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

Yasuo Imai,1 Etsuko Ishikawa,1 Sakiyo Asada,1 and Yoshikazu Sugimoto1,2

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 (E2) 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. E2 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 E2-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 E2-mediated BCRP down-regulation, suggesting that interaction of E2 and ERα is necessary for BCRP down-regulation. E2 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 E2-mediated BCRP down-regulation. These data indicate that estrogen down-regulates BCRP expression by novel posttranscriptional mechanisms. 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.

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 (E1) and 17β-estradiol (E2) 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 E2-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-[35S] in vitro Cell Labeling Mix (1-[[35S]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% CO2. 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.

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
SN-38 for 5 to 10 days, with the exception of T-47D cells, which were selected using 24 mmol/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, 100 mmol/L MgSO4, 2 mmol/L CaCl2, 1 mmol/L L-4(2-aminoethyl)-benzenesulfonyl fluoride] 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-ε-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 E2-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 mmol/L E2. The following procedure was the same as described above.

**Western Blot Analysis of ERα.** Cells (1.5 × 10⁴) 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 E2, exponentially growing MCF-7 or MCF-7/BCRP cells (3 × 10⁴/well) were seeded in a 12-well plates and cultured at 37°C in PRF-cDMEM supplemented with the indicated concentrations of CDFBS and E2 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 E2 on anticancer drug resistance, the cells were cultured in PRF-medium supplemented with the indicated concentrations of E2 for 4 days. The exponentially growing cells (3 × 10⁴) were then seeded in 12-well plates and cultured for a further 4 days in PRF-medium supplemented with the same concentration of E2 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. IC₅₀ 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 E2 on the cellular accumulation of topotecan were determined by flow cytometry. Cells were cultured in PRF-medium supplemented with the indicated concentrations of E2 for 4 days. After trypsinization, cells (5 × 10⁴) were incubated with 20 mmol/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 E2 on BCRP Expression in MCF-7/BCRP Cells Following Small Interfering RNA-induced ERα Knockdown.** Cells (2.5 × 10⁵/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 Dharmaco, 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 × 10⁶ 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 E2-mediated BCRP down-regulation, the culture medium was exchanged with fresh PRF-medium containing the indicated concentrations of E2, 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 × 10⁴) were incubated in PRF-medium with varying concentrations of E2 for 4 days. Extraction of total RNA and subsequent RT-PCR were done using an RNasea 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'-CAGTTGGAGCAAATCTCTGT-3' (forward) and 5'-ACACACACGATTACAGCTG-3' (reverse). As an internal control, amplification of GAPDH mRNA (551 bp fragment) was carried out with the primers 5'-ATCACACTTCCAGGGACG-3' (forward) and 5'-GCTTCACCCCTTCTTGATGT-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 × 10⁴) were incubated in PRF-medium with varying concentrations of E2 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 ³²P-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 × 10⁶/well) were cultured in PRF-medium in six-well plates for 4 days in the absence or presence of 3 nmol/L E2. After incubation in methionine- and cysteine-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 [³⁵S]S, for 0.5 and 1 hour. The cells labeled for 1 hour were subsequently chased for an additional 3 hours. For E2-pretreated cells, 3 nmol/L E2 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 MgCl₂, 1 mmol/L 4-(2-aminoethyl)-benzenesulfonyl fluoride, 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 E2 to the
levels in the absence of E2 are represented as the average ± SD from three independent experiments.

Next, BCRP pulse-chase labeling was done without E2 pretreatment, because 35S-labeled BCRP was hardly detectable and the half-life of BCRP could not be determined in MCF-7/BCRP cells pretreated with E2 for 4 days. Cells (2.5 × 10^5/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 [35S] 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 E2 on BCRP stability, 3 nmol/L of E2 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 E2-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 E1, E2, and diethylstilbestrol (Fig. 1A). Both E2 and diethylstilbestrol showed stronger suppressive effects on BCRP expression than E1 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 E2 for 1, 2, and 4 days, respectively, as compared with untreated MCF-7 cells (Fig. 1B). 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 E2 in A549 cells (Fig. 1A). Because MCF-7 cells are ERα-positive and estrogen-responsive but A549 cells are ERα-negative (Fig. 1C), these results suggest that estrogen-mediated BCRP down-regulation might depend on signaling pathways downstream of ERα.

Effects of E2 on Exogenous BCRP Expression in BCRP-Transduced Cells. We further studied the effects of E2 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 E2 (Fig. 2A). 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 E2 for 2, 3, and 4 days, respectively, as compared with untreated MCF-7/BCRP cells (Fig. 2B). In contrast, exogenous BCRP expression was not affected by E2 treatment in MDA-MB-231/BCRP and SKOV-3/BCRP cells (Fig. 2A). MCF-7 and T-47D cells are estrogen-responsive and express functional ERα (Fig. 2C), whereas MDA-MB-231 cells do not express ERα, and SKOV-3 cells, which only weakly express nonfunctional ERα, are estrogen-nonresponsive (Fig. 2C; 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.

E2-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). E2-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. E2, 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. 3A-I). The mitogenic activity saturated at concentrations of 0.03 nmol/L E2 or higher in both cell types (Fig. 3A-I). The effects of E2 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, E2 was found to marginally potentiate the cytotoxicity of SN-38, but not vincristine, in MCF-7 cells (Fig. 3A-2). The IC50 values for vincristine in the presence of 0.03 and 3 nmol/L E2 were 0.69 ± 0.01 and 0.65 ± 0.02 nmol/L in MCF-7 cells, respectively. For SN-38, IC50 values in the presence of 0.03 and 3 nmol/L E2
were 1.56 ± 0.15 and 1.22 ± 0.05 nmol/L in MCF-7 cells, respectively. Furthermore, exposure to 3 nmol/L E2 significantly potentiated the cytotoxicity of SN-38, but not vincristine, in comparison to 0.03 nmol/L E2 treatment in MCF-7/BCRP cells (Fig. 3A-2). The IC50 values for vincristine in the presence of 0.03 and 3 nmol/L E2 were 0.74 ± 0.03 and 0.65 ± 0.02 nmol/L in MCF-7/BCRP cells, respectively. The IC50 values for SN-38 at a 3 nmol/L E2 dose (2.65 ± 0.22 nmol/L) were significantly lower than the values at 0.03 nmol/L E2 dosage (5.18 ± 0.46 nmol/L; P < 0.01). Because mitogenic activity levels were saturated over the E2 concentration range that was used (from 0.03 to 3 nmol/L), we conclude that these results also suggest E2-mediated BCRP down-regulation in MCF-7/BCRP cells.

Intracellular Topotecan Uptake in MCF-7 and MCF-7/BCRP Cells. Effects of E2 on cellular accumulation of topotecan were investigated. Cellular accumulation of topotecan increased in MCF-7 cells treated with 0.03 nmol/L E2 as compared with untreated cells, whereas cellular accumulation of topotecan scarcely increased in MCF-7 cells treated with 3 nmol/L E2 when compared with cells treated with 0.03 nmol/L E2 (Fig. 3B). The results coincided with BCRP down-regulation in E2-treated MCF-7 cells (Fig. 1A). As for MCF-7/BCRP cells, intracellular topotecan accumulation only marginally increased in the presence of 0.03 nmol/L E2 as compared with untreated cells (Fig. 3B). Also, cellular accumulation of topotecan only marginally increased in MCF-7/BCRP cells treated with 3 nmol/L E2 when compared with those treated with 0.03 nmol/L E2 (Fig. 3B). 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 with 3 nmol/L E2.

Effects of Tamoxifen and ERα Knockdown by siRNA on E2-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. 4A, left). In MCF-7/BCRP cells, marginally higher levels of exogenous BCRP were produced by increasing dosages of tamoxifen (Fig. 4B, left), possibly by competition with residual estrogens in the culture medium. Tamoxifen was also found to partially reverse the E2-mediated down-regulation of either endogenous or exogenous BCRP in a dose-dependent manner (Fig. 4A and B, right). In these tamoxifen reversal experiments using MCF-7/BCRP cells, a concentration of 0.3 nmol/L E2 was used to down-regulate BCRP, because tamoxifen even at levels of 0.5 μmol/L failed to reverse 3 nmol/L E2-mediated BCRP down-regulation (data not shown). These results suggest that E2-mediated BCRP down-regulation in MCF-7 and MCF-7/BCRP cells may be associated with the interaction of E2 and ERα. We therefore did an experiment in which ERα expression was repressed using siRNA, and investigated the effects of this gene silencing on E2-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. 4C-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 E2-mediated BCRP down-regulation (Fig. 4C-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 E2 for 4 days did not affect the expression of endogenous BCRP mRNA (Fig. 5A and B, left). Similarly, the same treatment of MCF-7/BCRP cells with E2 for 4 days did not affect exogenous HAbCRP mRNA levels (Fig. 5B, right). Considering that these treatments dramatically reduce BCRP protein expression levels (up to 10-20% of control levels following exposure to 3 nmol/L E2), we speculated that the mechanism of E2-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. 6A. 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 BX-21, whereas the quantity of endogenous protein in parental MCF-7 cells is below the minimum detectable level (Fig. 6B and C). BCRP is initially detectable as a
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. 6B). Greater levels of metabolically labeled BCRP were observed in control MCF-7/BCRP cells, when compared with the E2-treated cells, throughout the pulse-chase period (Fig. 6B). The relative rate of labeled BCRP at the 4-hour time point in the presence of 3 nmol/L E2, over the levels measured in the absence of E2, was 0.24 ± 0.01. Because metabolically labeled BCRP was only detectable at very low levels in E2-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 E2-pretreatment. E2 (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 E2, over the levels measured in the absence of E2, was 0.84 ± 0.12. MCF-7/BCRP cells produced somewhat smaller amounts of labeled BCRP in the presence of 3 nmol/L E2 than in the absence of E2. The half-life of 35S-labeled BCRP in the absence or presence of 3 nmol/L E2 was similar, calculated as 35.6 ± 8.2 and 37.4 ± 6.3 hours, respectively (Fig. 6C). The relative rate of BCRP half-life in the presence of 3 nmol/L E2 to that in the absence of E2-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 E1, sulfated E2, 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 E1, E2, and diethylstilbestrol, down-regulate BCRP expression in MCF-7 cells (Fig. 1).

Figure 4. Effects of tamoxifen and ER\(\alpha\) 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 \(\mu\)g) was loaded in each lane. Endogenous BCRP in MCF-7 cells was detected using 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 \(\mu\)g) 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 ER\(\alpha\) knockdown by RNA interference on E\(2\)-mediated BCRP down-regulation in MCF-7/BCRP cells. Cells (2.5 \(\times\) 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 ER\(\alpha\) siRNA (SMARTpool GL3 Duplex for control; SMARTpool ESR1 for ER\(\alpha\)) using LipofectAMINE 2000. To confirm ER\(\alpha\) 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 \(\times\) 10\(^5\) cells were loaded in each lane. ER\(\alpha\) expression was detected by Western blotting using anti-ER\(\alpha\) monoclonal antibody, NCL-ER-6F11. To examine the effects of ER\(\alpha\) 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 ER\(\alpha\) expression. C-2, effects of ER\(\alpha\) 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 \(\mu\)g 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 \(\mu\)g (MCF-7) or 10 \(\mu\)g (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 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. 3A-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. 3A-I), 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. 3B). 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 coincident with BCRP protein expression levels in MCF-7 cells treated with E\(2\), in which BCRP down-regulation was most obvious.
In MCF-7/BCRP cells, the measured half-life of 35S-labeled BCRP in E2-treated MCF-7/BCRP cells produced far smaller quantities of endogenous BCRP trancripts in T47D A18 cells, established from T-47D cells by dilution cloning (9, 16). In our study, however, BCRP mRNA levels were unaffected by E2 (Fig. 5A and B, left) and endogenous BCRP protein levels were clearly reduced in response to E2 treatment in MCF-7 cells (Fig. 1A). Because T-47D cells compared (Fig. 3B). Because exogenous BCRP synthesis levels in MCF-7/BCRP cells treated with 3 nmol/L E2 are still greater than endogenous BCRP levels in E2-unreared MCF-7 cells, we suppose that down-regulated BCRP by 3 nmol/L E2 might still sufficiently efux topotecan out of the cells (Fig. 6).

MCF-7 and T-47D cells are estrogen-responsive cells that express ERs, 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). E2-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. 2A). Consistent with this, E2-mediated BCRP repression was not observed in SKOV-3/BCRP cells (Fig. 2A), 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 E2-mediated down-regulation of endogenous BCRP in MCF-7 cells and exogenous BCRP in MCF-7/BCRP cells (Fig. 4A and B). In addition, ERα knockout by RNA interference in MCF-7/BCRP cells also abolishes the E2-mediated down-regulation of exogenous BCRP (Fig. 4C). 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. 1A). 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 E2 treatment in MCF-7 cells (Fig. 5A and B, left). Furthermore, E2 exposure decreased endogenous 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 E2 treatment in MCF-7/BCRP cells (Fig. 5B, 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. 6B). In MCF-7/BCRP cells, the measured half-life of 35S-labeled BCRP in the absence or presence of 3 nmol/L E2 was similar, calculated as 35.6 ± 8.2 and 37.4 ± 6.3 hours, respectively (Fig. 6C). However, E2-treated MCF-7/BCRP cells produced far smaller quantities of 35S-labeled BCRP when compared with the control cells (Fig. 6D). In the pulse-chase experiments using MCF-7/BCRP cells pretreated with E2 for 4 days before experiments, the ratio of mature BCRP at the 4-hour time point in the presence of 3 nmol/L E2 to that in the absence of E2 was 0.24 ± 0.01 (Fig. 6B). These results suggested that E2 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 Eα-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, E2 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 E2 (Fig. 5A and B, left) and endogenous BCRP protein levels were clearly reduced in response to E2 treatment in MCF-7 cells (Fig. 1A). Because T-47D cells...
The authors of this study discussed whether this discrepancy in nine breast cancer samples has also been previously reported (24). Protein expression notwithstanding high observed in breast cancer cells (2, 23–25). The lack of BCRP drug-resistant MCF-7 variants, but its expression is rarely very early degradation of premature BCRP. Associated with maturation or trafficking may also have caused 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
Received 8/10/2004; revised 10/27/2004; accepted 11/9/2004.

The cost of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References


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

Yasuo Imai, Etsuko Ishikawa, Sakiyo Asada, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/2/596

Cited articles
This article cites 30 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/2/596.full#ref-list-1

Citing articles
This article has been cited by 17 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/2/596.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.