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*),

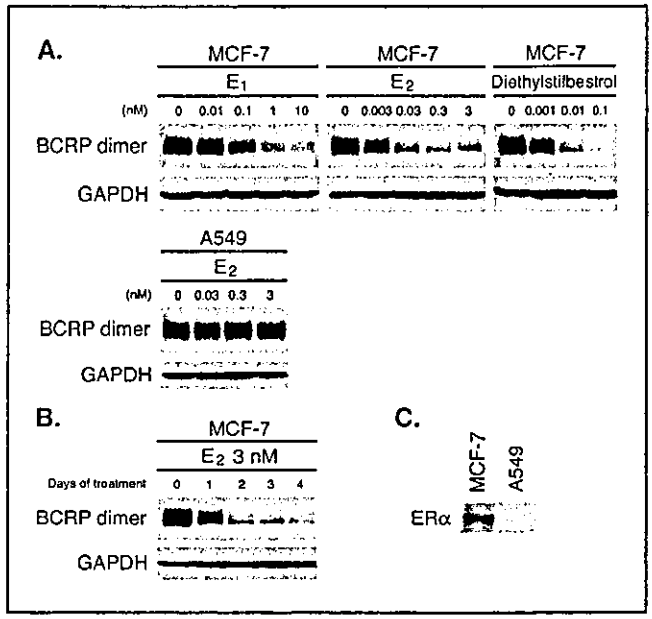

Figure 1. Effects of estrogens on endogenous BCRP expression in cancer cells. Cells were cultured in PRF-medium in the absence or presence of the indicated concentrations of estrogens for 4 days prior to harvesting. Western blot analysis was done under nonreducing conditions, such that the dimeric form 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. 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*.

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.

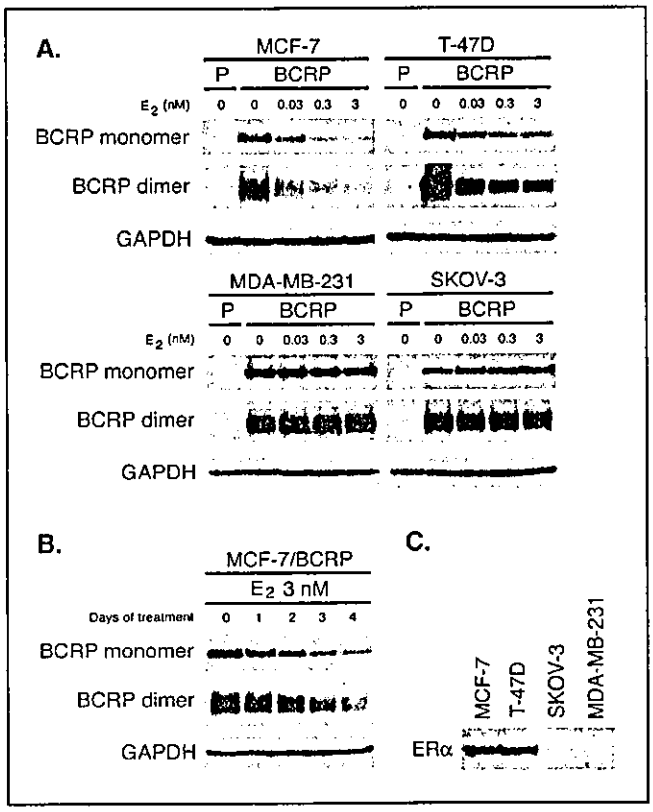

Figure 2. Effects of E_2 on exogenous BCRP expression in *BCRP*-transduced cancer cells. Cells were cultured in PRF-medium in the absence or presence of the indicated concentrations of E_2 for 4 days prior to harvesting. To see how soon the E_2-mediated BCRP down-regulation occurs, MCF-7/BCRP cells were 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

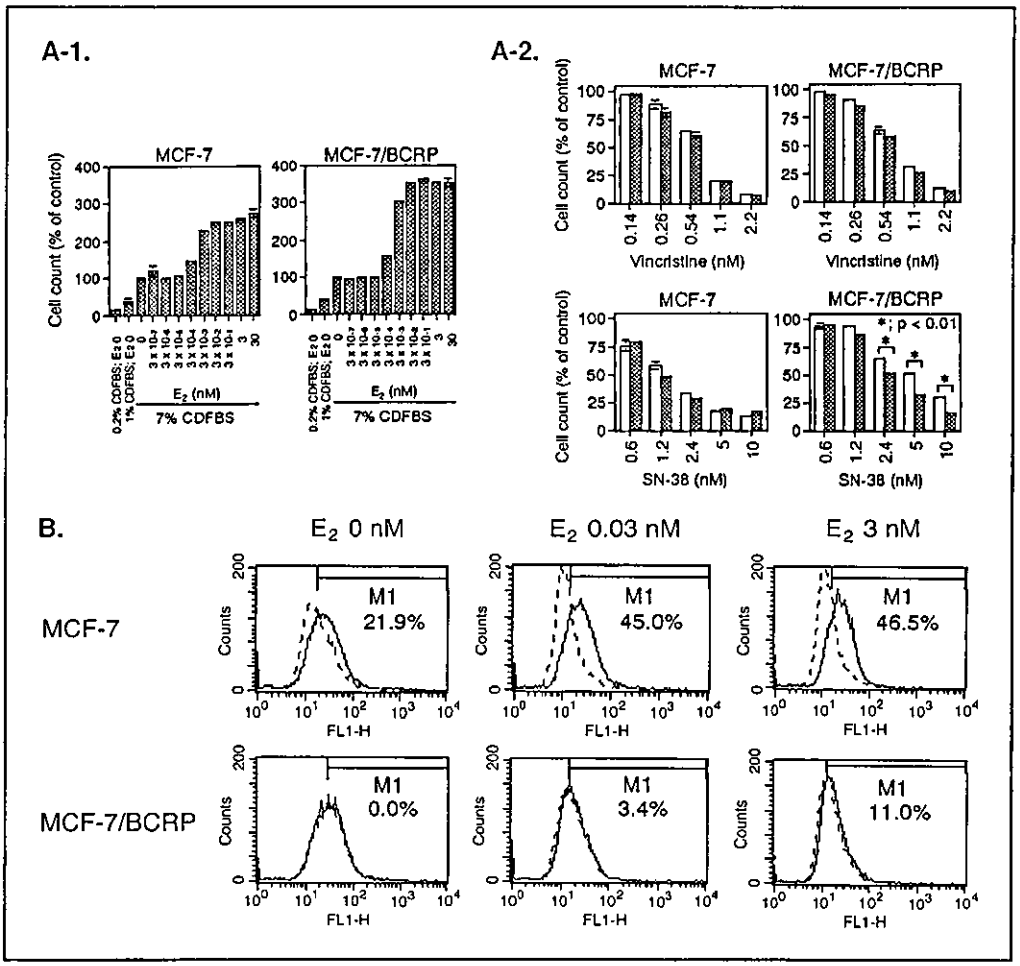

Figure 3. Cell growth studies and cellular topotecan uptake studies. *A*, cell growth studies. *A-1*, mitogenic effects of E_2 on MCF-7 and MCF-7/BCRP cells. Cells were cultured in PRF-DMEM supplemented with the indicated concentrations of CDFBS and E_2 for 4 days. Cell numbers were determined with a 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).

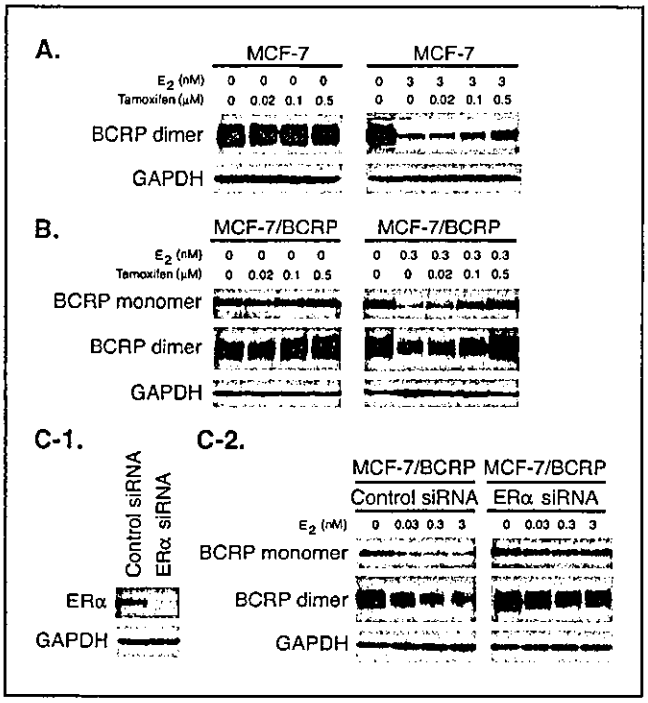

Figure 4. Effects of tamoxifen and ERα knockdown by RNA interference on E_2-mediated BCRP down-regulation. *A*, effects of tamoxifen on endogenous BCRP expression in MCF-7 cells. Cells were cultured in PRF-medium in the presence of indicated concentrations of compounds for 4 days prior to harvesting. 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.

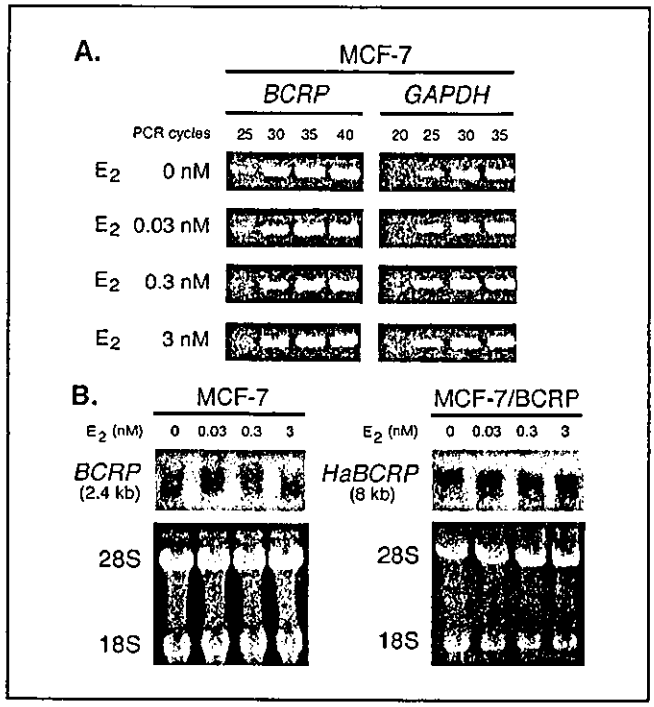

Figure 5. Expression analysis of *BCRP* mRNA in MCF-7 and MCF-7/BCRP cells. Cells were cultured in PRF-medium supplemented with the indicated concentrations of E_2 for 4 days. Exponentially growing cells were then harvested and total RNA was extracted. *A*, semi-quantitative RT-PCR of endogenous *BCRP* 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

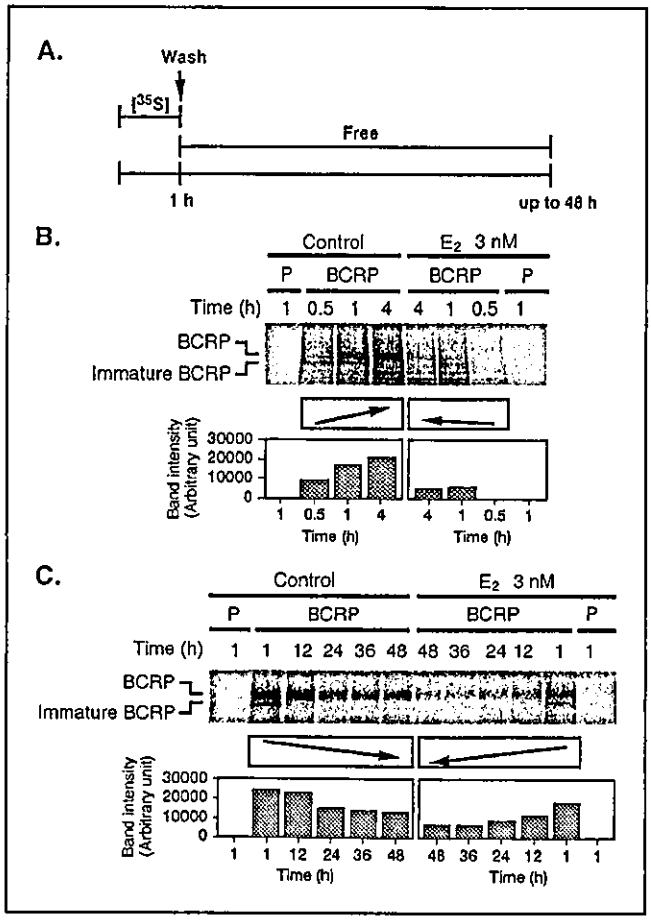

Figure 6. Metabolic labeling of BCRP in MCF-7/BCRP cells. *A*, an outline of the experimental procedure. *B*, biosynthesis of BCRP (0.5-4 h). Cells (1×10^6/well for control cells or 0.3×10^6/well for E_2-treated cells) were cultured in PRF-medium in a six-well plate for 4 days in the absence or presence 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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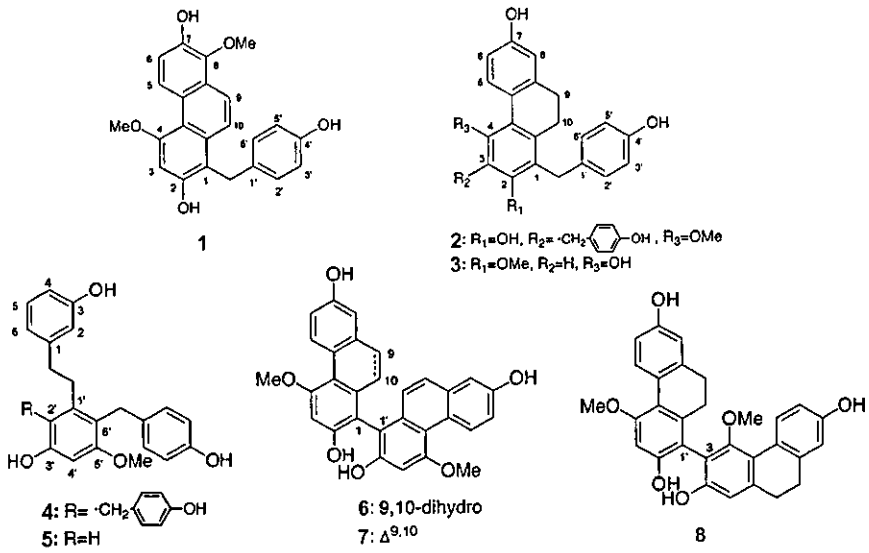


Generally antimitotic agents such as colchicines and vinblastine bind to either the colchicine binding site or the vinca alkaloid binding site. On the other hand, paclitaxel promotes the polymerization of tubulin by binding to and stabilizing the resulting microtubule polymer, which differs from those 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

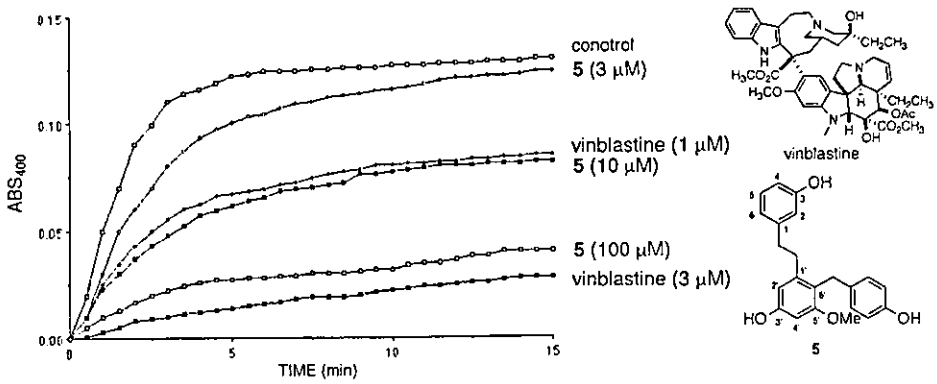


Figure 1. Inhibitory effects of bisbenzyl (**5**) and vinblastine to the polymerization of tubulin protein. Various concentrations of **5** were mixed with tubulin protein (1.5 mg/mL) at 0 °C and incubated at 37 °C. The absorbance at 400 nm was measured.

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

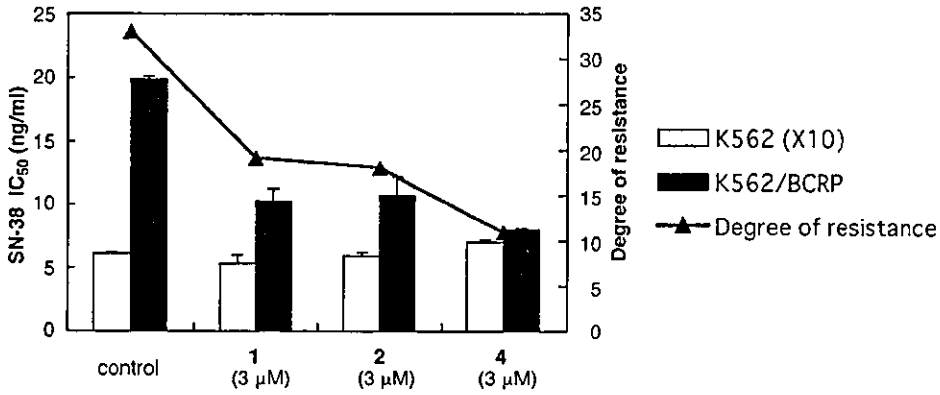
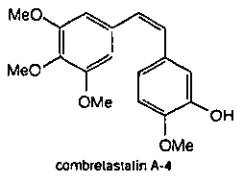


Figure 2. Inhibitory effects of stilbenoids (1), (2), and (4) on BCRP-mediated SN-38 resistance. K562 and K562/BCRP cells were cultured for five days in the absence or presence of 3 μM compound with increasing concentrations of SN-38. Cell numbers were determined using a cell counter, and then IC_{50} 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.



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.

Acknowledgements

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SELENOPROTEIN P, AS A PREDICTOR FOR EVALUATING GEMCITABINE RESISTANCE IN HUMAN PANCREATIC CANCER CELLS

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Gemcitabine is a new standard chemotherapeutic agent used in the treatment of pancreatic cancer, but the mechanisms of gemcitabine sensitivity are still controversial. In our study to determine a mechanism that regulates gemcitabine sensitivity, we carried out molecular analysis on the susceptibility of the pancreatic cancer cells. Using a gemcitabine-sensitive pancreatic cancer cell line KLM1, we established a resistant cell line KLM1-R exhibiting a 20-fold IC_{50}-value (the concentration of gemcitabine causing 50% growth inhibition). Microarray analysis of genes showed specific expression of *selenoprotein P*, one of the anti-oxidants, in the KLM1-R cell line but not in the KLM1 cell line. Administration of selenoprotein P inhibited the gemcitabine-induced cytotoxicity in the pancreatic cell lines. The levels of intracellular reactive oxygen species (ROS) were increased in the KLM1 cells by gemcitabine, but selenoprotein P suppressed the gemcitabine-induced ROS levels. Furthermore interferon-γ suppressed the expression of *selenoprotein P* mRNA and increased intracellular ROS level, leading to the recovery of the gemcitabine sensitivity in KLM1-R. These results suggest a novel mechanism that selenoprotein P reduces the intracellular ROS levels, resulting in the insusceptibility to gemcitabine.

Key words: *microarray; sensitivity; free radical scavenger; reactive oxygen species*

Pancreatic cancer is characterized by extensive local invasion and early lymphatic and hematogenous metastasis.[1] In fact, only 1–4% of all patients can survive 5 years after diagnosis as the carcinoma of pancreas.[2] Although surgical operations represent the only curative treatment available for the patients with pancreatic cancer, 5-year survival rates are <20% even after surgery,[3–5] indicating that effective adjuvant treatments are required. Among anticancer agents used in the treatment of pancreatic cancer, 5-fluorouracil (5-FU) has been known as a standard drug for the effective treatment of pancreatic cancer, but the response rate of 5-FU was quite low.[6–8]

During the last few years, a novel nucleoside analogue gemcitabine (2′, 2′-difluoro-deoxycytidine) has been reported to be an effective agent in the treatment of pancreatic cancer. Gemcitabine exhibited *in vitro* and *in vivo* the growth suppression of the human pancreatic cell lines that had previously shown insensitivity to multi-drugs such as 5-FU, doxorubicin or cisplatin.[9] Furthermore, gemcitabine improved the survival and clinical benefit responses compared to 5-FU.[10] Gemcitabine is now a standard first-line treatment for the patients with pancreatic cancer.[10,11]

Gemcitabine is metabolized sequentially to nucleoside monophosphate, diphosphate, and triphosphate by deoxycytidine kinase (dCK) after the entering cell. The difluoro-deoxycytidine triphosphate is incorporated into DNA, resulting in chain termination. Because gemcitabine is often effective in multi-drug resistant cells,[9] mechanisms of gemcitabine sensitivity have been investigated from several aspects. Buchler *et al.* reported that alterations of apoptosis-regulating genes, such as *bcl-2*, *bcl-x_L* and *bax*, regulate the sensitivity to gemcitabine.[12,13] Veronique *et al.*[14] identified the *dCK* deficiency as the genes responsible for the gemcitabine resistance. Indeed, the mechanisms of gemcitabine resistance are still controversial, although many studies were examined. Additionally, although there were reported individual gene expressions, any comprehensive expression change of the genes has not been investigated for gemcitabine sensitivity.

The cDNA microarray technique is one of methods that allow measurement of temporal changes in thousands of gene expression profiles during the development of anti-cancer drug resistance. Indeed, this methodology has been used to analyze alterations in the gene expression responsible for doxorubicin sensitivity[15] or paclitaxel sensitivity.[16] We established a human pancreatic cancer cell line showing decreased sensitivity to gemcitabine. Furthermore, cDNA microarray was utilized to monitor mRNA expression and to find a molecule, selenoprotein P, that might contribute to the gemcitabine-resistance. We showed that selenoprotein P suppressed the intracellular free radicals, with a potent cytotoxicity, induced by gemcitabine. Our study suggests a new mechanism for gemcitabine sensitivity in human pancreatic cancer cells.

MATERIAL AND METHODS

Cell cultures

Human KLM1, PK-45P and MIA Paca2 pancreas cancer cell lines were cultured in RPMI 1640, supplemented with 10% heat-inactivated FBS, streptomycin and penicillin at 37°C and 5% CO_2. A gemcitabine-resistant KLM1 cell line was established by exposure to gemcitabine as described previously.[17] The KLM1 cells cultured at an initial density of 1×10^6 cells on 6-well flat-bottomed plates containing 2 ml medium for 1 day were treated with 10 μg/ml gemcitabine for 1 week. Cells were then cultured in a gemcitabine-free medium for 2 weeks to recover cell density. After repeating the above treatment 4×, we established a cell line that exhibited stable characteristics with respect to growth rate, morphology and drug resistance.

The viability of cells after exposure to gemcitabine was determined by MTT assay as follow. The cells were seeded on 96-well flat-bottom plates. At 24 hr after seeding, the indicated amount of gemcitabine was added to the wells, and incubation was continued for another 72 hr. The colorimetric reaction was initiated by adding 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl [^{2}H]-tetrazolium bromide (MTT) at a concentration of 5 mg/ml. The formazan crystals were dissolved in 0.04 N acid-isopropanol, and the absorbance at 570 nm was quantified using a microtiter plate spectrophotometer. The 50% inhibitory concentration (IC_{50}) was estimated from in-

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dividual inhibition curves and represents the concentration of drug that inhibits cell proliferation by 50%.

The viability of cells after exposure to gemcitabine with selenoprotein P was determined by MTT assay as follow. The cells were seeded on 96-well flat-bottom plates. At 12 hr after seeding, 1 μg/ml selenoprotein P was added to the wells. After incubation for another 24 hr, the indicated amount of gemcitabine was added to the wells, and incubation was continued for another 72 hr. The numbers of viable cells was counted by MTT assay.

The viability of cells after exposure to H_2O_2 was determined as follows. Cells were seeded on 96-well and incubated for 24 hr. After incubation, the indicated amount of H_2O_2 was added to the wells, and incubation was continued for another 24 hr. The numbers of viable cells was counted by MTT assay.

The sensitivity of cells to 5FU, 1-β-D-arabinofuranosylcytosine, adriamycin, bleomycin, cisplatin, etoposide, paclitaxel and docetaxel was determined by MTT assays. The cells were seeded on 96-well and incubated for 24 hr. After incubation, each anticancer drug was added to the wells and incubation was continued for another 24 hr. The numbers of viable cells was counted by MTT assay.

KLM1-R cells were incubated for 24 hr with medium containing indicated amount of IFN-γ. After incubation, Total RNA was extracted from the cells using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacture's protocol. The sensitivity of cells to gemcitabine after IFN-γ treatment was determined as follows. The cells were seeded on 96-well and incubated for 24 hr. After incubation, 200 ng/ml IFN-γ was added to the wells. After an additional 24 hr incubation, the indicated amount of gemcitabine was added to the cells. The viability of the cells was determined using MTT assay.

cDNA microarray analysis

Human 1 cDNA Microarray Kit (Agilent Technologies, Palo Alto, CA) spotted 12,814 genes was used to analyze the different gene expression in the KLM1 and KLM1-R cells. Total RNA was extracted from the cells using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacture's protocol. The quality of RNA was assessed by running aliquots on agarose gels. Twenty micrograms of RNA were reverse transcribed for 1 hr at 42°C with Cyanine 3-dUTP or Cyanine 5-dUTP using Fluorescent Direct Labeling cDNA Synthesis Kit (Agilent Technologies). Hybridization experiments were carried out as a dye-swapped pair; *i.e.*, hybridization to one microarray with sample X labeled with cyanine 3 and sample Y labeled with cyanine 5, and to second microarray with sample X labeled with cyanine 5 and sample Y labeled with cyanine 3. Averaging the expression measurements obtained from the 2 dye-swapped hybridization should have minimized the impact of any dye-specific biases, enabling an accurate measurement of the differential expression levels. A cyanine3-/cyanine 5-labeled cDNA sample was resuspended in 7.5 μL of nuclease-free water. Cot-1 DNA was added in this cDNA sample for minimizing background fluorescence and incubates 98°C for 2 min to denature cDNA. After the sample was pipetted onto each microarray, the slide is placed in a hybridization chamber and incubated at 65°C for 17 hr. After incubation at 65°C, the slide was washed with wash solution 1 (0.5× SSC, 0.01% SDS) and wash solution 2 (0.06× SSC) and centrifuged to dry. The intensity of each hybridization signal was scanned in both Cy 3 and Cy 5 channels with Scan Array 4000 (GSI Lumonics, Billerica, MA) with a 10 μm resolution. The signal was converted into 16-bits-per-pixel resolution, yielding a 65,536-count dynamic range. A Quant Array soft (GSI Lumonics) was used for image analysis. The elements were determined by a gridding and region-detection algorithm. The area surrounding each element image was used to calculate a local background, which was subtracted from the total element signal. Background-subtracted element signals were used to calculate Cy3:Cy5 ratios. The Cy3:Cy5 ratio for each sample was calculated by global normalization.

RT-PCR

Single stranded DNA was synthesized in a reaction mixture containing 120 pmol random primers and Moloney murine leukemia virus transcriptase. PCR reaction mixture contained 10 pmol of forward and reverse primers and 2 U of TaqMan DNA polymerase (Takara, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The amplification and primer was shown as follows. Conditions were: initial denaturation step for 4 min at 94°C then 30 sec at 94°C, 30 sec at each annealing temperature, and 30 sec at 72°C followed by an elongation step for 5 min at 72°C. Selenoprotein P (sense: 5′-AAC AGA GAG CCA GGA CCA AA-3′ and antisense 5′-AAA GTT AGG AAG GAA AAA GG-3′, annealing temperature 53.7°C for 28 cycles), cytosolic glutathione peroxidase (sense: 5′-AGG AGA ACG CCA AGA ACG AA-3′ and antisense 5′-GGG ATC AAC AGG ACC AGC AC-3′, annealing temperature 59.4°C for 25 cycles), glutathione peroxidase-GI (sense: 5′-GTG AGG TGA ATG GGC AGA AC-3′ and antisense 5′-GGC AGA GGG GAA AGG CAA GG-3′, annealing temperature 57.0°C for 28 cycles), thioredoxin reductase1 (sense: 5′- TCG AAA TTA TGG ATG GAA AG-3′ and antisense 5′-CAG TAA GGC AAG GAG AAA AG-3′, annealing temperature 51.4°C for 25 cycles), selenoprotein W (sense: 5′-AAG AAG AAA GGC GAT GGC TAC-3′ and antisense 5′-AGG AGG GTG GGG TGG TGT GG -3′, annealing temperature 58.8°C for 28 cycles), dCK (sense: 5′-GCA TGA ATG AGA CAG AGT GG-3′ and antisense 5′-AGA TAA TCG AAG TTG GTT TT-3′, annealing temperature 49.1°C for 28 cycles), GAPDH (sense: 5′-GTC AAC GGA TTT GGT CGT ATT-3′ and antisense 5′-AGT CTT CTG CGT GGC ACT CAT-3′, annealing temperature 56.0°C for 25 cycles).

Dichlorofluorescein diacetate assay

One hundred micromoles (final concentration) dichlorofluorescein diacetate (DCF-DA) was added to wells 2 hr before cell harvest. At the time of the assay, the cells in each well were washed once and suspended in 5 ml of PBS. Then 0.5 ml of cell suspension from each well was diluted with 2.5 ml of PBS in a cuvette and the emission fluorescence intensity (FI) was taken at 520 nm using an excitation wavelength of 488 nm.

Purification of Selenoprotein P

Selenoprotein P was purified from human plasma using conventional chromatographic methods as described previously.[18]

Statistics

Statistical evaluations of numerical variables in viability of cells and DCF assay were carried out using Mann-Whitney's *U*-test. Significance was defined as $p < 0.05$.

RESULTS

Establishment of a pancreatic cell line showing insusceptibility

Resistance to gemcitabine was induced in a human pancreatic cancer cell line KLM1 by continuous exposure of 10 μg/ml gemcitabine (Fig. 1). The first variant was established after 4 weeks and termed KLM1-R. After repeated exposure to 10 μg/ml gemcitabine for 4 months, we established a cell line that exhibited stable characteristics with respect to growth rate, morphology and drug resistance. The KLM1-R cell line exhibited the viability to 3.5-fold in 10 μg/ml gemcitabine compared to KLM1 cells ($p < 0.05$). Based on the IC_{50} measurements, defined as the concentration of gemcitabine causing 50% growth inhibition, KLM1-R was found to be 20.06-fold resistant to gemcitabine compared to KLM1. Microscopically the parent cell line KLM1 and the resistant KLM1-R variant were not different. Cross-resistance was observed only for bleomycin, known as an agent generating intracellular reactive oxygen species (ROS),[19] although no cross-resistance was observed for other agents (Table I).

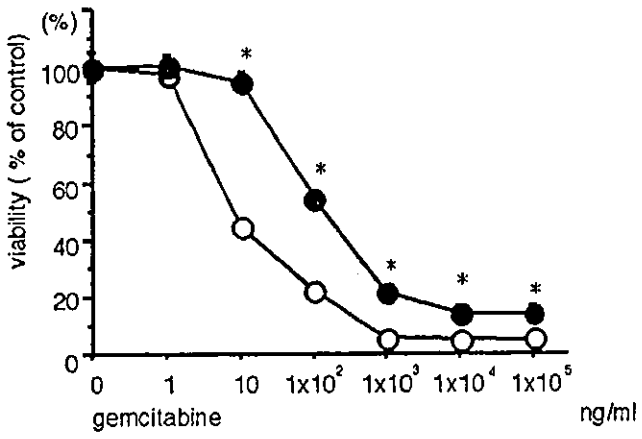

FIGURE 1 – Viability of KLM1 and KLM1-R cell line after exposure to gemcitabine. ○, KLM1 cells; ●, KLM1-R. KLM1-R cell lines exhibits lower sensitivity to gemcitabine compared to KLM1 cell line. *p lt; 0.05.

TABLE I – IC50 OF SEVERAL ANTICANCER DRUGS AS DETERMINED IN KLM1 AND KLM1-R CELLS[1]

	KLM1 (ng/ml)	KLM1-R (ng/ml)	Fold resistance
GEM	9.68	194.2	20.06
BLM	6.8×10^3	39.8×10^3	5.853
CDDM	3.1×10^2	5.1×10^2	1.645
5-FU	6.0×10^3	9.7×10^3	1.617
VP-16	77	99	1.286
TX	6.87	8.27	1.204
TXT	0.09	0.09	1.000
ADM	3.69	3.59	0.973
ara-C	9.9×10^2	9.3×10^2	0.939

[1]IC50 measured after 72 hr continuous expoure. GEM, gemcitabine; BLM, bleomycin; CDDP, cisplatin; 5-FU, 5- fluorouracil; VP-16, etoposide; TX, paclitaxel; TXT, docetaxel; ADM, adriamycin.

cDNA microarray to determine alteration of gene expression

The expression of alteration of 12,814 clones was analyzed by cDNA microarray between the KLM1 and KLM1-R cells (Fig. 2*a*). Table II demonstrates 25 genes that displayed the altered fluorescence ratios of >5-fold. Among the 25 differentially expressed genes, 5 genes were upregulated and 20 were downregulated in KLM1-R cells. It is noteworthy that the most of the upregulated gene was *selenoprotein P* (9.699-fold) in KLM1-R cells on the examined microarray. Selenoprotein P is one of the major selenium-rich extracellular proteins in human plasma.[18] The family of selenoproteins has been reported to play critical roles in anti-oxidative reaction.[20–23] Our microarray analysis clarified no alteration of the expression of bcl-2 related genes (*bcl-2*, *bfl-1*, *bag-1*, *bad*, *bak*, *bcl-x_L*) or multi-drug resistance genes (*MDR1*, *MDR3*, *MRP5*) in the gemcitabine resistant KLM1-R cells (data not shown).

Expression of selenoprotein P in pancreatic cancer cells

To evaluate expression of *selenoproteins* mRNA, RT-PCR analysis was assessed on KLM1 and KLM1-R cells. As shown in Figure 2*b*, the mRNA of *selenoprotein P* was clearly expressed in KLM1-R cells, but not in KLM1 cells. These results were compatible with the microarray results. There was no difference of mRNA expression in other selenoproteins (*cellular glutathione peroxidase, glutathione peroxidase-GI, selenoprotein W* and *thioredoxin reductase 1*) between KLM1 and KLM1-R cells. It should be mentioned that no different expression of *dCK*, which is the converting enzyme for gemcitabine and one of genes contributing to the gemcitabine resistance.[14] These data indicate that

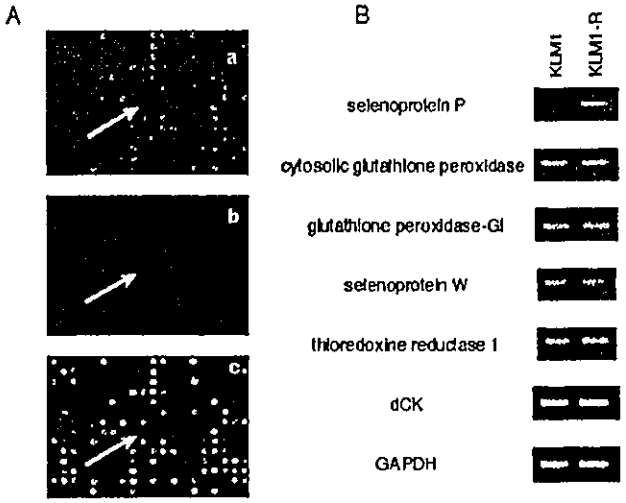

FIGURE 2 – (*a*) Microarray analysis between KLM1 and KLM1-R. *Inset a*, KLM1; *inset b*, KLM1-R; *inset c*, composition image of KLM1 and KLM1-R. The arrow indicates the spot of *selenoprotein P*. (*b*) RT-PCR in KLM1 and KLM1-R.

TABLE II – SPECIFICITY OF MICROARRAY[1]

Gene name	Ratio[2]
Up-regulated genes	
Selenoprotein P, plasma 1	9.699
Catalase	8.237
Emopamil-binding protein	5.656
TNF ligand superfamily, member 10	5.337
Prostaglandin-endoperoxidase synthase 2	5.053
Down-regulated genes	
Protein tyrosinephosphatase, non-receptoe type 2	0.188
Insulin-like growth factor binding protein 7	0.180
Metastasin	0.147
Melanoma antigen, family A, 9	0.146
Amphiregulin	0.145
Aldehyde dehydrogenase 3	0.140
Aldehyde dehydrogenase 6	0.130
S100 calcium-binding protein A2	0.111
N-acylsphingosine amidohydrolase	0.100
Four and a half LIM domains 1	0.082
Regulator of G-protein signaling 3	0.073
Calponin 3, acidic	0.068
Coagulation factor III	0.060
Transgelin	0.059
Dickkopf (Xenopus laevis) homolog 1	0.049
Kallikrein 5	0.033
Lymphocyte antigen 6 complex, locus D	0.027
Fructose- 1,6-bisphosphatase 1	0.024
Keratin 7	0.022
Cystein-rich protein 1 (intestinal)	0.016

[1]Gene expression profile in KLM1-R cells. –[2]Ratio means the expression in KLM1-R/that of KLM1.

selenoprotein P expression might be associated with the insusceptibility to gemcitabine in the pancreatic cancer cells.

Selenoprotein P improves the cell viability exposed to gemcitabine

To assess whether selenoprotein P protects the pancreatic cell lines from the toxicity of gemcitabine, cell survival was determined after exposure to gemcitabine. As demonstrated in Figure 3, the addition of 1 μg/ml selenoprotein P to the medium recovered the viability of pancreatic cancer cells after exposure of gemcitabine for 72 hr. These findings demonstrate important evidence that the selenoprotein P downregulates the susceptibility to gemcitabine in the pancreatic cancer cells.

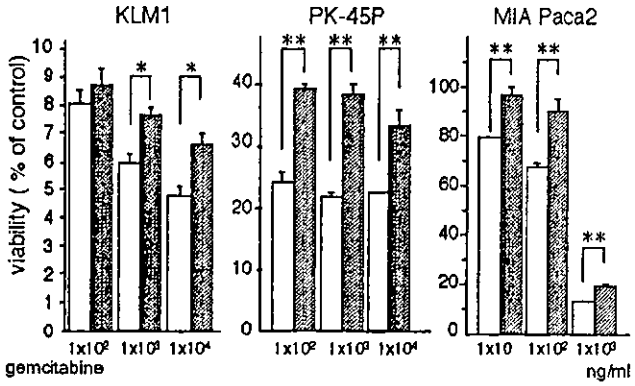

FIGURE 3 – The effect of selenoprotein P on viability after gemcitabine exposure in pancreatic cell lines. Selenoprotein P recovered the viability of pancreatic cell lines after exposure of gemcitabine for 72 hr. Control, with selenoprotein P. $*p < 0.05$, $**p < 0.01$.

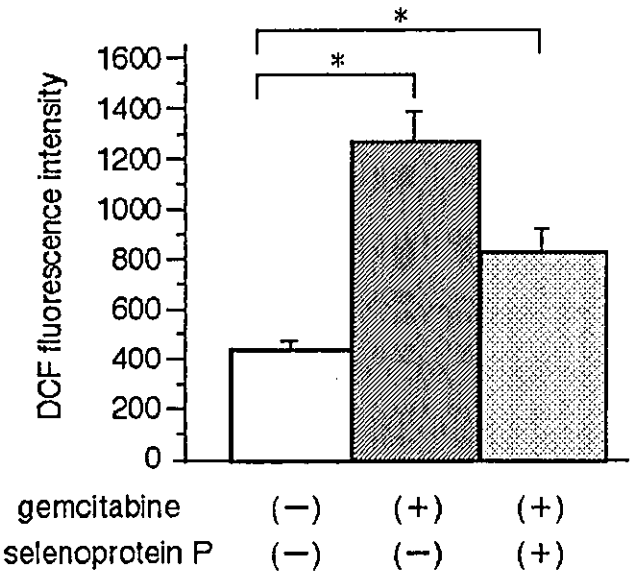

FIGURE 4 – The effect of selenoprotein P on ROS level after gemcitabine exposure. ROS induced by gemcitabine was significantly higher than that of control. Preincubation with selenoprotein P suppressed the increase of ROS induced by gemcitabine. $*p < 0.05$.

Selenoprotein P suppresses gemcitabine-induced intracellular ROS

Because selenoprotein P is known as an ROS scavenger,[18,23–25] the ROS generation was then analyzed in the pancreatic cancer cells after gemcitabine exposure. The intracellular ROS was measured in KLM1 cells by DCF-DA assay. Figure 4 showed that the ROS levels were increased 2.91-fold by 72-hr exposure to gemcitabine compared to the control ($p = 0.02$). In contrast, the administration of selenoprotein P induced only 1.89-fold ROS by gemcitabine exposure ($p = 0.08$). The addition of selenoprotein P suppressed the gemcitabine-induced intracellular free radicals that have extensive cytotoxic effects even on cancer.[26]

Susceptibility to oxidative stress in pancreatic cancer cells

To assess the susceptibility to oxidative stress in KLM1-R cell, survivals were determined after exposure to H_2O_2. Because H_2O_2 is readily converted to hydroxyl radicals, it is considered that a cytotoxic agent causes damage to many cellular components. After

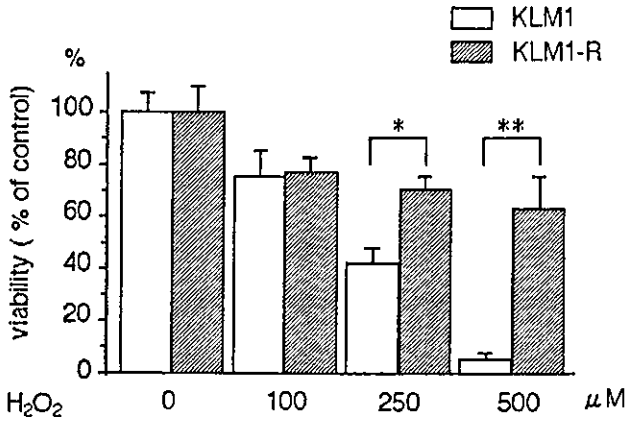

FIGURE 5 – Viability of the KLM1 and KLM1-R after exposure to H_2O_2. The cells were exposed to various concentrations of H_2O_2. The H_2O_2-mediated toxicity was suppressed in KLM1-R significantly. $*p < 0.05$, $**p < 0.01$.

exposure to H_2O_2, cell survivals were determined. As shown in Figure 5, the H_2O_2-mediated cytotoxicity was suppressed in KLM1-R. After exposure to 500 μM H_2O_2, the number of viable KLM1-R cells was 14.9-fold compared to that of KLM1 cells ($p < 0.01$). These findings indicate that the KLM1-R, which we established as a cell line less sensitive to gemcitabine, acquired potent resistance to oxidative stress.

IFN-γ suppresses mRNA expression and activity of selenoprotein P

Because negative regulation of *selenoprotein P* promoter by cytokines including IFN-γ was known,[27] IFN-γ was used to increase the cytotoxicity by gemcitabine through suppression of selenoprotein P. After treatment of IFN-γ for 24 hr, the mRNA expression of selenoprotein P was suppressed (Fig. 6*a*). Densitometric analysis of band intensities showed suppression in selenoprotein P-mRNA with 200 ng/ml IFN-γ (Fig. 6*b*). Although IFN-γ has no cytotoxicity (Fig. 6*c*), the cytotoxicity of gemcitabine to KLM1-R was increased after treatment of 200 ng/ml IFN-γ for 24 hr (Fig. 6*d*). Furthermore, the intracellular ROS was measured in KLM1-R after IFN-γ treatment. The administration of IFN-γ increased the intracellular ROS levels in KLM1-R (Fig. 6*e*). These findings indicated that IFN-γ suppressed the expression of selenoprotein P and increased the intracellular ROS levels, leading to improvement of the sensitivity to gemcitabine.

DISCUSSION

The novel nucleoside analogue gemcitabine is well known as one of the most effective anticancer agents, however, the mechanism of sensitivity has not been clearly understood. In our study, cDNA microarray analyses showed that the *selenoprotein P* gene was overexpressed in a gemcitabine-resistant pancreatic cancer cell line. Selenoprotein P is a member of the anti-oxidative selenoprotein family that is composed of more than 15 selenoproteins in mammalians, *i.e.*, glutathione peroxidase, thioredoxin reductase, and selenoprotein W. The selenoprotein family has been recognized as playing a survival role in suppression of free radicals.[20–22] In particular, selenoprotein P accounts for about 50% of total plasma selenium in humans, and differs from all other selenoproteins identified so far by its higher selenium content. Human selenoprotein P is predicted to contain 10 selenocysteine residues whereas other selenoproteins contain only 1 selenocysteine residue per subunit.

Previous studies demonstrated a role of selenoprotein P as a protective agent against the oxidation and nitration reactions mediated by peroxynitrite, a potent oxidant generated *in vivo*.[28] The

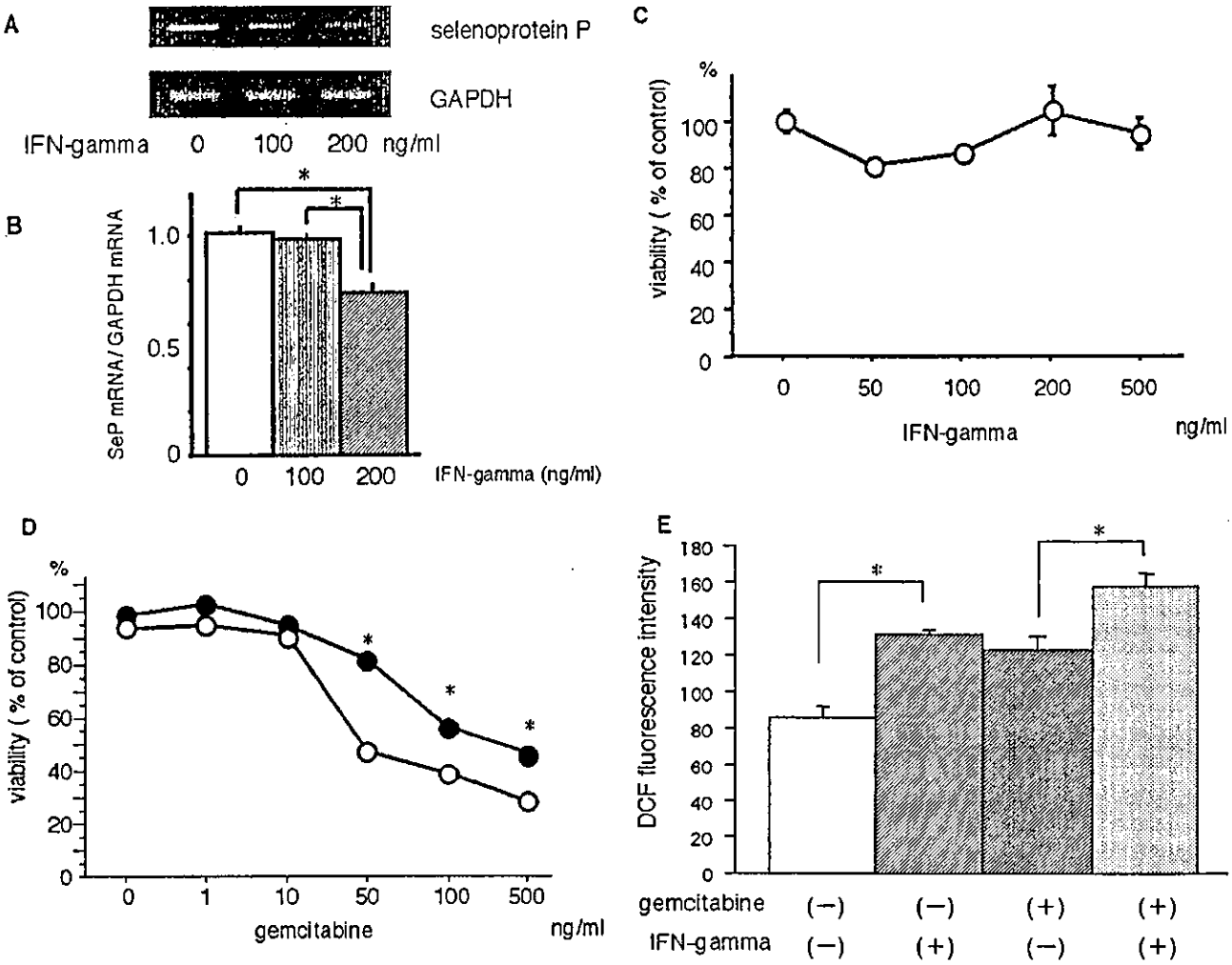

FIGURE 6 – The effect of IFN-γ on selenoprotein P. (*a*) 200 ng/ml IFN-γ suppressed the expression of selenoprotein P mRNA. (*b*) In hemiquantitative RT-PCR, selenoprotein P was suppressed after treatment of IFN-γ. $*p < 0.01$. (*c*) Viability of KLM1-R cell line after exposure of IFN-γ. (*d*) Viability of KLM1-R cell line after exposure to gemcitabine with and without IFN-γ. ○; with IFN-γ, ●; without IFN-γ. IFN-γ recovered sensitivity to gemcitabine. $*p < 0.01$. (*e*) IFN-γ increased the intracellular ROS level in the KLM1 cell line. $*p < 0.05$.

function of selenoprotein P was reported not only as an antioxidative enzyme but also as a selenium supplier to the cell.[29] Selenoprotein P-knockout mice exhibited the alteration of selenium distribution in organs that reflected activities of selenium-dependent enzyme.[30,31] The selenium levels in the brain and testis were completely reduced, and the activity of other selenoproteins was severely decreased in the selenoprotein P-knockout mice.[30,31] These reports suggested that selenoprotein P was an antioxidative enzyme in itself and protected tissues against oxidative stress. Furthermore, selenoprotein P activates the other selenoproteins though the delivery selenium to the organs. Selenoprotein P is an important molecule protecting tissues and organs against oxidative stress, cooperative with other selenoproteins.

The apoptosis-regulating genes of the bcl-2 family or multi-drug resistance genes were reported to have an important role in resistance of anti-cancer agents. Although these genes are thought to have a relation to gemcitabine resistance in our study, the result of our microarray clarified no alteration of the expression of these genes in the gemcitabine resistant KLM1-R. This was consistent with the result of no cross-resistance in other anticancer agents except bleomycin, known as an agent generating intracellular ROS in KLM1-R.

In our study, the exposure to gemcitabine induced approximately 3-fold intracellular free radicals that were inhibited by selenoprotein P. Intracellular free radicals occur in response to cytotoxic agents and this is related to the loss of mitochondrial inner membrane potential (MMP).[26] The loss of MMP releases mitochondrial mediators of apoptosis such as cytochrome *c* and induces apoptosis.[26] Intracellular free radicals have been noted as playing a contributing role in the cytotoxicity of anti-tumor agents including adriamycin,[32] bleomycin[19] and tumor necrosis factor.[33] Sylvia *et al.*[26] reported that gemcitabine-induced free radicals contributed to lethality through increasing the population with low MMP. Together with our results, the generation of free radicals is considered as one of the major cytotoxic mechanisms of gemcitabine. The 5′-flanking region of human selenoprotein P gene was shown to contain IFN-γ responsive elements and a negative regulation of selenoprotein P promoter using pro-inflammatory cytokines such as interleukin-1β and TNF-α was found.[27] Expression of selenoprotein P mRNA and protein in human hepatoma cells HepG2 is efficiently inhibited on a transcriptional level by the anti-inflammatory cytokine transforming growth factor (TGF)-β 1.[34] In our study, we examined the effect of IFN-γ to selenoprotein P, because IFN-γ is very common in the clinical environment.

IFN-γ suppressed the expression of selenoprotein P mRNA and improved the cytotoxicity of gemcitabine in the human pancreatic cancer cell line. The intracellular ROS level was increased by the administration of IFN-γ. This combination therapy, gemcitabine and IFN-γ, may provide a new approach to the treatment of pancreatic cancer.

Our findings showed selenoprotein P as a novel molecule responsible for gemcitabine resistance in human pancreatic cancer cells, through the suppression of free radical cytotoxicity. In addition, we established a gemcitabine-resistant cell line that suppressed the cytotoxicity induced not only by gemcitabine-mediated endogenous free radicals but also by H_2O_2-mediated exogenous free radicals. Because free radicals can act directly on intracellular apoptosis signaling, further studies on this mechanism are required before clinical application.

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