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Estrone and 17β-Estradiol Reverse Breast Cancer Resistance Protein-mediated Multidrug Resistance

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Breast cancer resistance protein (BCRP), an adenosine triphosphate-binding cassette transporter, confers resistance to a series of anticancer reagents, including mitoxantrone, SN-38 and topotecan. In the present study, we found that estrone and 17β-estradiol potentiated the cytotoxicity of mitoxantrone, SN-38 and topotecan in *BCRP*-transduced K562 cells (K562/BCRP). These estrogens showed only a marginal effect, or none, in parental K562 cells. Estrone and 17β-estradiol increased the cellular accumulation of topotecan in K562/BCRP cells, but not in K562 cells, suggesting that these estrogens inhibit the BCRP-mediated drug efflux and overcome drug resistance.

Key words: BCRP — Estrone — 17β-Estradiol — MDR — Reversal of drug resistance

Multidrug drug resistance (MDR) is a common phenomenon in cancer cells. Cancer cells selected for resistance to a certain chemotherapeutic agent often reveal cross-resistance to structurally unrelated antitumor agents.[1)] Family members of adenosine triphosphate-binding cassette (ABC) transporters, such as *MDR*1 gene product P-glycoprotein and multidrug resistance-associated protein (MRP) 1, are involved in multidrug resistance by pumping out a variety of structurally and functionally diverse antitumor drugs from cells.[2, 3)] Breast cancer resistance protein (BCRP), also called ABCP or MXR, is a newly discovered ABC transporter. It mediates resistance to such chemotherapeutic agents as mitoxantrone, SN-38 (an active metabolite of CPT-11) and topotecan, presumably by pumping these reagents out of the cell to lower the cytotoxic effects.[4–8)] Overcoming MDR is a major problem in the clinical management of cancer. In order to identify agents which reverse BCRP-mediated drug resistance, we examined the effect of various organic compounds on the toxicity of mitoxantrone, SN-38 and topotecan in BCRP-expressing K562 cells. Among the compounds tested, estrone and 17β-estradiol were found to reverse BCRP-mediated drug resistance.

BCRP retrovirus-producing PA/MycBCRP cells were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. K562 human leukemic cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C with 5% CO_2. K562 cells were transduced with the retrovirus supernatant for one day and cultured further for 2 days in the same medium without the retrovirus supernatant. Cells were then treated with SN-38 (20 ng/ml) for 5 days to enrich the transduced cells. The expression of BCRP in *BCRP*-transduced K562 (K562/BCRP) cells was detected with the anti-BCRP polyclonal antibody 3488.[9)]

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

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

The expression of BCRP in K562/BCRP cells was observed in *BCRP*-transduced K562/BCRP cells, but not in parental K562 cells. In the presence of reducing agents, BCRP was recognized as a 70-kDa protein by western blot analysis (Fig. 1). K562/BCRP cells showed significantly higher resistances to mitoxantrone, SN-38, and topotecan than K562 cells (Table I). K562 and K562/BCRP cells showed similar sensitivity to camptothecin, vincristine, and paclitaxel. K562 and K562/BCRP cells expressed

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very small amounts of *MDR*1, *MRP1*, *MRP2*, and *MRP3* mRNAs as determined by reverse transcription-polymerase chain reaction. BCRP transduction did not affect the expression of these transporter mRNAs (data not shown).

The reversal activities of steroids were examined by using fixed doses of steroids (3 and 10 μM) in combination with increasing concentrations of the antitumor agents (Fig. 2). These steroids alone showed only a marginal growth-inhibitory effect, or none, on either K562 or K562/BCRP cells at the doses used in Fig. 2 (Table II). Experimental data for 10 μM 17β-estradiol are not shown because of the excessive growth-inhibitory effect. Estrone enhanced the cytotoxicity of mitoxantrone, SN-38 and topotecan on K562/BCRP cells in a dose-dependent manner, while it did not affect the cytotoxicity of any of these drugs on K562 cells. The reversal activities (the ratios of IC_{50} values in the absence or presence of the steroid) of 10 μM estrone were 7.5-fold for mitoxantrone, 3.6-fold for SN-38 and 4.1-fold for topotecan. Similarly, 17β-estradiol enhanced the cytotoxicity of the antitumor agents on K562/BCRP cells, but not K562 cells. Drug resistance was slightly overcome by estriol and pregnenolone in a dose-dependent manner. However, it is not clear whether this reversal was solely attributable to the inhibition of BCRP-mediated drug transport, because estriol and pregnenolone also potentiated the cytotoxicity of these antitumor reagents in parental K562 cells. Progesterone had little effect on the drug sensitivity of K562/BCRP cells.

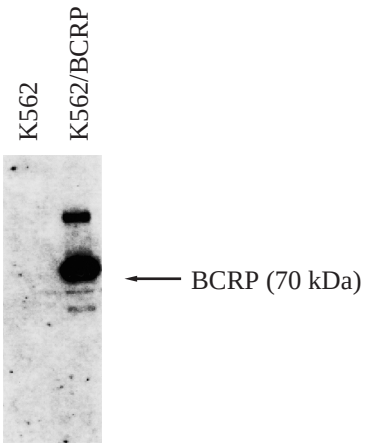

Fig. 1. Expression of BCRP in K562/BCRP cells. Western blot analysis was performed under reducing conditions, revealing BCRP as a 70-kDa band (arrow).

Table I. Drug Resistance of K562/BCRP Cells

Drug	IC_{50} (ng/ml)		Degree of resistance
	K562	K562/BCRP	
Mitoxantrone	0.27	3.0	11
SN-38	0.58	12	21
Topotecan	3.3	37	11

Degree of resistance is the ratio of IC_{50} value for K562/BCRP cells divided by that for K562 cells. Data are mean values of triplicate determinations. Standard deviations are less than 10% of the mean values.

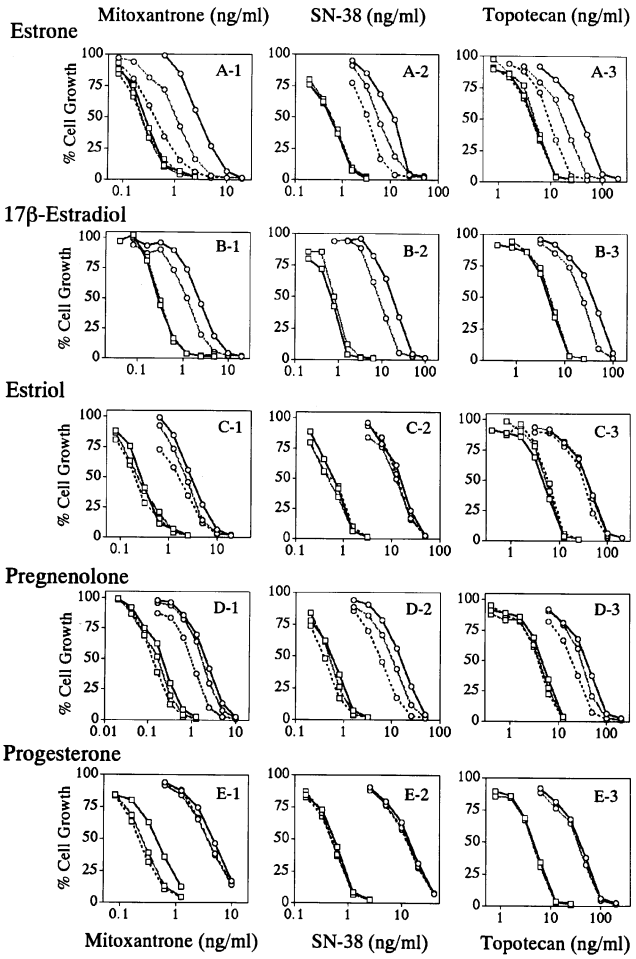

Fig. 2. Reversal effect of steroids on antitumor drug resistance. K562 (□) and K562/BCRP (○) cells were cultured for 5 days in the absence (——) or presence of 3 μM (·····), 10 μM (----) steroid with increasing doses of antitumor drugs: estrone (A), 17β-estradiol (B), estriol (C), pregnenolone (D), progesterone (E). Antitumor agents are mitoxantrone (*N*-1), SN-38 (*N*-2), and topotecan (*N*-3) (*N*: A–E). (Each point is an average of triplicate determinations). Cell numbers were counted with a Coulter counter. For 17β-estradiol, data for 10 μM are omitted because of high cytotoxicity.

Table II. Steroid Sensitivity of K562/BCRP Cells

Steroid	IC_{50} (μM)		Degree of resistance
	K562	K562/BCRP	
Estrone	>100	>100	ND
17β-Estradiol	5.9	4.8	0.8
Estriol	19.4	16.7	0.86
Pregnenolone	32.3	21.9	0.68
Progesterone	10.6	9.2	0.87

Degree of resistance is the ratio of IC_{50} value for K562/BCRP cells divided by that for K562 cells. Data are mean values of triplicate determinations. Standard deviations are less than 10% of the mean values. ND: not determined.

Interestingly, the cytotoxicity of mitoxantrone to parental K562 cells was enhanced in the presence of progesterone, but that of the other antitumor reagents was not. The reason for this result is unclear, but progesterone and mitoxantrone might have synergistic effects on K562 cells.

Some steroids and their metabolites are substrates of P-glycoprotein and MRP1. P-Glycoprotein excretes hydrocortisone and aldosterone.[10] Glucuronides such as 17β-estradiol-17β-D-glucuronide are substrates of MRP1.[11] However, hydrocortisone, aldosterone and 17β-estradiol-17β-D-glucuronide did not overcome drug resistance of K562/BCRP cells. K562 and K562/BCRP cells showed similar and marginal expression of *MDR*1 and *MRP1* mRNAs by reverse transcription-polymerase chain reaction. These results suggest that estrone and 17β-estradiol interact specifically with BCRP and overcome drug resistance of K562/BCRP cells.

In order to determine whether this reversal might be associated with increased drug transport, the cellular accumulation of topotecan was evaluated in the absence or presence of steroid hormones by flow cytometric analysis. Intracellular accumulation of topotecan increased in the presence of estrone in a dose-dependent manner in K562/BCRP cells, whereas the levels were not altered in K562 cells (Fig. 3). In addition, increased cellular accumulation of topotecan was also observed in the presence of 17β-estradiol and estriol. Pregnenolone and progesterone showed marginal effects on topotecan uptake. These results indicate that estrone and 17β-estradiol reversed topotecan resistance by increasing the cellular levels of topotecan in BCRP-expressing cells. The other steroids, especially estriol, induced little reversal of the drug resistance as detected in the growth inhibition assay, although estriol strongly enhanced topotecan uptake in K562/BCRP cells. This discrepancy could be attributable to the differences in the concentrations of the steroids tested, and resistance to these drugs might have been overcome in the growth inhibition assay if higher concentrations of steroids had been used. Intracellular topotecan accumulation in

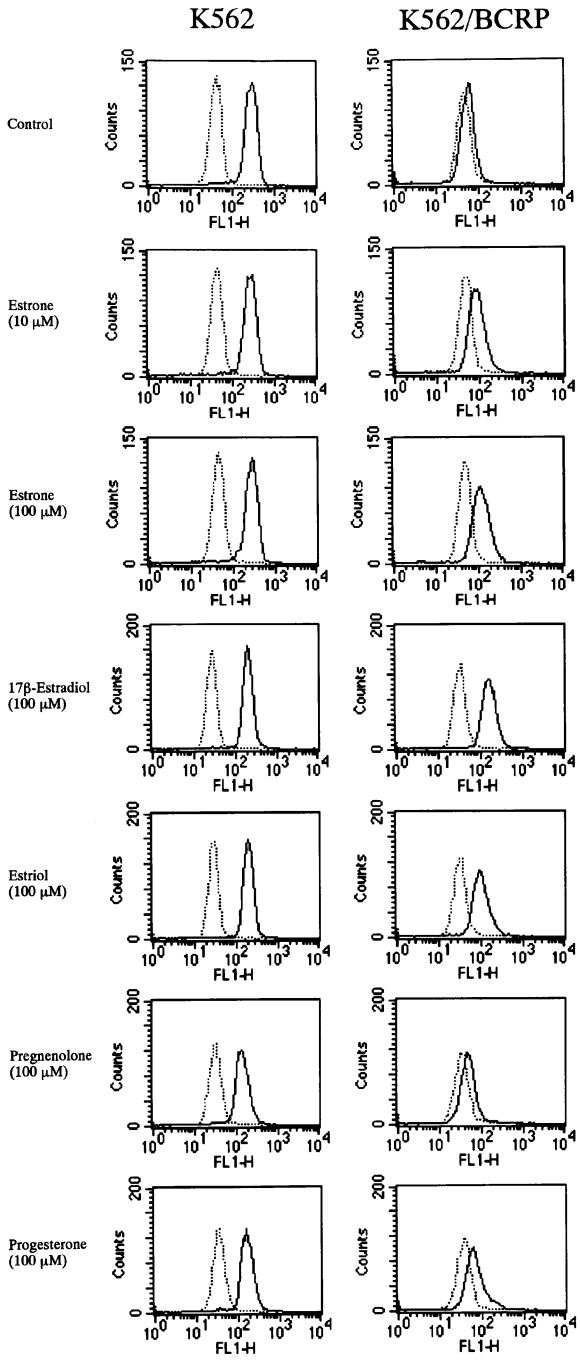

Fig. 3. Effect of steroids on the intracellular uptake of topotecan in K562 and K562/BCRP cells. Cells were incubated with or without 20 μM topotecan in the presence or absence of the steroid: (——), with topotecan; (----), without topotecan. Uptake of topotecan was measured by FACS. In K562/BCRP cells, a fluorescence peak shift to the right indicates cellular uptake of topotecan in the presence of steroids, while no shift occurred in the absence of steroids. In contrast, fluorescence peak shifts to the right were observed in K562 cells, irrespective of the status of steroids.

K562/BCRP cells slightly increased in the presence of pregnenolone, so that the enhanced cytotoxicity of antitumor drugs observed in the growth inhibition assay might be, in part, explained by the inhibition of BCRP-mediated drug efflux.

Therefore, we infer that steroid hormones, especially estrone and 17β-estradiol, may be physiological substrates for BCRP and act to competitively inhibit drug transport. Among normal tissues, the highest levels of BCRP expression are recognized in the placenta, and BCRP is presumed to play a role in protecting the fetus against toxic compounds. In addition, BCRP expression has been observed in the syncytiotrophoblast of the placenta that produces the steroid hormones evaluated in this study.[12] Although K562 and K562/BCRP cells showed similar sensitivity to the steroids, as shown in Table II, our preliminary experiments suggest that BCRP transports estrone and 17β-estradiol, but not progesterone, in the basal to apical direction in *BCRP*-transduced LLC-PK1 cells (manuscript in preparation). A detailed investigation is currently under way to confirm the hypothesis that these steroids are physiological substrates for BCRP.[10, 13]

BCRP is overexpressed in some cancer cell lines other than those of the genital organs, such as lung A549 and colon KM12 cells, and may underlie the natural resistance to antitumor agents.[14] GF120918 and fumitremorgin C have been found to reverse BCRP-mediated drug resistance.[15, 16] Estrone and 17β-estradiol are the first endogenous compounds shown to exert strong reversal activity. Steroid analogs without estrogenic or anti-estrogenic activity might be used to overcome BCRP-mediated drug resistance without significant side effects.

In summary, estrogens were found to restore drug sensitivity in K562/BCRP cells by increasing the cellular accumulation of anticancer drugs. These findings should assist practical therapy and the design of more effective and safer reagents to circumvent drug resistance, as well as having implications for the physiological role of BCRP.

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