed ER-α (Fig. 1b) but were found not to express appreciable amounts of P-gp (Fig. 1a). We

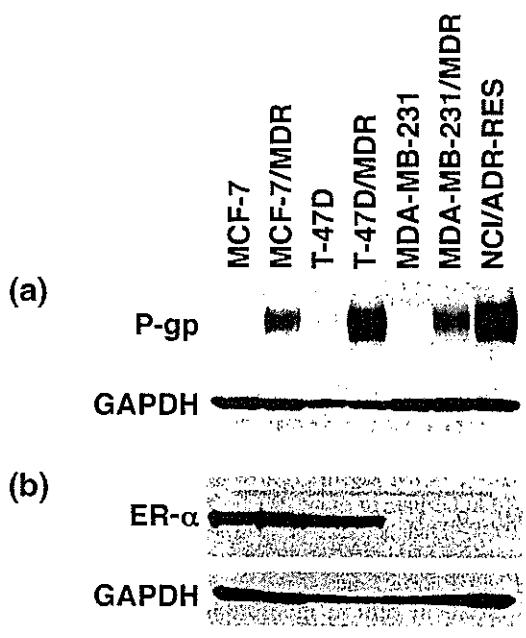

Fig. 1. P-glycoprotein (P-gp) and estrogen receptor α (ER-α) expression levels in cancer cell lines. For the detection of P-gp, cell lysates (40 μg) were subjected to western blot analysis with the anti-P-gp monoclonal antibody, C219. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were assessed as a loading control using the anti-GAPDH monoclonal antibody. For the detection of ER-α, whole cell lysates were subjected to western blot analysis with the anti-ER-α monoclonal antibody, NCL-ER--6F11, and anti-GAPDH monoclonal antibody.

therefore established stable *MDR1*-transduced human breast cancer cell lines to examine the effects of estrogens upon the expression levels of P-gp. In addition, we used two ER-α-negative cancer cells as controls. MDA-MB-231, which does not express P-gp, and NCI/ADR-RES, a doxorubicin-selected MDR cell line[22,27] that expresses endogenous P-gp (Fig. 1).

The MCF-7/MDR, T-47D/MDR and MDA-MB-231/MDR cell lines were established after transduction of the corresponding parental cells with a HaMDR retrovirus and subsequent selection of the transduced cells with vincristine. Mixed populations of the transduced cells were found to express P-gp (Fig. 1a). In addition, the P-gp expression levels in the transduced cells were found to be stable for at least several months.

MCF-7, MCF-7/MDR, T-47D and T-47D/MDR cells express ER-α (Fig. 1b), indicating that *MDR1* transduction did not affect the expression levels of this gene in these cells. In contrast, MDA-MB-231, MDA-MB-231/MDR and NCI/ADR-RES cells do not express ER-α (Fig. 1b). Furthermore, MCF-7/MDR, T-47D/MDR and MDA-MB-231/MDR cells showed significantly higher resistance to vincristine and doxorubicin than their parental cells (Table 1).

Estradiol down-regulates P-gp expression in *MDR1*-transduced cells. We next examined whether estrogens would down-regulate P-gp in the MCF-7/MDR, T-47D/MDR, MDA-MB-231/MDR and NCI/ADR-RES cells. As shown in Fig. 2, exogenous P-gp expression was predictably decreased in MCF-7/MDR and T-47D/MDR cells in a dose-dependent manner, following treatment with 10 pM-10 nM E_2. MCF-7/MDR or T-47D/MDR cells that had been cultured in the absence of E_2, and those treated with 1 pM E_2, show similar levels of P-gp expression, suggesting that this very low dose of E_2 does not affect the expression levels of P-gp. The P-gp expression levels in MCF-7/MDR cells treated with 100 pM E_2 were between 10- and 20-fold less than those in the same cells that were cultured without E_2. In contrast, E_2 did not alter the expression levels of P-gp in either the MDA-MB-231/MDR or NCI/ADR-RES cells (Fig. 2). These results suggest that ER-α expression may be required for the E_2-mediated down-regulation of P-gp. Furthermore, since it is exogenous P-gp that is suppressed by E_2, this down-regulation is likely to be independent of the suppression of the endogenous *MDR1* promoter.

The effects of estradiol on the cell surface expression of P-gp. Because Western blot analysis revealed that E_2 suppresses the expression of P-gp in a dose-dependent manner (Fig. 2), we further examined its effects on the cell surface expression levels of P-gp, also by FACS analysis. Our subsequent findings demonstrated that the expression levels of P-gp on the cell surface were considerably decreased in MCF-7/MDR cells in a dose-dependent manner following treatment with 10 pM-10 nM E_2 (Fig. 3). It is noteworthy

Table 1. Drug resistance levels in *MDR1*-transduced cells

Cell line	Degree of resistance†		Estrogen receptor α expression
	Vincristine	Doxorubicin	
MCF-7/MDR	149 ± 2.1	18 ± 1.8	+
T-47D/MDR	87 ± 2.1	26 ± 2.4	+
MDA-MB-231/MDR	220 ± 1.8	24 ± 0.58	–

†The degree of drug resistance is calculated as the IC_{50} value of the resistant cells, divided by this measurement in the parental cells. The data shown are the mean values ± SD from tripricate determinations.

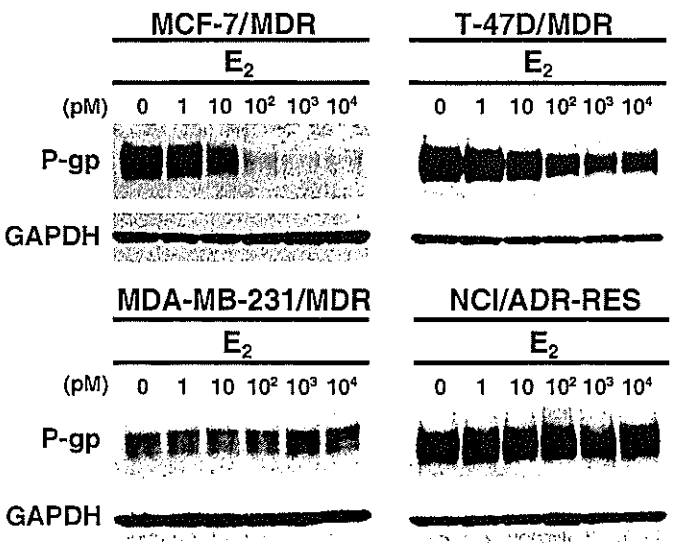

Fig. 2. The effects of E_2 on P-glycoprotein (P-gp) expression. The indicated cells were cultured in basal medium in the absence or presence of the indicated concentrations of E_2 for 4 days. Cell lysates (40 μg) were then subjected to western blot analysis with the anti-P-gp monoclonal antibody, C219. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were assessed as a loading control using the anti-GAPDH monoclonal antibody.

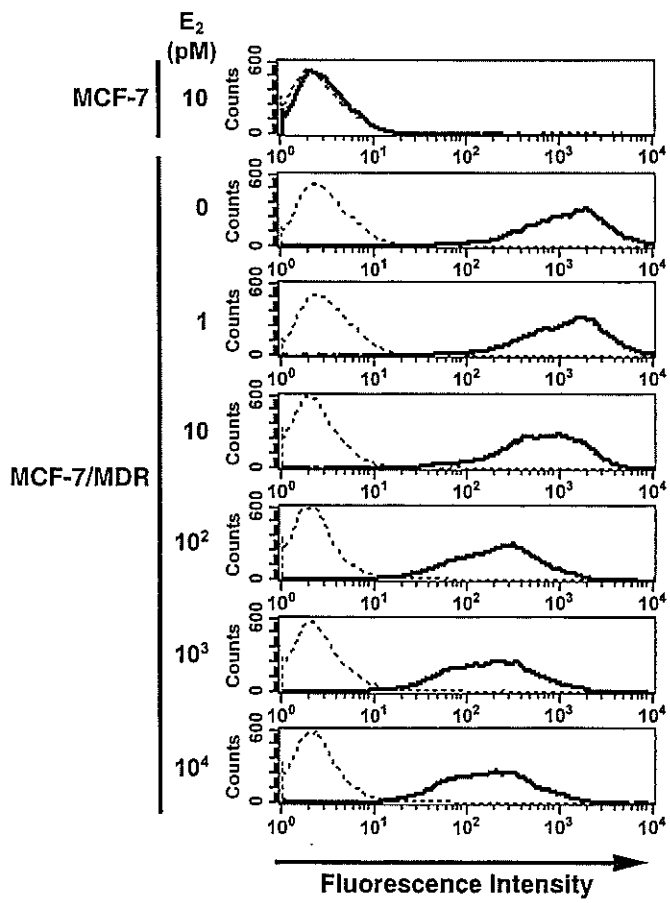

Fig. 3. Analysis of the cell surface expression of P-glycoprotein (P-gp) using fluorescence-activated cell sorting (FACS) analysis. MCF-7/MDR cells were cultured in basal medium in the absence or presence of the indicated concentrations of E_2 for 4 days. MCF-7/MDR cells were then harvested, incubated with or without the biotinylated $F(ab')_2$ fragment of the anti-P-gp monoclonal antibody, MRK16, and then incubated with R-phycoerythrin-conjugated streptavidin. After washing the cells, the fluorescence intensities were determined using FACSCalibur. The bold and dotted lines indicate that the cells were incubated with and without MRK16, respectively.

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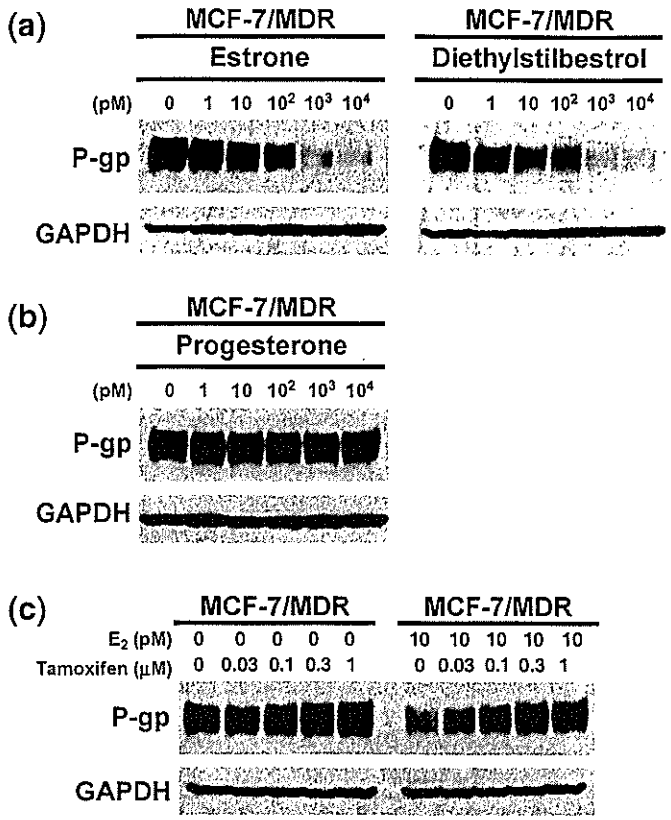

Fig. 4. The effects of estrogens, progesterone and tamoxifen on P-glycoprotein (P-gp) expression levels. MCF-7/MDR cells were cultured in basal medium in the absence or presence of the indicated concentrations of each agent for 4 days. Cell lysates (40 μg) were then subjected to western blot analysis with the anti-P-gp monoclonal antibody, C219, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody to normalize for protein loading control. (a) Effects of estrogens on P-gp expression. (b) Effects of progesterone on P-gp expression. (c) Effects of tamoxifen on P-gp expression in the absence of E_2 (left panel) and the reversal effects of tamoxifen on the E_2-mediated down-regulation of P-gp (right panel).

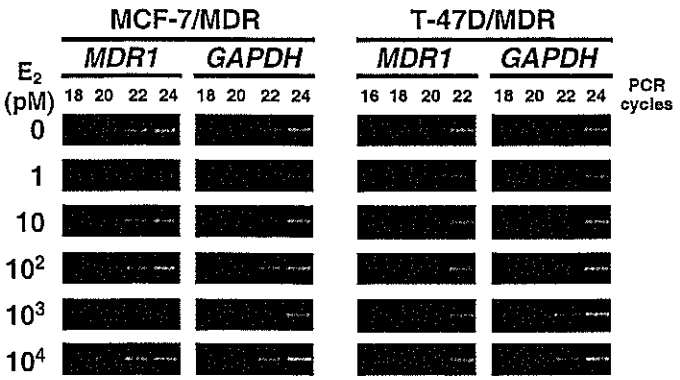

Fig. 5. Reverse transcription polymerase chain reaction (RT-PCR) analysis of the *MDR1* mRNA expression levels in both MCF-7/MDR and T-47D/MDR cells. Cells were cultured in basal medium in the absence or presence of the indicated concentrations of E_2 for 4 days. Exponentially growing cells were harvested and total RNA was extracted. First-strand cDNAs were synthesized with 0.3 μg of total RNA, and an *MDR1* cDNA fragment (702 bp) was subsequently amplified by PCR using the indicated cycles. The amplification of *GAPDH* mRNA (551 bp fragment) was performed as an internal control.

that the cells treated with 100 pM-10 nM E_2 in this experiment still expressed significant amounts of P-gp but we speculate that this may be due to the high sensitivity of the fluorescence signals when using a biotin-streptavidin amplification system.

The effects of estrogen and antiestrogen on P-gp expression. We examined the effects of other estrogenic compounds, and of progesterone, upon P-gp expression in MCF-7/MDR cells. The expression levels of P-gp in these cells were observed to have decreased in a dose-dependent manner following treatment with either estrone or diethylstilbestrol (Fig. 4a). However, slightly higher concentrations of estrone or diethylstilbestrol were required to achieve similar levels of P-gp down-regulation, compared to E_2 (Figs 2, 4a). On the other hand, progesterone did not show any suppressive effects on P-gp expression in MCF-7/MDR cells (Fig. 4b).

We next examined whether tamoxifen, an antiestrogen, could reverse the estrogen-mediated down-regulation of P-gp in MCF-7/MDR cells. In the absence of E_2, tamoxifen slightly increased P-gp expression. Significantly, in the presence of 10 pM E_2, tamoxifen was found to reverse the E_2-mediated down-regulation of P-gp in a dose-dependent manner (Fig. 4c). These data further suggest that estrogenic activity is required for the down-regulation of P-gp expression.

Estradiol does not alter *MDR1* mRNA levels. We next examined the effects of E_2 on *MDR1* mRNA levels. RT-PCR analyzes were carried out following the incubation of both MCF-7/MDR and T-47D/MDR cells in the absence or presence of various concentrations of E_2 for four days. Our subsequent results revealed that the *MDR1* mRNA levels were not affected by E_2-treatment in either cell type (Fig. 5). This indicates that the E_2-mediated down-regulation of P-gp may depend on either translational or post-translational processes.

The effects of estradiol on P-gp-mediated transport and resistance. We examined the effects of either 1 or 100 pM E_2 treatment on the cellular accumulation of rhodamine 123 and vincristine in the MCF-7 and MCF-7/MDR cells. MCF-7/MDR cells treated with 100 pM E_2 showed higher rhodamine 123 uptake, compared with 1 pM E_2 treatment (Fig. 6a). In contrast, MCF-7 cells treated with either 1 or 100 pM E_2 showed similar levels of rhodamine 123 uptake (Fig. 6a). MCF-7 cells showed higher rhodamine 123 uptake than MCF-7/MDR cells for both E_2 treatments.

We additionally examined vincristine uptake in E_2-treated cells. MCF-7/MDR cells treated with 100 pM E_2 showed higher [^{3}H]vincristine uptake compared to the 1 pM E_2 treatment (Fig. 6b). In contrast, MCF-7 cells treated with either 1 or 100 pM E_2 showed similar levels of [^{3}H]vincristine uptake, which was at higher levels than MCF-7/MDR cells in both cases (Fig. 6b). These results therefore suggest that the down-regulation of P-gp by E_2 results in an increase of the cellular uptake of P-gp substrates.

We then examined the effects of E_2 on cellular drug resistance. As shown in Figure 7a, MCF-7/MDR cells treated with 100 pM E_2 show a higher sensitivity to vincristine than the same cells treated with 1 pM E_2. The IC_{50} values for vincristine in MCF-7 cells in the presence of 1 and 100 pM E_2 were determined to be 0.35 ± 0.04 and 0.34 ± 0.01 nM, respectively (Fig. 7a). In contrast, the IC_{50} values for vincristine in MCF-7/MDR cells in the presence of 1 and 100 pM E_2 were measured at 68.7 ± 2.9 and 8.4 ± 0.2 nM, respectively (Fig. 7a). Hence, MCF-7/MDR cells treated with 100 pM E_2 showed an eight-fold higher sensitivity to vincristine than the same cells treated with 1 pM E_2. The timecourse of the P-gp expression profile in the E_2-treated MCF-7/MDR cells further supports our hypothesis that the suppression of P-gp expression by E_2 leads to the sensitization of cells to anticancer agents (Fig. 7b).

Discussion

In our previous study, we reported for the first time that the physiological levels of estrogens (10–100 pM) down-regulate

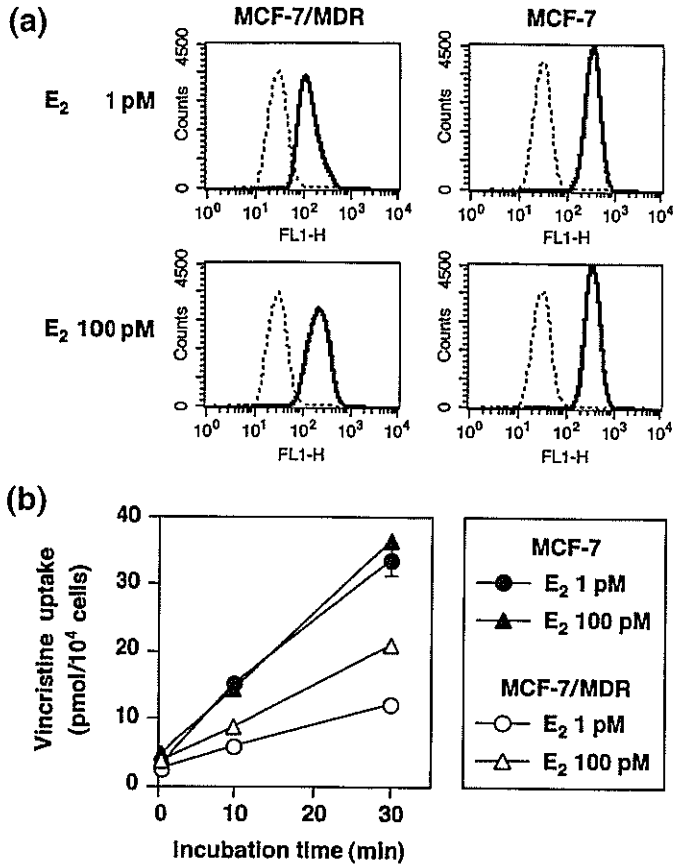

Fig. 6. Analysis of rhodamine 123 and [^{3}H]vincristine uptake in E_2-treated MCF-7/MDR cells. (a) Rhodamine 123 uptake. MCF-7 and MCF-7/MDR cells were cultured in basal medium in the presence of either 1 or 100 pM E_2 for 4 days. After trypsinization, the cells were incubated with (—) or without (---) 100 nM rhodamine 123 in basal medium supplemented with the same concentrations of E_2 for 20 min. After washing of the cells, the cellular uptake of rhodamine 123 was measured by FACSCalibur. (b) [^{3}H]Vincristine uptake. MCF-7 (▲ ●) and MCF-7/MDR (△ ○) cells were cultured in basal medium in the presence of either 1 pM (● ○) or 100 pM (▲ △) E_2 for 4 days. The cells were then washed and incubated with 50 nM [^{3}H]vincristine for 0.5, 10, and 30 min in basal medium supplemented with the same concentration of E_2. After washing of the cells, the cells were lyzed, and [^{3}H]vincristine uptake was measured by liquid scintillation. Data are the mean ± SD values from triplicate determinations. Where a horizontal bar is not shown, the SD is low and contained within the symbol.

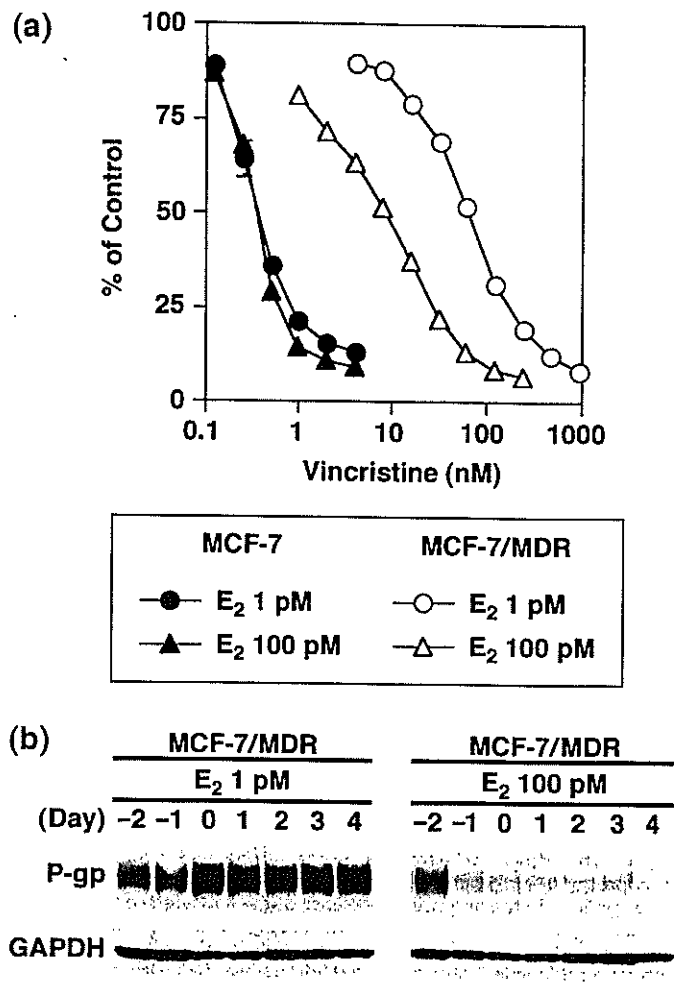

Fig. 7. Drug sensitivity levels of E_2-treated MCF-7/MDR cells. (a) Drug sensitivity to vincristine. MCF-7 cells (● ▲) and MCF-7/MDR cells (○ △) were cultured in basal medium in the presence of either 1 pM (● ○) or 100 pM (▲ △) E_2 for 2 days. Vincristine was then added to the cultures and the cells were incubated for an additional 4 days. Cell numbers were determined using a cell counter. Data are the mean ± SD values from triplicate determinations. Where a horizontal bar is not shown, the SD is low and contained within the symbol. (b) Timecourse analysis of the P-gp expression levels in E_2-treated MCF-7/MDR cells. MCF-7/MDR cells were cultured in basal medium in the presence of either 1 or 100 pM E_2 for up to 6 days. Cell pellets were obtained each day and cell lysates were subjected to western blot analysis with the anti-P-glycoprotein (P-gp) monoclonal antibody, C219.

BCRP expression by post-transcriptional processes without affecting the *BCRP* transcript levels.[18] Because P-gp also belongs to the ABC transporter superfamily, in addition to BCRP, we examined the effects of estrogens on P-gp expression in our current study and demonstrate that E_2-mediated down-regulation of P-gp expression occurs in ER-α-positive human breast cancer cells.

It is significant that E_2 down-regulates P-gp expression only in the ER-α-positive cells, MCF-7/MDR and T-47D/MDR, but not in the ER-α-negative cells, MDA-MB-231/MDR and NCI/ADR-RES (Fig. 2). Estrone and diethylstilbestrol were also found to down-regulate P-gp expression in MCF-7/MDR cells (Fig. 4a), but progesterone did not cause such effects (Fig. 4b). Moreover, the antiestrogen drug, tamoxifen, strongly reverses the E_2-mediated down-regulation of P-gp in MCF-7/MDR cells (Fig. 4c). These results suggest that a functional ER-α and its downstream pathways are important for estrogen-mediated P-gp down-regulation.

In a previous study we demonstrated that estrogens down-regulate BCRP expression without affecting its gene promoter activity.[18] In this study we also screened the 60 cell line panel of the NCI for ER-α- and P-gp-positive cells. However, the ER-α-positive MCF-7 and T-47D cells that we used in our present experiments do not express endogenous P-gp. Moreover, NCI/ADR-RES, a cell line in the NCI panel that does overexpress P-gp, is likely to be of ovarian origin, and thus lacks any expression of ER-α.[22] We therefore established *MDR1*-transduced cells, MCF-7/MDR and T-47D/MDR, to enable us to examine the effects of estrogens on P-gp expression in ER-α-positive cells. We show herein that estrogens down-regulate P-gp expression in *MDR1*-transduced cells, but there remains a possibility that estrogens may not exert such an effect in ER-α-positive human breast cancer cells, if they were to activate the *MDR1* promoter. To clarify this point, the effects of estrogen on P-gp expression in ER-α-positive human breast cancer cells that express endogenous P-gp will need to be assessed in the near future, when such cell lines become available.

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It has been reported that murine *mdr* mRNA is expressed at high levels in the gravid uterus and that these levels dramatically increase during pregnancy, compared with the relatively low levels of expression observed in the non-gravid uterus.[28] In addition, it was previously shown that *mdr* mRNA and murine P-gp are induced at high levels in the uterine secretory epithelium by a combination of estrogen and progesterone treatments.[29] In humans, the high expression levels of P-gp in the syncytiotrophoblast of the placenta has been reported during the early phase of pregnancy.[30] However, although these reports suggest a possible role for estrogen/progesterone in the control of P-gp expression, most studies have focused only on the regulation of *MDR1/mdr* mRNA expression. In our present study, we have shown that estrogens down-regulate P-gp expression in ER-α-positive human breast cancer cells, without affecting *MDR1* transcription levels. It is not clear at present whether our observations are in any way linked with the findings of previous studies of *MDR1* regulation in the uterus, but our present data do suggest the possibility of a role for estrogens in the control of P-gp expression in breast cancer cells and possibly also in normal breast tissues.

Clinical trials of P-gp inhibitors have now been conducted against various malignancies, including advanced breast cancers.[31–34] Tidefelt *et al.* examined the effects of the cyclosporinee derivative, PSC 833, on the concentration of daunorubicin in leukemic blast cells *in vivo* during treatment.[35] They have shown that PSC 833 treatment results in a higher increase of cellular daunorubicin concentrations in P-gp-positive leukemic cells than in P-gp-negative leukemic cells. These results suggest that PSC 833, by interacting with P-gp, can increase the cellular uptake of daunorubicin in leukemic blasts *in vivo*.[35] However, the results of clinical trials using verapamil or other P-gp inhibitors, in combination with either doxorubicin, vincristine, paclitaxel or epirubicin, against breast cancers have resulted in only minor responses, with PR rates of 10–20%. The evidence provided so far is therefore not entirely sufficient to evaluate the possible effectiveness of P-gp inhibition during chemotherapies against breast cancer.

Tamoxifen has also been reported to overcome P-gp-mediated drug resistance[36] and clinical trials of high oral doses of tamoxifen have been conducted to examine for possible MDR-reversing effects.[37–41] The earliest studies were conducted using tamoxifen alone, and subsequent studies used tamoxifen in combination with other MDR modulators. Unfortunately, these trials proved to be only moderately successful. The patients enrolled in these studies had presented with either colorectal or renal carcinomas, and it may thus be difficult to discuss the results of these studies in the context of our present analyzes. Human breast cancer cells grow in the presence of estrogens, which are supplied from the ovary and placenta. In the presence of estrogens, tamoxifen may increase the P-gp expression of ER-α-positive cells. Hence, the possible effects of tamoxifen on the P-gp expression levels in human breast cancer cells *in vivo* is an important area for future studies.

In conclusion, we find that estrogen decreases P-gp expression in *MDR1*-transduced, ER-α-positive human breast cancer cells *in vitro* via post-transcriptional processes. Furthermore, E_2-mediated P-gp down-regulation induces a reduction in the cellular resistance to anticancer agents. These results may therefore serve to provide a better understanding of the expression control of ABC transporters. In addition, the modulation of P-gp expression in breast cancer cells by small molecules may be a useful strategy for sensitizing such cells to anticancer agents.

Acknowledgments

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Estrogen-Mediated Post transcriptional Down-regulation of Breast Cancer Resistance Protein/ABCG2

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Abstract

Breast cancer resistance protein (BCRP)/ABCG2 mediates concurrent resistance to chemotherapeutic agents, such as 7-ethyl-10-hydroxycamptothecin (SN-38), mitoxantrone, and topotecan, by pumping them out of cells. We previously reported that BCRP transports sulfated estrogens. In the present study, we show that at physiologic levels, estrogens markedly decrease endogenous BCRP expression in the estrogen-responsive and estrogen receptor α (ERα)–positive human breast cancer MCF-7 cells, but not in estrogen-nonresponsive human cancer cells. 17 β-Estradiol (E_2) also significantly reduces exogenous BCRP expression, driven by a constitutive promoter, in *BCRP*-transduced estrogen-responsive and ERα-positive MCF-7 (MCF-7/BCRP) and T-47D cells, but not in *BCRP*-transduced estrogen-nonresponsive MDA-MB-231 and SKOV-3 cells. E_2 potentiates the cytotoxicity of SN-38, but not vincristine, in MCF-7/BCRP cells significantly, and increases cellular topotecan uptake in MCF-7 and MCF-7/BCRP cells. Antiestrogen tamoxifen partially reverses E_2-mediated BCRP down-regulation in MCF-7 and MCF-7/BCRP cells and treatment of MCF-7/BCRP cells with an ERα small interfering RNA abolished E_2-mediated BCRP down-regulation, suggesting that interaction of E_2 and ERα is necessary for BCRP down-regulation. E_2 does not affect endogenous *BCRP* mRNA levels in MCF-7 cells or exogenous *BCRP* mRNA levels in MCF-7/BCRP cells. The results from pulse-chase labeling experiments with MCF-7/BCRP cells suggest that decreased protein biosynthesis and maturation, but not alterations in protein turnover, might underlie E_2-mediated BCRP down-regulation. These data indicate that estrogen down-regulates BCRP expression by novel posttranscriptional mechanisms. This is the first report of small molecules that can affect BCRP protein expression in cells and may therefore assist in establishing new strategies for regulating BCRP expression. (Cancer Res 2005; 65(2): 596-604)

Introduction

Breast cancer resistance protein (BCRP), also known as ABCG2, is a half-size ATP-binding cassette transporter with a molecular weight of 80 kDa (1–3). BCRP mediates concurrent resistance to chemotherapeutic agents, such as 7-ethyl-10-hydroxycamptothecin (SN-38, an active metabolite of CPT-11), mitoxantrone and topotecan, presumably by pumping these compounds out of the cell and thus lowering their cytotoxic effects (1–5). The expression of BCRP in cancer cells may therefore be an important determinant of the efficacy of anticancer agents. We previously reported that estrone (E_1) and 17β-estradiol (E_2) circumvent BCRP-mediated drug resistance and that BCRP transports sulfated estrogens as physiologic substrates (6, 7). In our present study, we have examined the possible effect of estrogens on BCRP expression in cancer cells.

The structure and characterization of the *BCRP* promoter has previously been reported (8). More recently, the identification of an estrogen response element in the *BCRP* promoter and an E_2-mediated increase in *BCRP* mRNA expression in T47D:A18 cells have been shown (9). These findings therefore suggested that estrogens might induce BCRP expression in estrogen-responsive cells.

In the present study, however, we show that BCRP expression is negatively regulated by estrogen at the protein level in MCF-7 and T-47D cells, both of which are estrogen responsive. In addition, we present data suggesting that estrogen down-regulates BCRP expression by posttranscriptional inhibition of protein biosynthesis. This is the first report showing that small molecules can modulate BCRP protein expression in cells and our findings provide new insights on the regulation of BCRP expression in the cell.

Materials and Methods

Reagents. The anti-BCRP mouse monoclonal antibody, BXP-21, was purchased from Chemicon (Temecula, CA) and the anti–c-*myc* mouse monoclonal antibody, 9E10, was obtained from Roche Diagnostics (Mannheim, Germany). PRO-MIX L-[^{35}S] *in vitro* Cell Labeling Mix (L-[^{35}S] Methionine > 1,000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom).

Cell Cultures. Human breast cancer cell lines MCF-7, T-47D, MDA-MB-231, ovarian cancer SKOV-3 cells, and lung cancer A549 cells were maintained in DMEM supplemented with 7% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO_2. MCF-7, A549, and MDA-MB-231 cell clones were established by a limiting dilution method. MCF-7 clone 3, A549 clone 8, and MDA-MB-231 clone 4 were used for further analyses. Hereinafter in the text of this report, MCF-7, A549, and MDA-MB-231 cells represent MCF-7 clone 3, A549 clone 8, and MDA-MB-231 clone 4, respectively, unless ouherwise stated. T-47D cells were obtained from American Type Culture Collection (Rockville, MD) and immediately used for the experiments. To investigate the effects of estrogens upon BCRP expression levels, cells were cultured in the absence or presence of the indicated concentrations of reagents for 4 days in phenol red–free (PRF)-medium containing 93% PRF-DMEM (Roche) and 7% charcoal/dextran-treated FBS (CDFBS; HyClone, Logan, UT).

Establishment of MCF-7/BCRP, T-47D/BCRP, MDA-MB-231/BCRP, and SKOV-3/BCRP Cells. MCF-7/BCRP, T-47D/BCRP, MDA-MB-231/BCRP, and SKOV-3/BCRP cells were established by transduction of MCF-7, T-47D, MDA-MB-231, and SKOV-3 cells, respectively, with a HaBCRP retrovirus, bearing a myc-tagged human *BCRP* cDNA (10). Subsequent selection for the enrichment of transduced cells was done using 50 nmol/L

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SN-38 for 5 to 10 days, with the exception of T-47D cells, which were selected using 24 nmol/L SN-38 for 13 days. The mixed populations of stably transduced cells that were generated by selection were used in subsequent experiments. The levels of myc-tagged BCRP protein in each transduced cell line were unchanged for at least 2 months.

Western Blot Analysis of BCRP. Cells were cultured in the absence or presence of the indicated reagent concentrations for 4 days in PRF-medium. Exponentially growing cells were harvested, washed, and lysed in T buffer [10 mmol/L Tris-HCl (pH 8.0), 0.1% Triton-X 100, 10 mmol/L $MgSO_4$, 2 mmol/L $CaCl_2$, 1 mmol/L 4-(2-aminoethyl)-benzenesulfonylfluoride] with or without 1 mmol/L DTT. After centrifugation, the cell lysates were solubilized with 2% SDS, 50 mmol/L Tris-HCl (pH 7.5), in the absence or presence of 5% 2-mercaptoethanol and resolved by 5% to 20% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes, and blots were then incubated with either 5 μg/mL of the anti-BCRP mouse monoclonal antibody BXP-21 for detection of endogenous BCRP or with 10 μg/mL of the anti-c-*myc* mouse monoclonal antibody 9E10 for detection of exogenous BCRP. After washing, the blots were incubated with the anti-mouse peroxidase-conjugated secondary antibody (Amersham Pharmacia). Membrane-bound peroxidase was visualized on Kodak XAR film (Rochester, NH) after enhancement using a chemiluminescence detection kit (Amersham Pharmacia).

To see how soon the E_2-mediated BCRP down-regulation occurs in MCF-7 and MCF-7/BCRP cells, cells were cultured for 1, 2, 3, and 4 days in PRF-medium in the presence of 3 nmol/L E_2. The following procedure was the same as described above.

Western Blot Analysis of ERα. Cells (1.5×10^5) were solubilized in sample buffer (62 mmol/L Tris, 2% SDS, 10% glycerol) and resolved by 5% to 20% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes, and blots were incubated with the anti-ERα monoclonal antibody, NCL-ER-6F11 (1:30 dilution). The ensuing procedure was the same as described for Western blotting of BCRP.

Cell Growth Studies. To investigate the mitogenic activity of E_2, exponentially growing MCF-7 or MCF-7/BCRP cells (3×10^4/well) were seeded in a 12-well plates and cultured at 37°C in PRF-DMEM supplemented with the indicated concentrations of CDFBS and E_2 for 4 days. Cell numbers were then determined using a cell counter (Sysmex, Kobe, Japan), and presented as percentages relative to those of control cells cultured in PRF-medium. To investigate the effects of E_2 on anticancer drug resistance, the cells were cultured in PRF-medium supplemented with the indicated concentrations of E_2 for 4 days. The exponentially growing cells (3×10^4) were then seeded in 12-well plates and cultured for a further 4 days in PRF-medium supplemented with the same concentration of E_2 used in the pretreatment, in the absence or presence of increasing doses of specific anticancer agents. Cell numbers were determined using a cell counter and presented as percentages relative to those of control cells cultured in the absence of anticancer agents. IC_{50} values (drug dosages that cause 50% inhibition of cell growth) were determined from the growth inhibition curves.

Intracellular Topotecan Uptake in MCF-7 and MCF-7/BCRP Cells. The effects of E_2 on the cellular accumulation of topotecan were determined by flow cytometry. Cells were cultured in PRF-medium supplemented with the indicated concentrations of E_2 for 4 days. After trypsinization, cells (5×10^5) were incubated with 20 μmol/L topotecan for 30 minutes at 37°C, washed in ice-cold PBS, and subjected to fluorescence analysis using FACSCalibur (Becton Dickinson, San Jose, CA). The data are representative of two independent experiments.

Effects of E_2 on BCRP Expression in MCF-7/BCRP Cells Following Small Interfering RNA-induced ERα Knockdown. Cells (2.5×10^5/well) were cultured in PRF-medium in six-well plates for 24 hours and transfected with 100 nmol/L of small interfering RNA (siRNA; for ERα knockdown, ESR1; for control, Luciferase GL3 Duplex, both obtained from Dharmacon, Lafayette, CO) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To confirm subsequent ERα knockdown, the culture medium was exchanged with fresh PRF-medium 6 hours after transfection. After 48 hours, whole cell lysates of 1.5×10^5 cells were subjected to Western blotting. ERα expression was detected with the anti-ERα antibody, NCL-ER-6F11. To investigate the effects of ERα knockdown on E_2-mediated BCRP down-regulation, the culture medium was exchanged with fresh PRF-medium containing the indicated concentrations of E_2, 6 hours after transfection. After 96 hours, cells were harvested and exogenous BCRP expression was determined by Western blotting.

Semi-quantitative Reverse Transcription-PCR Analysis of *BCRP* Expression in MCF-7 Cells. *BCRP* mRNA expression in MCF-7 cells was examined by reverse transcription (RT)-PCR. Cells (5×10^5) were incubated in PRF-medium with various concentrations of E_2 for 4 days. Extraction of total RNA and subsequent RT-PCR were done using an RNeasy Mini kit (Qiagen, Valencia, CA) and an LA-RT-PCR kit (TaKaRa, Kyoto, Japan), according to the manufacturer's instructions, respectively. First-strand cDNA was synthesized with 0.3 μg of total RNA and a 315-bp *BCRP* cDNA fragment was amplified with the primers 5′-CAGGTGGAGGCAAATCTTCGT-3′ (forward) and 5′-ACACACCACGGATAAACTGA-3′ (reverse). As an internal control, amplification of *GAPDH* mRNA (551 bp fragment) was carried out with the primers 5′-ATCACCATCTTCCAGGAGCGA-3′ (forward) and 5′-GCTTCACCACCTTCTTGAT GT-3′ (reverse). The PCR conditions were as follows: 95°C for 9 minutes, then increasing cycle numbers of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and a final 15-min incubation at 72°C. Data are representative of two independent experiments.

Northern Blot Analysis of *BCRP* Expression in MCF-7 and MCF-7/BCRP Cells. Cells (5×10^5) were incubated in PRF-medium with varying concentrations of E_2 for 4 days. Either 20 μg (MCF-7) or 10 μg (MCF-7/BCRP) of total RNA was fractionated on a 1% agarose-formaldehyde gel and transferred to Hybond-N+ (Amersham Pharmacia). The blot was hybridized at 42°C for 16 hours with a 456-bp fragment, from nucleotides 574 to 1029 of *BCRP* cDNA, which was ^{32}P-labeled with a High Prime Probe Labeling Kit (Roche) according to the manufacturer's instructions. The membrane was thoroughly washed and exposed to Kodak XAR film for either 21 days (MCF-7) or 7 days (MCF-7/BCRP). The presented data are representative of two independent experiments.

Metabolic Labeling of BCRP in MCF-7/BCRP Cells. First, cells (1×10^6/well for E_2-treated cells, respectively) were cultured in PRF-medium in six-well plates for 4 days in the absence or presence of 3 nmol/L E_2. After incubation in methionine- and cystine-free DMEM (Roche) supplemented with 7% CDFBS (labeling medium) for 1.5 hours just before beginning the experiment, the resulting 70% to 80% confluent cells were incubated in labeling medium, supplemented with 300 μCi/mL of [^{35}S], for 0.5 and 1 hour. The cells labeled for 1 hour were subsequently chased for an additional 3 hours. For E_2-pretreated cells, 3 nmol/L E_2 was present in the medium throughout the experiment. Cells were then harvested, lysed in T buffer without DTT, and centrifuged. The supernatant was supplemented with 1% of Triton-X and the protein concentration was measured by the Bradford method. Cell lysates (100 μg) were incubated with 0.5 μg of the anti-BCRP antibody, BXP-21, for 30 minutes on ice, and further incubated for an additional 30 minutes on ice after the addition of 5% (v/v) Protein A-Sepharose (Amersham Pharmacia). The immune complex precipitated with Protein A-Sepharose was then washed six times with wash buffer [10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L 4-(2-aminoethyl)-benzenesulfonylfluoride, 1% Aprotinin, 0.1% Triton-X 100], and the pellets were resuspended in 2% SDS, 5% 2-mercaptoethanol, 50 mmol/L Tris-HCl (pH 7.5). The labeled protein was subjected to SDS-PAGE and autoradiographed. The relative rates of labeled BCRP after 4 hours in the presence of E_2 to the

levels in the absence of E_2 are represented as the average ± SD from three independent experiments.

Next, BCRP pulse-chase labeling was done without E_2 pretreatment, because ^{35}S-labeled BCRP was hardly detectable and the half-life of BCRP could not be determined in MCF-7/BCRP cells pretreated with E_2 for 4 days. Cells (2.5×10^6/well) were cultured in PRF-medium for 2 days and the resulting 70% to 80% confluent cells were incubated in labeling medium for 1.5 hours just before beginning the experiment, and then incubated in labeling medium containing 300 μCi/mL of [^{35}S] for 1 hour. The labeling medium was then replaced with fresh PRF-medium and the cells were lysed after 12, 24, 36, and 48 hours from the start of metabolic labeling. To investigate the effect of E_2 on BCRP stability, 3 nmol/L of E_2 was added to the medium in one set of the experiment and was present throughout the pulse-chase experiments. The subsequent procedure was the same as described for E_2-pretreated cells, except that one-fifth of the total immunoprecipitated protein was subjected to SDS-PAGE and autoradiography. The intensities of the bands representing metabolically labeled BCRP were quantified with the NIH-Image densitometric program. The BCRP half-life under each set of experimental conditions is represented as the average ± SD from three independent experiments.

Statistical Analysis. Statistical significance between the two sets of data was evaluated by using the two-sided unpaired Student's *t* test.

Results

Effects of Estrogens on Endogenous BCRP Expression. Effects of estrogens on endogenous BCRP expression were investigated by Western blotting under nonreducing conditions, as this generates stronger BCRP signals. Under the nonreducing conditions, BCRP was detected as a dimer of 160 kDa. Endogenous BCRP protein expression in MCF-7 cells decreased in a dose-dependent manner following treatment with E_1, E_2, and diethylstilbestrol (Fig. 1*A*). Both E_2 E_2 and diethylstilbestrol showed stronger suppressive effects on BCRP expression than E_1 did. MCF-7 cells expressed approximately 2-fold, 5-fold, and 10-fold less amounts of endogenous BCRP protein after treatment with 3 nmol/L E_2 for 1, 2, and 4 days, respectively, as compared with untreated MCF-7 cells (Fig. 1*B*). The inhibitory effect of estrogens on endogenous BCRP expression in MCF-7 cells was also observed in other MCF-7 clones (data not shown). In contrast, endogenous BCRP protein expression was not affected by E_2 in A549 cells (Fig. 1*A*). Because MCF-7 cells are ERα-positive and estrogen-responsive but A549 cells are ERα-negative (Fig. 1*C*), these results suggest that estrogen-mediated BCRP down-regulation might depend on signaling pathways downstream of ERα.

Effects of E_2 on Exogenous BCRP Expression in *BCRP*-Transduced Cells. We further studied the effects of E_2 on exogenous BCRP expression, driven by a constitutive long terminal repeat promoter, in MCF-7/BCRP, T-47D/BCRP, MDA-MB-231/BCRP, and SKOV-3/BCRP cells. Western blotting was done under both nonreducing and reducing conditions, in which BCRP was detected as a dimer of 160 kDa and as a monomer of 80 kDa, respectively. Exogenous BCRP expression decreased in MCF-7/BCRP and T-47D/BCRP cells in a dose-dependent manner following treatment with physiologic levels of E_2 (Fig. 2*A*). MCF-7/BCRP cells expressed approximately 2-fold, 4-fold, and 8-fold less amounts of exogenous BCRP protein after treatment with 3 nmol/L E_2 for 2, 3, and 4 days, respectively, as compared with untreated MCF-7/BCRP cells (Fig. 2*B*). In contrast, exogenous BCRP expression was not affected by E_2 treatment in MDA-MB-231/BCRP and SKOV-3/BCRP cells (Fig. 2*A*). MCF-7 and T-47D cells are estrogen-responsive and express functional ERα (Fig. 2*C*),

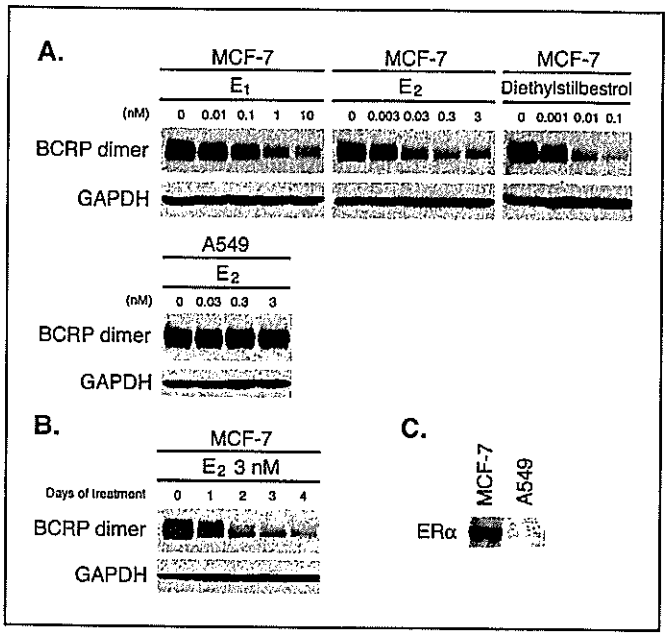

Figure 1. Effects of estrogens on endogenous BCRP expression in cancer cells. Cells were cultured in PRF-medium in the absence or presence of the indicated concentrations of estrogens for 4 days prior to harvesting. Western blot analysis was done under nonreducing conditions, such that the dimeric form of BCRP was detected as a band of approximately 160 kDa. Protein sample (30 μg) was loaded in each lane. BCRP was detected using the anti-BCRP monoclonal antibody, BXP-21. For ERα expression analysis, whole cell lysates consisting of 1.5×10^5 cells were loaded in each lane, and expression was detected by Western blotting using the anti-ERα monoclonal antibody, NCL-ER-6F11. To see how soon the E_2-mediated BCRP down-regulation occurs, MCF-7 cells were cultured for 1, 2, 3, and 4 days in PRF-medium in the presence of 3 nmol/L E_2. The following procedure was the same as described above. *A*, effects of estrogens on endogenous BCRP expression in MCF-7 cells and A549 cells. GAPDH expression was analyzed as a loading control. *B*, time course of E_2-mediated down-regulation of endogenous BCRP in MCF-7 cells. *C*, ERα expression in MCF-7 and A549 cells. The data are representative of at least three independent experiments.

whereas MDA-MB-231 cells do not express ERα, and SKOV-3 cells, which only weakly express nonfunctional ERα, are estrogen-nonresponsive (Fig. 2*C*; refs. 11, 12). These results also suggested that estrogen-mediated BCRP down-regulation may be dependent on ERα function, which may influence posttranscriptional processes rather than the transcription of *BCRP*.

E_2-mediated BCRP down-regulation was more remarkable in MCF-7/BCRP cells than in T-47D/BCRP cells, although MCF-7 cells and T-47D cells expressed similar amounts of ERα (Fig. 2). E_2-mediated BCRP down-regulation would therefore be affected not only by ERα expression levels but by other factors, such as signaling pathways downstream of ERα, in estrogen-responsive, ERα-positive cells.

Cell Growth Studies. E_2, at concentrations of 3×10^{-4} nmol/L or higher, induces mitogenic activity in MCF-7 and MCF-7/BCRP cells cultured in PRF-medium (Fig. 3*A-1*). The mitogenic activity saturated at concentrations of 0.03 nmol/L E_2 or higher in both cell types (Fig. 3*A-1*). The effects of E_2 on anticancer drug sensitivity were therefore investigated within this concentration range. At a concentration of 3 nmol/L, when compared with a 0.03 nmol/L dose, E_2 was found to marginally potentiate the cytotoxicity of SN-38, but not vincristine, in MCF-7 cells (Fig. 3*A-2*). The IC_{50} values for vincristine in the presence of 0.03 and 3 nmol/L E_2 were 0.69 ± 0.01 and 0.65 ± 0.02 nmol/L in MCF-7 cells, respectively. For SN-38, IC_{50} values in the presence of 0.03 and 3 nmol/L E_2

were 1.56 ± 0.15 and 1.22 ± 0.05 nmol/L in MCF-7 cells, respectively. Furthermore, exposure to 3 nmol/L E_2 significantly potentiated the cytotoxicity of SN-38, but not vincristine, in comparison to 0.03 nmol/L E_2 treatment in MCF-7/BCRP cells (Fig. 3*A-2*). The IC_{50} values for vincristine in the presence of 0.03 and 3 nmol/L E_2 were 0.74 ± 0.03 and 0.65 ± 0.02 nmol/L in MCF-7/BCRP cells, respectively. The IC_{50} values for SN-38 at a 3 nmol/L E_2 dose (2.65 ± 0.22 nmol/L) were significantly lower than the values at the 0.03 nmol/L E_2 dosage (5.18 ± 0.46 nmol/L; $P < 0.01$). Because mitogenic activity levels were saturated over the E_2 concentration range that was used (from 0.03 to 3 nmol/L), we conclude that these results also suggest E_2-mediated BCRP down-regulation in MCF-7/BCRP cells.

Intracellular Topotecan Uptake in MCF-7 and MCF-7/BCRP Cells. Effects of E_2 on cellular accumulation of topotecan were investigated. Cellular accumulation of topotecan increased in MCF-7 cells treated with 0.03 nmol/L E_2 as compared with untreated cells, whereas cellular accumulation of topotecan scarcely increased in MCF-7 cells treated with 3 nmol/L E_2 when compared with cells treated with 0.03 nmol/L E_2 (Fig. 3*B*). The results coincided with BCRP down-regulation in E_2-treated MCF-7 cells (Fig. 1*A*). As for MCF-7/BCRP cells, intracellular topotecan accumulation only marginally increased in the presence of 0.03 nmol/L E_2 as compared with untreated cells (Fig. 3*B*). Also, cellular accumulation of topotecan only marginally increased in MCF-7/BCRP cells treated with 3 nmol/L E_2 when compared with those treated with 0.03 nmol/L E_2 (Fig. 3*B*). The results suggest that down-regulation of exogenous BCRP in MCF-7/BCRP cells would not be enough for abrogation of topotecan efflux out of the cells, even after treatment xwith 3 nmol/L E_2.

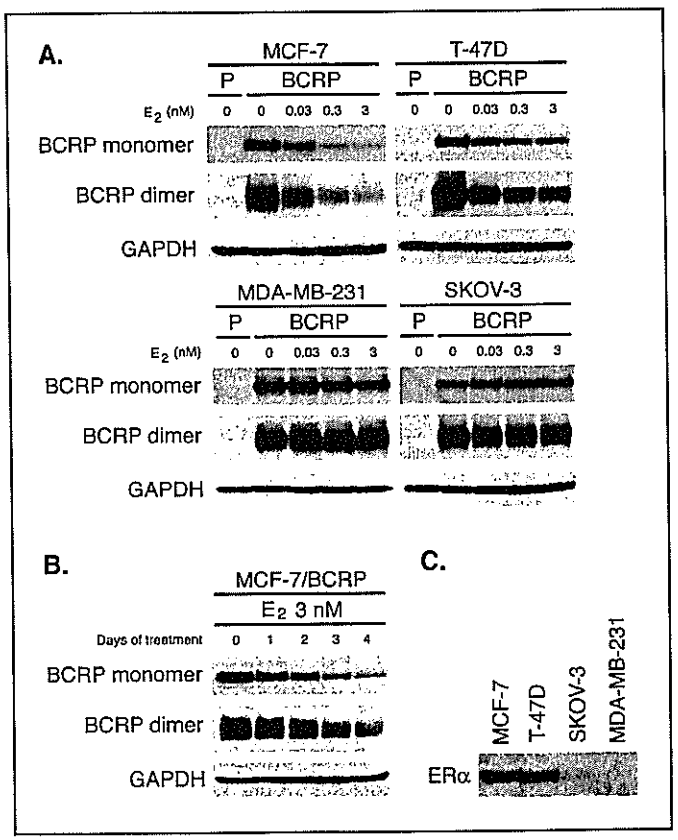

Figure 2. Effects of E_2 on exogenous BCRP expression in *BCRP*-transduced cancer cells. Cells were cultured in PRF-medium in the absence or presence of the indicated concentrations of E_2 for 4 days prior to harvesting. To see how soon the E_2-mediated BCRP down-regulation occurs, MCF-7/BCRP cells were cultured for 1, 2, 3, and 4 days in PRF-medium in the presence of 3 nmol/L E_2. The following procedure was the same as described above. *A*, Western blot analysis of exogenous BCRP expression. The monomeric form of BCRP was detected as an approximately 80 kDa band under reducing conditions, and the dimeric form of BCRP as an approximately 160 kDa band under nonreducing conditions. Protein sample (20 μg) was loaded in each lane. Exogenous BCRP tagged with c-*myc* was detected using anti-c-*myc* antibody, 9E10. GAPDH expression was analyzed as a loading control. *P* and *BCRP* indicate parental cells and *BCRP*-transduced cells, respectively. The data are representative of at least three independent experiments. *B*, time course of E_2-mediated down-regulation of exogenous BCRP in MCF-7/BCRP cells. *C*, ERα expression in MCF-7, T-47D, MDA-MB-231, and SKOV-3 cells. Whole cell lysates consisting of 1.5×10^5 cells were loaded in each lane. ERα expression was detected by Western blotting using the anti-ERα monoclonal antibody, NCL-ER-6F11.

Effects of Tamoxifen and ERα Knockdown by siRNA on E_2-mediated BCRP Down-regulation in MCF-7 and MCF-7/BCRP Cells. MCF-7 cells expressed similar amounts of endogenous BCRP in the presence of increasing concentrations of tamoxifen (Fig. 4*A*, *left*). In MCF-7/BCRP cells, marginally higher levels of exogenous BCRP were produced by increasing dosages of tamoxifen (Fig. 4*B*, *left*), possibly by competition with residual estrogens in the culture medium. Tamoxifen was also found to partially reverse the E_2-mediated down-regulation of either endogenous or exogenous BCRP in a dose-dependent manner (Fig. 4*A* and *B*, *right*). In these tamoxifen reversal experiments using MCF-7/BCRP cells, a concentration of 0.3 nmol/L E_2 was used to down-regulate BCRP, because tamoxifen even at levels of 0.5 μmol/L failed to reverse 3 nmol/L E_2-mediated BCRP down-regulation (data not shown). These results suggest that E_2-mediated BCRP down-regulation in MCF-7 and MCF-7/BCRP cells may be associated with the interaction of E_2 and ERα. We therefore did an experiment in which ERα expression was repressed using siRNA, and investigated the effects of this gene silencing on E_2-mediated modification of BCRP expression. Transfection of 100 nmol/L ERα siRNA resulted in a nearly complete loss of ERα expression in MCF-7/BCRP cells after 48 hours (Fig. 4*C-1*). In addition, this down-regulation of ERα expression persisted for at least 6 days after the siRNA transfections (data not shown). Gene silencing of ERα in MCF-7/BCRP cells by RNA interference was also found to attenuate E_2-mediated *BCRP* down-regulation (Fig. 4*C-2*), indicating that ERα is necessary for the repression of BCRP.

Semi-quantitative RT-PCR and Northern Blot Analysis of *BCRP* Expression in MCF-7 and MCF-7/BCRP Cells. RT-PCR and Northern blot analyses revealed that the treatment of MCF-7 cells with E_2 for 4 days did not affect the expression of endogenous *BCRP* mRNA (Fig. 5*A* and *B*, *left*). Similarly, the same treatment of MCF-7/BCRP cells with E_2 for 4 days did not affect exogenous *HaBCRP* mRNA levels (Fig. 5*B*, *right*). Considering that these treatments dramatically reduce BCRP protein expression levels (up to 10-20% of control levels following exposure to 3 nmol/L E_2), we speculated that the mechanism of E_2-mediated inhibition would be a posttranscriptional process.

Metabolic Labeling of BCRP in MCF-7/BCRP Cells. The biosynthesis and degradation of BCRP was further investigated by pulse-chase experiments. An outline of the experimental procedure is presented in Fig. 6*A*. MCF-7/BCRP cells produce a large amount of exogenous BCRP, driven by a constitutive long terminal repeat promoter, which could be successfully immunoprecipitated with the anti-BCRP antibody BXP-21, whereas the quantity of endogenous protein in parental MCF-7 cells is below the minimum detectable level (Fig. 6*B* and *C*). BCRP is initially detectable as a

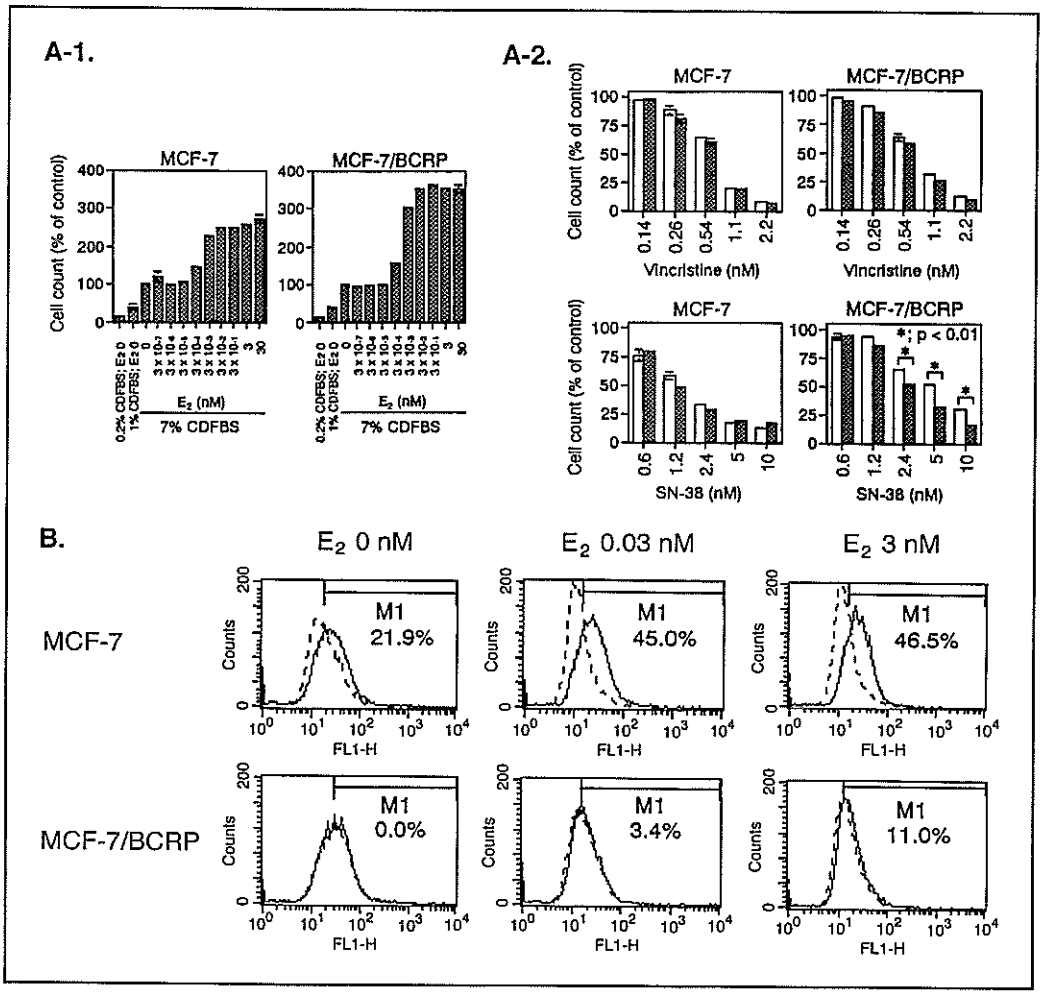

Figure 3. Cell growth studies and cellular topotecan uptake studies. *A*, cell growth studies. *A-1*, mitogenic effects of E_2 on MCF-7 and MCF-7/BCRP cells. Cells were cultured in PRF-DMEM supplemented with the indicated concentrations of CDFBS and E_2 for 4 days. Cell numbers were determined with a cell counter and presented as percentages relative to those of control cells cultured in PRF-medium. The given data are means ± SD of triplicate determinations. Invisible error bars are present within the symbols. The data are representative of two independent experiments. *A-2*, effects of E_2 on anticancer drug sensitivities in MCF-7 and MCF-7/BCRP cells. Cells were cultured in PRF-medium with 0.03 or 3 nmol/L E_2 for 4 days, and the cells (3×10^4) were then seeded into 12-well plates and cultured in PRF-medium with the same concentrations of E_2 used in pretreatments, in the absence or presence of increasing doses of specific anticancer agents for a further 4 days. Cell numbers were determined with a cell counter, and presented as percentages relative to those of control cells cultured in the absence of anticancer agents. *Clear columns*, cells cultured with 0.03 nmol/L E_2. *Dotted columns*, cells cultured with 3 nmol/L E_2. The given data are means ± SD of triplicate determinations, and are representative of three independent experiments. Where a vertical bar is not shown, the SD is within the bar. *, $P < 0.01$. *B*, effects of E_2 on cellular topotecan uptake. Cells were cultured in PRF-medium in the absence or presence of indicated concentrations of E_2 for 4 days. After trypsinization, cells (5×10^5) were incubated with (*solid line*) or without (*dotted line*) 20 μmol/L topotecan for 30 minutes. After washing, cellular uptake of topotecan was measured by fluorescence-activated cell sorting. Under each set of experimental conditions, 20,000 events were analyzed. Ratio (%) represents a fraction of topotecan-treated cells in the M1 area subtracted by that of control cells in the M1 area.

premature protein (66 kDa) which has a lower molecular size than the commonly observed *N*-glycosylated mature form (80 kDa; refs. 2, 13). During 1 hour of pulse labeling, the levels of mature protein gradually increased above the levels of the precursor molecule, and following 3 hours of chase period, only mature BCRP were measurable (Fig. 6*B*). Greater levels of metabolically labeled BCRP were observed in control MCF-7/BCRP cells, when compared with the E_2-treated cells, throughout the pulse-chase period (Fig. 6*B*). The relative rate of labeled BCRP at the 4-hour time point in the presence of 3 nmol/L E_2, over the levels measured in the absence of E_2, was 0.24 ± 0.01. Because metabolically labeled BCRP was only detectable at very low levels in E_2-treated cells and the half-life of synthesized BCRP could not be determined under these experimental conditions, the BCRP half-life was measured in cells without a 4-day E_2-pretreatment. E_2 (3 nmol/L) was added to the labeling medium and was present throughout the 48-hour pulse-chase period. The relative rate of labeled BCRP at the 1-hour time point in the presence of 3 nmol/L E_2, over the levels measured in the absence of E_2, was 0.84 ± 0.12. MCF-7/BCRP cells produced somewhat smaller amounts of labeled BCRP in the presence of 3 nmol/L E_2 than in the absence of E_2. The half-life of ^{35}S-labeled BCRP in the absence or presence of 3 nmol/L E_2 was similar, calculated as 35.6 ± 8.2 and 37.4 ± 6.3 hours, respectively (Fig. 6*C*). The relative rate of BCRP half-life in the presence of 3 nmol/L E_2 to that in the absence of E_2-treatment was 1.08 ± 0.27.

Discussion

We have recently reported several findings that provide evidence of interactions between BCRP and estrogens (6, 7, 14, 15). BCRP has been shown to export sulfated E_1, sulfated E_2, and genistein aglycone which has weak estrogenic activity (7, 15).

These data prompted us to investigate whether estrogens in fact regulate BCRP expression and we have now elucidated, contrary to our expectation that estrogens might augment BCRP expression, that physiologic levels of estrogens, such as E_1, E_2, and diethylstilbestrol, down-regulate BCRP expression in MCF-7 cells (Fig. 1).

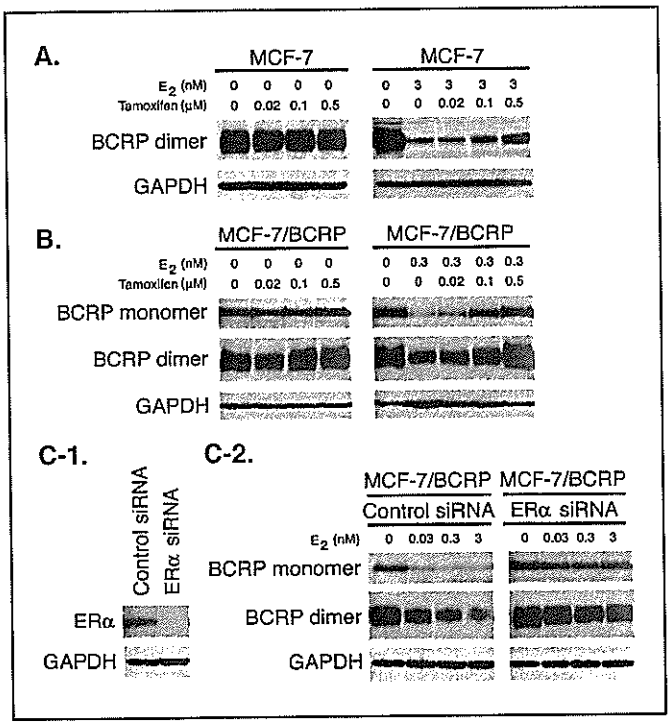

Figure 4. Effects of tamoxifen and ERα knockdown by RNA interference on E_2-mediated BCRP down-regulation. *A*, effects of tamoxifen on endogenous BCRP expression in MCF-7 cells. Cells were cultured in PRF-medium in the presence of indicated concentrations of compounds for 4 days prior to harvesting. Dimeric form of BCRP was detected as an approximately 160 kDa band under nonreducing conditions. Protein sample (30 μg) was loaded in each lane. Endogenous BCRP in MCF-7 cells was detected using the anti-BCRP antibody, BXP-21. *Left*, effects of tamoxifen on endogenous BCRP expression. *Right*, reversal effects of tamoxifen on E_2-mediated down-regulation of endogenous BCRP. GAPDH expression was analyzed as a loading control. The data are representative of two independent experiments. *B*, effects of tamoxifen on exogenous BCRP expression in MCF-7/BCRP cells. Cells were cultured in PRF-medium in the presence of the indicated concentrations of compounds for 4 days prior to harvesting. Dimeric form of BCRP was detected as an approximately 160 kDa band under nonreducing conditions, and the monomeric form of BCRP was detected as an approximately 80 kDa band under reducing conditions by Western blotting. Protein sample (20 μg) was loaded in each lane. Exogenous BCRP in MCF-7/BCRP cells was detected using the anti-c-*myc* antibody, 9E10. *Left*, effects of tamoxifen on exogenous BCRP expression. *Right*, reversal effects of tamoxifen on E_2-mediated down-regulation of exogenous BCRP. GAPDH expression was analyzed as a loading control. The data are representative of two independent experiments. *C*, effects of ERα knockdown by RNA interference on E_2-mediated BCRP down-regulation in MCF-7/BCRP cells. Cells (2.5×10^5/well) were cultured in PRF-medium in six-well plates for 24 hours and then transfected with 100 nmol/L of either control or ERα siRNA (SMARTpool GL3 Duplex for control; SMARTpool ESR1 for ERα) using LipofectAMINE 2000. To confirm ERα knockdown, the culture medium was exchanged with fresh PRF-medium 6 hours after transfection. After 48 hours, the cells were harvested and whole cell lysates consisting of 1.5×10^5 cells were loaded in each lane. ERα expression was detected by Western blotting using anti-ERα monoclonal antibody, NCL-ER-6F11. To examine the effects of ERα knockdown on E_2-mediated BCRP down-regulation, the culture medium was exchanged with fresh PRF-medium containing the indicated concentrations of E_2 6 hours after transfection. After 96 hours, cells were harvested and exogenous BCRP expression was examined by Western blotting as described above. *C-1*, siRNA-induced knockdown of ERα expression. *C-2*, effects of ERα knockdown on E_2-mediated BCRP down-regulation.

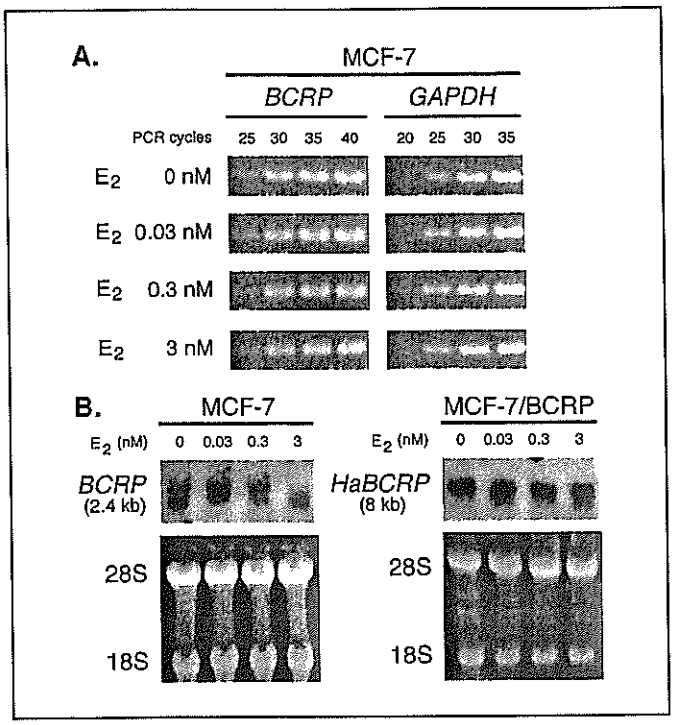

Figure 5. Expression analysis of *BCRP* mRNA in MCF-7 and MCF-7/BCRP cells. Cells were cultured in PRF-medium supplemented with the indicated concentrations of E_2 for 4 days. Exponentially growing cells were then harvested and total RNA was extracted. *A*, semi-quantitative RT-PCR of endogenous *BCRP* mRNA in MCF-7 cells. First-strand cDNA was synthesized with 0.3 μg of total RNA and a *BCRP* cDNA fragment (315 bp) was amplified by PCR using the indicated cycle numbers. Amplification of *GADPH* mRNA (551 bp fragment) was carried out as an internal control. The data are representative of two independent experiments. *B*, Northern blotting of endogenous *BCRP* mRNA in MCF-7 cells (*left*) and exogenous *HaBCRP* mRNA in MCF-7/BCRP cells (*right*). Either 20 μg (MCF-7) or 10 μg (MCF-7/BCRP) of total RNA was loaded in each lane. The blot was hybridized with a ^{32}P-labeled internal *BCRP* cDNA probe and then exposed to X-ray film for either 21 days (MCF-7) or 7 days (MCF-7/BCRP). Endogenous *BCRP* mRNA was detected as a band of approximately 2.4 kb in size, and exogenous *HaBCRP* mRNA as a band of approximately 8 kb in size. Under the experimental conditions used for MCF-7/BCRP cells, endogenous *BCRP* mRNA was not detected. Ethidium bromide staining of total RNA is presented as a loading control. *28s* and *18s*, 28S and 18S rRNA, respectively. The data are representative of two independent experiments.

Furthermore, E_2 strongly reduces the levels of exogenous BCRP in MCF-7/BCRP and T-47D/BCRP cells, the expression of which is constitutively transcribed by a Harvey long terminal repeat promoter (Fig. 2). Moreover, MCF-7/BCRP cells in the presence of 3 nmol/L E_2 were significantly more sensitive to SN-38, but not vincristine, than the same cells treated with 0.03 nmol/L E_2 (Fig. 3*A-2*). Because E_2 at a concentration ranging from 0.03 to 3 nmol/L shows similar mitogenic properties in MCF-7/BCRP cells (Fig. 3*A-1*), this further suggests that E_2 mediates the down-regulation of BCRP in these cells.

In proportion to BCRP down-regulation in MCF-7 cells, cellular accumulation of topotecan was found to increase by E_2-treatment (Fig. 3*B*). The increase in cellular topotecan uptake was most obvious when comparisons were made between untreated MCF-7 cells and MCF-7 cells treated with 0.03 nmol/L E_2. The results were coincident with BCRP protein expression levels in MCF-7 cells treated with E_2, in which BCRP down-regulation was most obvious when comparison was made between treatment with 0 nmol/L E_2 and that with 0.03 nmol/L E_2 (Fig. 1*A*). By contrast, the increase in cellular topotecan uptake was minimal even when untreated MCF-7/BCRP cells and MCF-7/BCRP cells treated with 3 nmol/L E_2 were

compared (Fig. 3*B*). Because exogenous BCRP synthesis levels in MCF-7/BCRP cells treated with 3 nmol/L E_2 are still greater than endogenous BCRP levels in E_2-untreated MCF-7 cells, we suppose that down-regulated BCRP by 3 nmol/L E_2 might still sufficiently efflux topotecan out of the cells (Fig. 6).

MCF-7 and T-47D cells are estrogen-responsive cells that express ERα, and it was significant that the estrogen-mediated down-regulation of endogenous BCRP was not observed in A549 cells, which do not express this receptor (Fig. 1). E_2-mediated down-regulation of exogenous BCRP was observed in MCF-7/BCRP and T-47D/BCRP cells, but not in MDA-MB-231/BCRP cells which also do not express ERα (Fig. 2*A*). Consistent with this, E_2-mediated BCRP repression was not observed in SKOV-3/BCRP cells (Fig. 2*A*), which express a small amount of nonfunctional ERα, possibly due to the disruption of downstream signaling pathways or an inactivating mutation within the ERα gene (11, 12). The antiestrogen drug tamoxifen partially reverses the E_2-mediated down-regulation of endogenous BCRP in MCF-7 cells and exogenous BCRP in MCF-7/BCRP cells (Fig. 4*A* and *B*). In addition, ERα knockdown by RNA interference in MCF-7/BCRP cells also abolishes the E_2-mediated down-regulation of exogenous BCRP (Fig. 4*C*). These results suggest that functional expression of ERα and the activity of its associated downstream pathways are important for estrogen-mediated BCRP down-regulation.

We first found that estrogens down-regulated BCRP expression at the protein level in MCF-7 cells (Fig. 1*A*). This was evident in experiments with three independent MCF-7 clones (data not shown). Subsequent semi-quantitative RT-PCR and Northern blotting analyses revealed that endogenous *BCRP* transcript levels were not reduced by E_2 treatment in MCF-7 cells (Fig. 5*A* and *B*, *left*). Furthermore, E_2 exposure decreased exogenous BCRP expression in MCF-7/BCRP and T-47D/BCRP cells, both constitutively expressing BCRP, driven by a Harvey long terminal repeat promoter. In addition, exogenous *HaBCRP* transcript levels were not reduced by E_2 treatment in MCF-7/BCRP cells (Fig. 5*B*, *right*). These data strongly argue for the existence of an estrogen-mediated posttranscriptional BCRP regulation mechanism, such as the degradation of translation products. We therefore did a pulse-chase experiment using MCF-7/BCRP cells. BCRP is a glycoprotein, containing four potential *N*-glycosylation sites (2, 13). BCRP was initially detectable as a premature protein of approximately 66 kDa in size at the 30-minute time point from the start of the pulse labeling, and a mature protein product of 80 kDa was then predominantly detected after 1 hour of the pulse labeling (Fig. 6*B*). In MCF-7/BCRP cells, the measured half-life of ^{35}S-labeled BCRP in the absence or presence of 3 nmol/L E_2 was similar, calculated as 35.6 ± 8.2 and 37.4 ± 6.3 hours, respectively (Fig. 6*C*). However, E_2-treated MCF-7/BCRP cells produced far smaller quantities of ^{35}S-labeled BCRP when compared with the control cells (Fig. 6*B*). In the pulse-chase experiments using MCF-7/BCRP cells pretreated with E_2 for 4 days before experiments, the ratio of mature BCRP at the 4-hour time point in the presence of 3 nmol/L E_2 to that in the absence of E_2 was 0.24 ± 0.01 (Fig. 6*B*). These results suggested that E_2 suppresses the biosynthesis of mature BCRP.

The sequence and characterization of the *BCRP* gene promoter has previously been reported (8). Very recently, an estrogen responsive element was identified in the *BCRP* promoter, and E_2-mediated activation of the *BCRP* promoter in a luciferase reporter system has been shown in ERα-negative ovarian cancer PA-1 cells, upon cotransfection with an ERα expression vector (9). In addition, E_2 has been shown to induce the increased expression of endogenous *BCRP* transcripts in T47D:A18 cells, established from T-47D cells by dilution cloning (9, 16). In our study, however, *BCRP* mRNA levels were unaffected by E_2 (Fig. 5*A* and *B*, *left*) and endogenous BCRP protein levels were clearly reduced in response to E_2 treatment in MCF-7 cells (Fig. 1*A*). Because T-47D cells

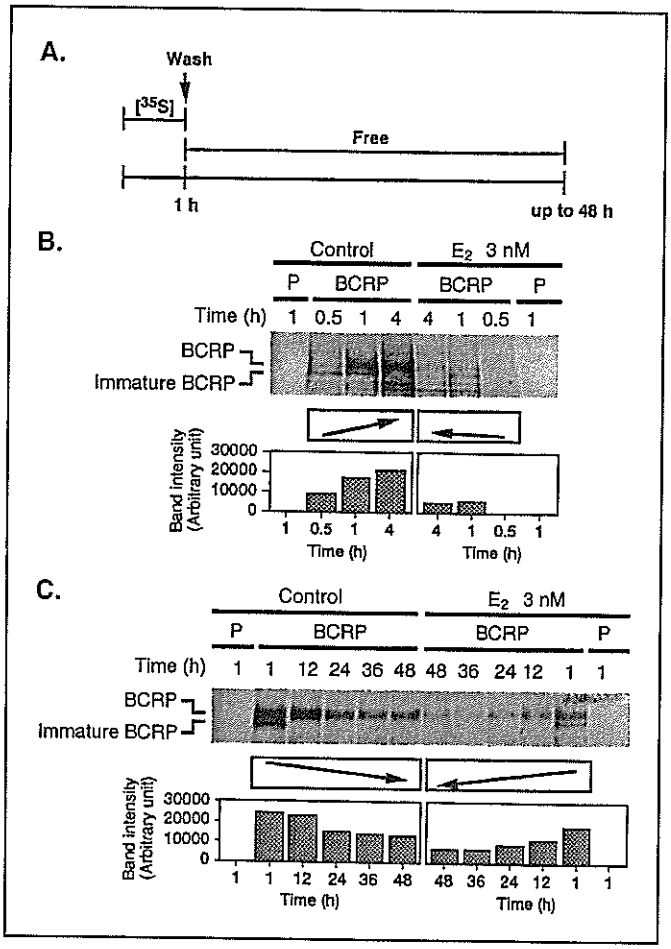

Figure 6. Metabolic labeling of BCRP in MCF-7/BCRP cells. *A*, an outline of the experimental procedure. *B*, biosynthesis of BCRP (0.5-4 h). Cells (1×10^6/well for control cells or 0.3×10^6/well for E_2-treated cells) were cultured in PRF-medium in a six-well plate for 4 days in the absence or presence of 3 nmol/L E_2. Exponentially growing cells were then incubated in methionine-free and cystine-free DMEM supplemented with 7% CDFBS (labeling medium) for 1.5 hours just prior to beginning the experiment. The cells were then metabolicallylabeled with 300 μCi/mL of [^{35}S] for both 0.5 and 1 hour periods. After 1 hour of pulse labeling, the labeling medium was replaced with fresh PRF-medium and the cells were chased for an additional 3 hours. For E_2-pretreated cells, 3 nmol/L E_2 was added to the medium and was present throughout the pulse-chase period. After preparation of cell lysates, ^{35}S-labeled BCRP was immunoprecipitated from 100 μg of the cell lysate with 0.5 μg BXP-21, subjected to SDS-PAGE, and autoradiographed. The band intensities representing metabolically labeled BCRP were quantified with NIH-Image. The data are representative of three independent experiments. *P* and *BCRP*, parental and MCF-7/BCRP cells, respectively. *C*, pulse-chase experiment of BCRP (1-48 h). Cells (2.5×10^6/well) were cultured in PRF-medium in six-well plates for 2 days. After incubation in labeling medium for 1.5 hours just before beginning the experiment, cells were metabolically labeled with 300 μCi/mL of [^{35}S] for 1 hour. The labeling medium was then replaced with fresh PRF-medium. The cells were lysed at 12, 24, 36, and 48 hours from the start of metabolic labeling. To investigate the effect of E_2 on BCRP stability, 3 nmol/L of E_2 was added to the medium in one set of experiments and was present in the medium throughout the pulse-chase periods. The following procedure in this case was identical to the one already described above, except that one-fifth of the total immunoprecipitated protein was subjected to SDS-PAGE and autoradiography. The band intensities, representing metabolically labeled BCRP, were quantified with NIH-Image. The data are representative of three independent experiments. *P* and *BCRP*, parental and MCF-7/BCRP cells, respectively.

express very low levels of endogenous BCRP, we could not examine the effect of E_2 on these levels by Western blotting. However, E_2 clearly reduced exogenous BCRP protein expression, which was driven by a constitutive promoter, in T-47D/BCRP cells (Fig. 2*A*), probably by inhibiting the biosynthesis of BCRP. Thus, E_2 up-regulates BCRP via transcriptional activation in some T-47D cells (T47D:A18 cells), and E_2 down-regulates BCRP expression via posttranscriptional mechanisms in some T-47D cells (T-47D/BCRP cells). Although both T47D:A18 cells and T-47D/BCRP cells were established from T-47D cells obtained from the same supplier, T47D:A18 cells were established by dilution cloning after repeated passages and T-47D/BCRP cells, a mixed population of stable *BCRP*-transduced cells, were used for the experiments shortly after supplied (16). The factors underlying distinct E_2-mediated BCRP regulation between these two T-47D derived cells remain to be elucidated.

Based upon global analyses of estrogen responsive genes in MCF-7 cells by cDNA microarray, many of these factors were determined to be growth- or transcription-related genes but no genes associated with protein translation have thus far been identified (17, 18). Among the candidate genes in these microarray screens, quiescin Q6, a FAD-dependent sulfhydryl oxidase, was reported to be estrogen-repressed (17). Quiescin Q6 products are expressed in the endoplasmic reticulum, Golgi, and extracellular spaces, and catalyze disulfide-bond formation in specific proteins (19, 20). Although it is currently unknown whether this protein interacts with BCRP, the maturation of BCRP by dimerization through bridge formation by disulfide bonds might well be necessary for stable BCRP expression. In addition, impaired protein maturation (glycosidation) or trafficking may also cause early degradation of the BCRP protein, as shown for multidrug resistance-related protein 2 (21, 22). Undetermined proteins associated with maturation or trafficking may also have caused very early degradation of premature BCRP.

BCRP had been initially isolated as an overexpressed protein in drug-resistant MCF-7 variants, but its expression is rarely observed in breast cancer cells (2, 23–25). The lack of BCRP protein expression notwithstanding high *BCRP* mRNA levels in nine breast cancer samples has also been previously reported (24). The authors of this study discussed whether this discrepancy might be due to the contribution of nontumor lactiferous ducts and blood vessels, both expressing BCRP, included in the tumor samples. However, we speculate that the low levels of BCRP protein in breast cancer cells might be explained by the inhibition of protein biosynthesis because a majority of primary breast cancers express ERα (26).

BCRP has been implicated in the cellular transport of several organic compounds (15, 27–29), and we have previously shown that it transports sulfated estrogens (7). Because mammary glands are one of the target organs of estrogens, we reasoned that BCRP in mammary glands might export sulfated estrogens and that, accordingly, estrogens may enhance its expression levels. MCF-7 cells also inactivate estrogens by sulfate conjugation (data not shown), and BCRP would most likely efflux them out of the cells. Moreover, it has been recently reported that BCRP expression was decreased in MCF-7 cells maintained in low folate medium (30). Because BCRP has been shown to transport methotrexate using membrane vesicle transport assays, the finding that BCRP expression is down-regulated was not considered to be surprising. However, in the case of estrogen treatment, this did not increase but considerably decreased BCRP expression in MCF-7 cells. Estrogen-mediated regulation of BCRP might therefore be responsible for the accumulation of estrogen in breast cancer cells.

In conclusion, our findings in this study show that estrogen posttranscriptionally decreases BCRP expression in estrogen-responsive cancer cells. This is also the first report showing that small molecules could modulate BCRP expression in cells, and our data therefore provide new insights into the regulation of BCRP expression and may assist in establishing new strategies for the reversal of BCRP-mediated multidrug resistance.

Acknowledgments

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Antimitotic activity and reversal of breast cancer resistance protein-mediated drug resistance by stilbenoids from *Bletilla striata*

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Abstract—Eight stilbenoids, 1-(*p*-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (**1**), 2,7-dihydroxy-1,3-bis(*p*-hydroxybenzyl)-4-methoxy-9,10-dihydrophenanthrene (**2**), 4,7-dihydroxy-1-(*p*-hydroxybenzyl)-2-methoxy-9,10-dihydrophenanthrene (**3**), 3,3′-dihydroxy-2′,6′-bis(*p*-hydroxybenzyl)-5-methoxybibenzyl (**4**), 3′,5-dihydroxy-2-(*p*-hydroxybenzyl)-3-methoxybibenzyl (**5**), blestriarenes B (**6**) and C (**7**), and blestrianol A (**8**) have been isolated by the guidance of inhibitory effect of tubulin polymerization from the tubers of *Bletilla striata* (Orchidaceae). Among them, both of bisbenzyls **4** and **5** inhibited the polymerization of tubulin at IC_{50} 10 μM, respectively. Furthermore bisbenzyl **4** potentiated the cytotoxicity of SN-38 in BCRP-transduced K562 (K562/BCRP) cells.

Antimitotic agents that inhibit the microtubule formation and the mitotic arrest of eucaryotic cells, such as paclitaxel and vinblastine, are important components of current anticancer therapy.[1] Paclitaxel is potent inhibitor of cell proliferation and arrest cells in mitosis, but in contrast to vinblastine, promote the polymerization of purified tubulin, causing stabilization and bundling of microtubules.[2] The antimitotic agents have potential applications in drug development. Recently much effort has been directed to the isolation and synthesis of new antimitotic drugs that target the tubulin/microtubule system and display efficacy against drug-refractory carcinomas.[3]

Multidrug-resistance (MDR) is a phenomenon in which cancer cells display cross-resistance to structurally unrelated drugs. Breast cancer resistance protein (BCRP), also called ABCG2, mediates concurrent resistance to chemotherapeutic agents such as SN-38 (an active metabolite of CPT-11), mitoxantrone, and topotecan, presumably by pumping these compounds out of cell and thus decreasing their cytotoxic effects.[4]

During our search for bioactive compounds targeting the tubulin/microtubules from medicinal plants,[5] we found that the extract from the tubers of *Bletilla striata* remarkably inhibited the polymerization of tubulin. The tubers of *B. striata* (Orchidaceae) have been used as traditional medicine to treat pulmonary tuberculosis and as hemostatic agent.[6] Our efforts on identifying new agents that target tubulin resulted in the isolation of eight known stilbenoids: 1-(*p*-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (**1**),[7] 2,7-dihydroxy-1,3-bis(*p*-hydroxybenzyl)-4-methoxy-9,10-dihydrophenanthrene (**2**),[8] 4,7-dihydroxy-1-(*p*-hydroxybenzyl)-2-methoxy-9,10-dihydrophenanthrene (**3**),[9] 3,3′-dihydroxy-2′,6′-bis(*p*-hydroxybenzyl)-5-methoxybibenzyl (**4**),[9] 3′,5-dihydroxy-2-(*p*-hydroxybenzyl)-3-methoxybibenzyl (**5**),[8] blestriarenes B (**6**)[10] and C (**7**),[10] and blestrianol A (**8**),[11] whose structures were established by spectroscopic data. This paper describes effects of these stilbenoids (**1–8**) on tubulin assembly as well as inhibitory effects of the stilbenoids on BCRP-mediated SN-38 resistance was also described.

The tubers of *B. striata* were extracted with MeOH, and the MeOH extract was in turn partitioned with hexane, EtOAc, $CHCl_3$, and *n*-BuOH. EtOAc-soluble materials inhibiting the polymerization of tubulin were subjected to a silica gel column ($CHCl_3$/MeOH, 1:0 → 0:1) followed by a C_{18} column (CH_3CN/0.1% TFA, 2:3) to afford stilbenoids **1–8**.

Keywords: Stilbenoid; Antimitotic activity; Reversal of breast cancer resistance protein-mediated drug resistance.
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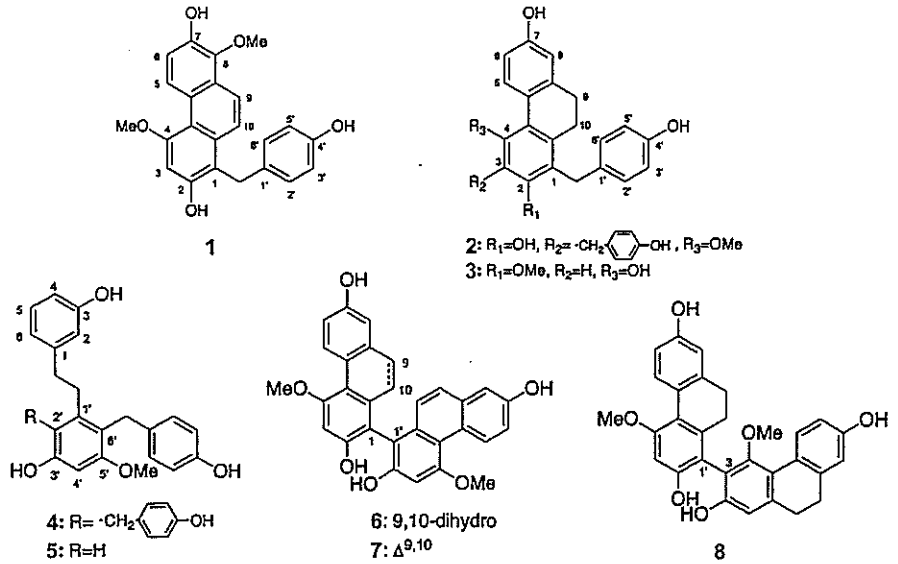


Generally antimitotic agents such as colchicines and vinblastine bind to either the colchicine binding site or the vinca alkaloid binding site. On the other hand, paclitaxel promotes the polymerization of tubulin by binding to and stabilizing the resulting microtubule polymer, which differs from those of colchicines, podophyllotoxin, and the vinca alkaloids.[1] Microtubules polymerized in the presence of paclitaxel are resistant to depolymerization by Ca^{2+} ions.

In this study, it was found that bisbenzyls **4** and **5** remarkably inhibited the polymerization of tubulin. Microtubule polymerization and depolymerization were monitored by the increase and the decrease in turbidity. Inhibitory effects of bisbenzyl **5** to tubulin polymerization are shown in Figure 1, in which tubulin polymerization was inhibited in a concentration-dependent manner. On the other hand, phenanthrene and dihydrophenanthrene with a benzyl moiety (**1** and **2**) and dimeric phenanthrenes (**6–8**) were found to be three times less potent (IC_{50}, 30 μM, respectively) than bisbenzyls **4** and **5**, indicating that the restricted biaryl ring system of phenanthrenes is unfavorable for tubulin binding. Substitution of the hydroxy group at C-4 is also critical as shown in Table 1. There was no difference for inhibitory effects of the polymerization of tubulin between phenanthrenes and dihydrophenanthrenes. The presence of *p*-hydroxy benzyl at C-2′ of bisbenzyl **4** had no influence in the polymerization of tubulin. In addition, inhibitory effects of the polymerization of tubulin by dimeric phenanthrenes was comparable with those by monomeric ones.

Antimitotic activity of stilbene related compounds have been well studied so far.[11] Especially combretastatin A-4 (CA-4) isolated from a South African willow tree *Combretum caffrum* is one of the most potent antimitotic agent and strongly inhibits the polymerization of tubulin by binding at the colchicine binding site (CLC site) (IC_{50}, 1.9 μM).[12] Common elements can be found in the structures of the active combretastatin congeners and of other well-known CLC site ligands such as colchicine,[13] steganacin,[14] and podophyllotoxin.[15] Common structural features among these compounds are the presence of two aromatic rings, which can be connected directly or through one or two atoms bridge spacer of single or double bond. Orientation of the two aromatic rings is required to be *cis*. In addition, the appropriate chiral torsion may be important in the conformation of the two aromatic rings. These structural features correspond

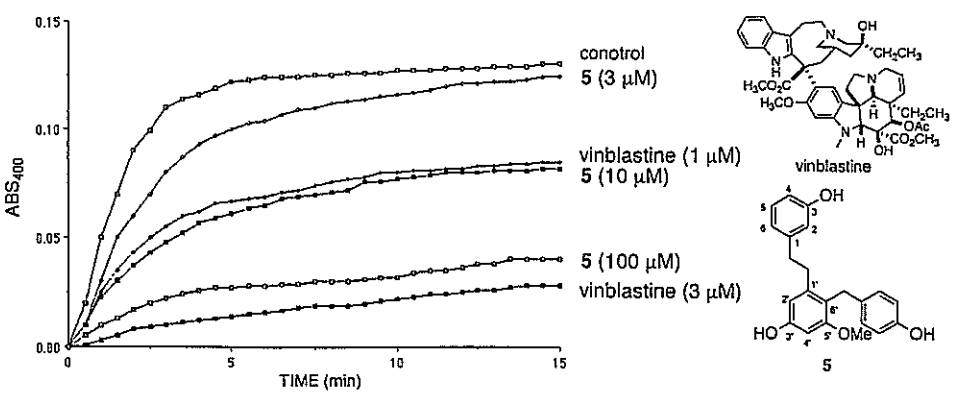


Figure 1. Inhibitory effects of bisbenzyl (**5**) and vinblastine to the polymerization of tubulin protein. Various concentrations of **5** were mixed with tubulin protein (1.5 mg/mL) at 0 °C and incubated at 37 °C. The absorbance at 400 nm was measured.

Table 1. Inhibitory effects of stilbenoids (1–8) and vinblastine to the polymerization of tubulin

Compounds	1	2	3	4	5	6	7	8	Vinblastine
IC_{50} (μM)	30	30	300	10	10	30	30	30	1

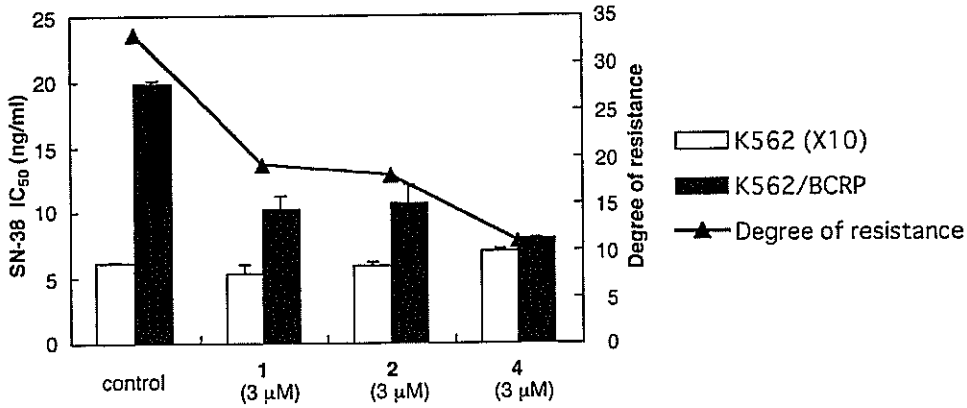
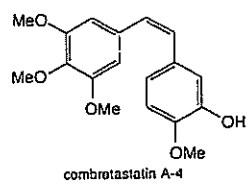


Figure 2. Inhibitory effects of stilbenoids (1), (2), and (4) on BCRP-mediated SN-38 resistance. K562 and K562/BCRP cells were cultured for five days in the absence or presence of 3 μM compound with increasing concentrations of SN-38. Cell numbers were determined using a cell counter, and then IC_{50} values were measured. Open bar represents nonresistance cells and black bar denotes resistance cells. The degree of resistance is the ratio of IC_{50} values of the cells to that of K562 cells under the indicated experimental conditions.

to the fact that, in this experiment, bisbenzyls with high flexibility such as 4 and 5, which only showed high affinity to the tubulin protein.



combretastatin A-4

On the other hand, Estrone, 17β-estradiol, estrogen agonists, and estrogen antagonists reverse BCRP-mediated drug resistance. Recently, phytoestrogens with weak estrogenic activity such as flavonoids were also reported to potentiate the cytotoxicity of 7-ethyl-10-hydroxycamptothecin (SN-38) and mitoxantrone in BCRP-transduced K562 (K562/BCRP) cells.[4] In this study, we examined the potential reversal effects of stilbenoids from *B. striata*, since stilbenoids such as diethylstilbestrol and tamoxifen also interact with the same drug-binding site of BCRP.[4] As shown in Figure 2, 3 μM of **1**, **2**, and **4** strongly enhanced the cytotoxicity of SN-38 in K562/BCRP cells but not in K562 cells. Reversal indexes (ratios of IC_{50} measurements in the absence of reversing agents divided by levels in the presence of reversing agents) of 3 μM **1**, **2**, and **4** for SN-38 were 1.7, 1.8, and 3.0, respectively. These results suggested that stilbenoids sensitized K562/BCRP cells to SN-38 by inhibiting BCRP function.

In this work, we found that stilbenoids such as phenanthrene, dihydrophenanthrene, dimeric phenanthrene, and bisbenzyls from the tubers of *B. striata*, which have been used as traditional medicine, showed antimitotic activity and inhibited BCRP-mediated drug resistance.

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