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BCRP/MXR/ABCP Expression in Topotecan-Resistant Human Breast Carcinoma Cells

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ABSTRACT. We have previously described a mitoxantrone-resistant MCF7 cell line that is cross-resistant to topotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin (CPT-11), and 9-aminocamptothecin, but not to camptothecin. A novel mechanism that resulted in decreased topotecan accumulation in MCF7/MX cells was proposed (Yang *et al. Cancer Res* 55: 4004–4009, 1995). We now have developed a topotecan-resistant cancer cell line from wild-type MCF7 cells. MCF7/TPT300 cells were 68.9-fold resistant to topotecan, 68.3-fold to 10-hydroxy-7-ethylcamptothecin (SN-38), and 116-fold to mitoxantrone, but only 4.1-fold to camptothecin. Topotecan efflux was increased in MCF7/TPT300 cells compared with MCF7/WT cells, and this increase was reversed upon ATP depletion by sodium azide, suggesting an energy-dependent drug efflux mechanism. However, MCF7/TPT300 cells did not overexpress P-glycoprotein or the multidrug resistance-associated protein (MRP1). In contrast, overexpression of the breast cancer resistance protein (BCRP/MXR/ABCP) was observed in MCF7/TPT300 cells as well as DNA topoisomerase I down-regulation. Our data suggest that enhanced topotecan efflux contributes partly to topotecan resistance in MCF7/TPT300 cells, possibly mediated by BCRP/MXR/ABCP. BIOCHEM PHARMACOL **60**;6:831–837, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. topotecan; BCRP; mitoxantrone; multidrug resistance

Topoisomerase I inhibitors have become important anticancer agents during the last 10 years. Topotecan is effective in the treatment of ovarian and small cell lung cancer patients, and 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin (CPT-11) is useful in treating colorectal cancer patients. Both topotecan and CPT-11 show much therapeutic promise in anti-cancer treatment, whereas the parent compound camptothecin itself is not useful in the clinic due to its high toxicity profile. Several resistance mechanisms to camptothecin and its derivatives have been described, including mutations in topoisomerase I [1], decreased levels of topoisomerase I [2], and decreased topotecan accumulation in P-glycoprotein-overexpressing cells [3]. DNA topoisomerase I mutations and down-regulation of topoisomerase I were the two most frequently described phenomena in camptothecin-resistant cancer cells and affected all analogs similarly [4]. In contrast, our previous study has shown that human breast carcinoma cells selected for mitoxantrone resistance (MCF7/MX) [5] develop cross-resistance to topotecan, CPT-11, and 10-hydroxy-7-ethylcamptothecin (SN-38), but not to camptothecin, suggesting that topotecan resistance in these cells may be different from classical camptothecin resistance. A non-P-glycoprotein, non-MRP1**-mediated topotecan accumulation defect was proposed [6]. To determine if topotecan selection could induce a resistance pattern different from typical camptothecin resistance, but similar to the one observed in the mitoxantrone-selected cells, a topotecan-resistant subline from MCF7 wild-type cells was selected by stepwise elevation of topotecan concentration in the culture medium. In the present study, the resistance pattern of MCF7/TPT300 cells, the expression of ATP-binding cassette proteins, the expression of DNA topoisomerases, and topotecan efflux in sensitive and resistant cells were demonstrated.

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** *Abbreviations*: MRP1, multidrug resistance-associated protein 1; DMEM, Dulbecco's Modified Eagle's Medium; HBSS, Hanks' Balanced Salt Solution; RT–PCR, reverse transcription–polymerase chain reaction; TBE, Tris–borate–EDTA; DIG, digoxigenin; VP-16, etoposide; 5-FU, 5-fluorouracil; and BCRP, breast cancer resistance protein.

MATERIALS AND METHODS

Materials

Topotecan was provided by E. Schneider (Wadsworth Center). SN-38 was provided by Rhone Poulenc Rorer. Mitoxantrone was purchased from Lederle Parenterals Inc. Sulforhodamine B and camptothecin were obtained from the Sigma Chemical Co. Topotecan and mitoxantrone were dissolved in water. SN-38 and camptothecin were dissolved in 100% alcohol.

Cell Lines

MCF7/WT, MCF7/MX, MCF7/VP, MCF7/MTX, and MCF7/ADR cells were gifts from Dr. Kenneth Cowan (National Cancer Institute). MCF7/TPT50 and MCF7/TPT300 cells were developed in our laboratory as described in Results. Cells were grown in DMEM (Gibco) containing 10% fetal bovine serum (Gibco) at 37° in 5% CO_2 in a humidified incubator.

Growth Rate Determination

MCF7/WT, MCF7/TPT50, and MCF7/TPT300 cells were cultured in 12-well plates. Viable cells from individual plates in triplicate were counted daily for 7 days using trypan blue exclusion staining in a counting chamber.

Cytotoxicity Assay

MCF7/WT, MCF7/TPT50, and MCF7/TPT300 cells were seeded in 96-well microtiter plates. Various concentrations of drugs were added to the wells. Cells were incubated at 37° for 4 days in a humidified incubator. Cell growth was determined using the sulforhodamine B assay as described previously [7]. Concentrations of chemotherapeutic agents that inhibited growth by 50% (IC_{50}) were determined from survival curves. The relative resistance factor is defined as IC_{50} of resistant cells divided by IC_{50} of MCF7/WT cells.

Topotecan Accumulation and Efflux

Topotecan accumulation was measured by flow cytometry as described previously [3]. MCF7/WT, MCF7/MX, MCF7/TPT50, and MCF7/TPT300 cells were trypsinized and washed with PBS. Cells (10^5 cells/mL) were resuspended in HBSS and incubated with topotecan (50 μM) at 37° for 20 min. At the end of the incubation, each sample was subjected to flow cytometry on a FACScan (Becton Dickinson). The accumulation of topotecan was determined by fold-of-increase of the mean fluorescent units of the topotecan-incubated cells [(mean fluorescent units of topotecan incubated cells –mean fluorescent units of control cells)/mean fluorescent units of control cells]. The efflux of topotecan was determined after the steady state of accumulation of topotecan was achieved. The topotecan-accumulated cells were centrifuged and resuspended in HBSS to measure topotecan efflux. After resuspension in HBSS for different periods of time, each sample was subjected to flow cytometric measurement. Approximately 1000 events were recorded at each time point.

ATP Depletion by Sodium Azide

MCF7/TPT300 cells were incubated in glucose-free PBS containing 15 mM sodium azide (Merck) for 30 min before being subjected to flow cytometric measurement of topotecan efflux.

RT–PCR for mdr-1

One microgram of total RNA isolated from MCF7/WT, MCF7/TPT, and MCF7/ADR cells was subjected to RT–PCR using a 0.2 mM concentration of mdr-1 primers (5′-GCCTGGCAGCTGGAAGACAAATACACAAA-ATT-3′ and 5′-CAGACAGCAGCTGACAGTCCAA-GAACAGGACT-3′) as described previously [8]. Ten-microliter samples of the reaction products were separated on a 2% agarose gel in TBE buffer and visualized under a UV light.

Northern Blot for MRP1 and BCRP/MXR/ABCP

Total RNA from MCF7/WT, MCF7/MX, MCF7/VP, MCF7/MTX, MCF7/TPT50, and MCF7/TPT300 cells was isolated by the guanidinium/acid phenol method [9]. Ten micrograms of total RNA from each cell line was separated on a formaldehyde–agarose gel, transferred to a nylon membrane, and hybridized with an 870-bp DIG-labeled BCRP/MXR/ABCP probe or a DIG-labeled MRP1 probe. The BCRP/MXR/ABCP and MRP1 probes were made by purification of an RT–PCR product made with specific primer pairs for the *BCRP/MXR/ABCP* or *MRP1* gene, respectively. Bound probe was detected by chemiluminescence (Roche-Boehringer Mannheim).

Western Blotting

Membrane proteins were isolated from MCF7/WT, MCF7/MX, MCF7/VP, MCF7/MTX, MCF7/TPT50, and MCF7/TPT300 cells and separated by denaturing polyacrylamide gel electrophoresis. BCRP/MXR/ABCP levels were measured by using a 1:2000 dilution of an anti-MXR (BCRP) antibody provided by Drs. Tito Fojo and Susan Bates (National Cancer Institute, U.S.A.). Topoisomerase I and II levels were measured by western blot as described previously [6]. The total protein was incubated overnight with 1:1000 dilution of Topo I antibody (Topogen) or 1:500 Topo II antibody (Calbiochem). Bound antibody was visualized by a chemiluminescence kit (NEN Life Science).

TABLE 1. IC_{50} values and relative resistance factors of chemotherapeutic agents in MCF7 cell line variants

	IC_{50}* (nM)							
	Camptothecin	Mitoxantrone	SN-38	Topotecan	5-FU	Doxorubicin	VP-16	Paclitaxel
MCF7/WT	11.5 ± 1.6	4.40 ± 0.64	4.54 ± 0.36	16.8 ± 1.1	1510 ± 80	7.60 ± 0.76	203 ± 29	0.87 ± 0.10
MCF7/TPT50	18.2 ± 2.3 (1.6)†	205 ± 44 (46.5)†	70.0 ± 10.0 (15.4)†	199 ± 22 (11.9)†	1870 ± 77 (1.2)†	26.0 ± 5.6 (3.4)†	565 ± 59 (2.8)†	1.23 ± 0.12 (1.4)†
MCF7/TPT300	46.7 ± 4.8 (4.1)†	512 ± 101 (116)†	310 ± 14 (68.3)†	1150 ± 30 (68.9)†	ND‡	41 ± 15 (5.4)†	890 ± 560 (4.4)†	3.2 ± 1.4 (3.7)†
MCF7/MX	(3.2)†§	(3932)†‖	(101)†§	(180)†§	(1.1)†¶	(11.4)†¶	(10.6)†¶	(1.0)†¶

*Means ± SEM from at least three independent measurements.
†Relative resistance factor: IC_{50} of resistant cells divided by IC_{50} of MCF7/WT cells.
‡ND, not determined.
§Ref. 6.
‖Ref. 5.
¶Schneider *et al.*, unpublished.

RESULTS

Establishment of MCF7/TPT Cells

MCF7/WT cells were grown as an attached monolayer in DMEM containing 10% fetal bovine serum at 37° in 5% CO_2 in a humidified incubator. Five nanomolar topotecan was added to the medium. After the cells started to divide actively, they were exposed to the next higher concentration of topotecan (starting from 5 nM, then escalating to 10, 20, 30, 40, and finally 50 nM). After 18 months, and about 100 passages, selected MCF7 cells were able to grow in 50 nM topotecan. Cells were diluted in medium, and individual clones were picked out. One such clone (MCF7/TPT50) was used to develop cells with resistance to 300 nM topotecan (MCF7/TPT300 cells) following 4 months of additional selection. The resistant cells were cloned to reduce heterogeneity within selected cells and kept in drug-free medium for over 2 months before analysis. Doubling times calculated from growth curves for MCF7/WT, MCF7/TPT50, and MCF7/TPT300 cells were 23.2, 25.8, and 25.0 hr, respectively.

Drug Sensitivity of MCF7/TPT Cells

The 50% growth inhibiting concentrations (IC_{50} values) and relative resistance factors are shown in Table 1. MCF7/TPT50 cells were cross-resistant to SN-38 and topotecan but not to camptothecin. MCF7/TPT50 cells were also slightly cross-resistant to doxorubicin and VP-16 but not to 5-FU or paclitaxel. When MCF7/TPT50 cells were selected further in higher concentrations of topotecan, the resulting MCF7/TPT300 cells were highly resistant to topotecan (68.9-fold), SN-38 (68.3-fold), and mitoxantrone (116-fold), whereas there was only mild cross-resistance to camptothecin (4.1-fold).

Topotecan Accumulation and Efflux

Representative topotecan efflux curves of MCF7/WT, MCF7/MX, and MCF7/TPT cells are shown in Fig. 1. Time points 0 of Fig. 1A show topotecan accumulation in MCF7/WT, MCF7/MX, MCF7/TPT50, and MCF7/TPT300 cells, respectively. The accumulation of topotecan by MCF7/WT cells was 8-fold higher than the baseline fluorescence, whereas that of MCF7/MX and MCF7/TPT300 cells was only 2.5-fold higher. During topotecan efflux, MCF7/WT cells loaded with topotecan retained the drug for up to 10 min (Fig. 1A). In contrast, there was no detectable topotecan remaining in MCF7/MX or MCF7/TPT300 cells even at the first time point (1 min, Fig. 1A). The efflux curve from MCF7/TPT50 cells fell between those of MCF7/WT and MCF7/TPT300 cells.

ATP Depletion in MCF7 Cells

An energy-dependent efflux pump was postulated to account for the enhanced topotecan efflux in MCF7/TPT300 cells. As shown in Fig. 1B, when MCF7/TPT300 cells were depleted of ATP by incubating cells in glucose-free solution containing 15 mM sodium azide, the topotecan efflux was inhibited profoundly. The topotecan efflux curve of MCF7/TPT300 cells after ATP depletion was similar to that of MCF7/WT cells without ATP depletion.

P-glycoprotein, MRP1, and BCRP/MXR/ABCP Expression

Several ATP-binding cassette family proteins have been shown to enhance drug efflux and confer resistance to chemotherapeutic agents. In the present study, expression of P-glycoprotein, MRP1, and the recently described BCRP/MXR/ABCP was examined in MCF/TPT cells using RT–PCR and northern and western blots, respectively. As shown in Fig. 2A, MDR1 mRNA was not detected in MCF7/WT or MCF/TPT300 cells, whereas the P-glycoprotein-overexpressing MCF7/ADR cells used as a control gave a clear band. Also not detectable in MCF/WT and MCF7/TPT300 cells was MRP1 mRNA, as shown in Fig. 2B, in contrast to the MRP1-overexpressing MCF7/VP cells used as a positive control. In contrast, Fig. 2B shows that BCRP/MXR/ABCP mRNA was highly overexpressed

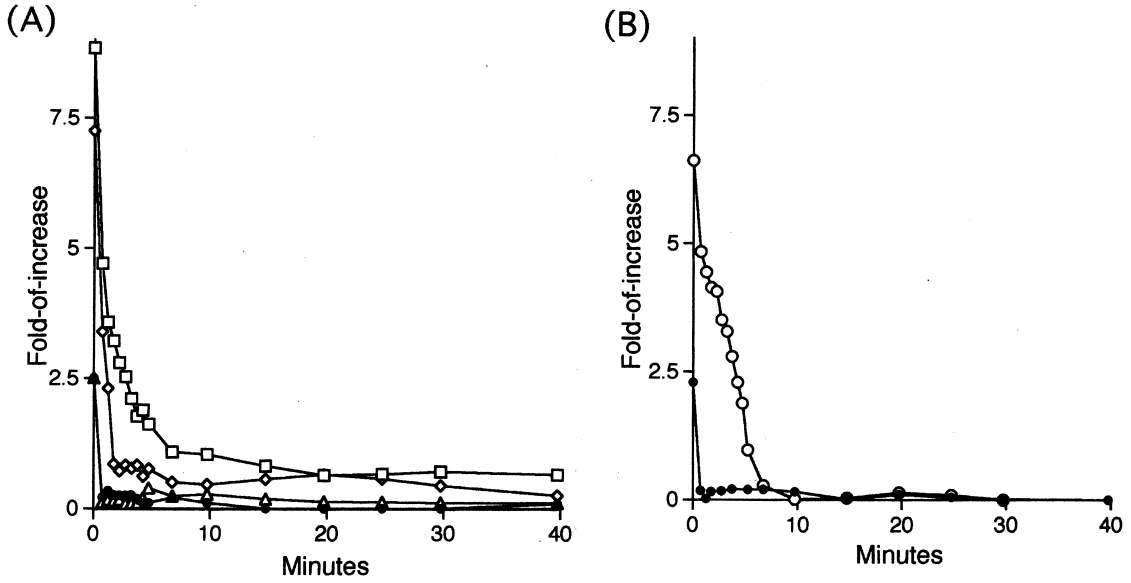

FIG. 1. Representative topotecan efflux curves from at least three independent experiments. (A) Topotecan efflux curves in MCF7/WT(□), MCF7/MX(Δ), MCF7/TPT50 (◇), and MCF7/TPT300 cells (●). Cells were loaded with 50 μM topotecan, and topotecan efflux was measured from time 0. The ordinate shows intracellular topotecan levels determined by fold-of-increase of basal FL-2 units in flow cytometry. Note that topotecan efflux in MCF7/TPT300 and MCF7/MX cells was much faster than that of MCF7/WT cells. (B) Effect of ATP depletion on topotecan efflux in MCF7/TPT300 cells (○) as compared with control MCF7/TPT300 cells (●).

in MCF/MX cells and weakly overexpressed in MCF/TPT300 cells (less than 5% of the level in MCF7/MX cells). None of the other cells, including MCF/TPT50, exhibited detectable BCRP/MXR/ABCP mRNA expression. The expression of BCRP/MXR/ABCP in these drug-sensitive and -resistant cells was confirmed by a western blot (Fig. 2C).

Topoisomerase I and II Expression

Figure 3A shows DNA topoisomerase I protein levels in MCF7/WT, MCF7/TPT50, and MCF7/300 cells. Topoisomerase I was down-regulated only slightly in MCF7/TPT50 cells, but was reduced markedly in MCF7/TPT300 cells. In contrast, DNA topoisomerase II levels were similar in these cell lines (Fig. 3B).

DISCUSSION

DNA topoisomerase I inhibitors are a new class of chemotherapeutic agents that have shown promising anticancer activity. However, both intrinsic resistance and acquired resistance to topoisomerase I inhibitors are frequently observed in patients treated with topotecan or CPT-11. Acquired camptothecin resistance in tissue culture cells has been described in the literature. Mutation of DNA topoisomerase I and down-regulation of topoisomerase I in resistant cancer cells are observed most frequently under these experimental conditions [4]. However, topoisomerase I alterations have not been associated with clinical camptothecin resistance [10].

There are only a few studies that have used topotecan to select for resistance in cancer cells. For instance, Sorensen *et al.* selected small cell lung cancer cells in topotecan. The resulting NYH/TPT cells were 6-fold resistant against topotecan compared with their drug-sensitive parental cells. Down-regulation of topoisomerase I and up-regulation of topoisomerase II were found in these cells. There was only minor cross-resistance to other chemotherapeutic agents [11]. Decreased drug accumulation was associated with resistance to topotecan (29-fold) and SN-38 (51-fold) in a topotecan-resistant ovarian cancer cell line IGROV(T100r). IGROV(T100r) cells were also 32-fold cross-resistant to mitoxantrone, but only 3-fold resistant to camptothecin [12].

Cross-resistance to topotecan was observed in P-glycoprotein-overexpressing cells. Hendricks *et al.* demonstrated that P-glycoprotein-positive, colchicine-resistant CHRC5 cells were 3.2-fold resistant to topotecan compared with parental P-glycoprotein-negative AuxB1 Chinese hamster ovary cells. Topotecan accumulation was reduced in these P-glycoprotein-expressing resistant cells [3]. Chen *et al.* [13] showed that topotecan cytotoxicity was reduced in P-glycoprotein-expressing KB V1 cells, whereas camptothecin toxicity was not affected.

The mechanism of topotecan resistance in MCF7/TPT cells described in this report seems to be different from classical camptothecin resistance in that MCF7/TPT cells were only slightly cross-resistant to the DNA topoisomerase I inhibitor camptothecin. In addition, mitoxantrone cross-resistance has not been described in classical camptothecin resistance. Rapid efflux of topotecan was noted in MCF7/TPT300 cells but not in MCF7/WT cells. ATP depletion inhibited topotecan efflux profoundly in MCF7/TPT300

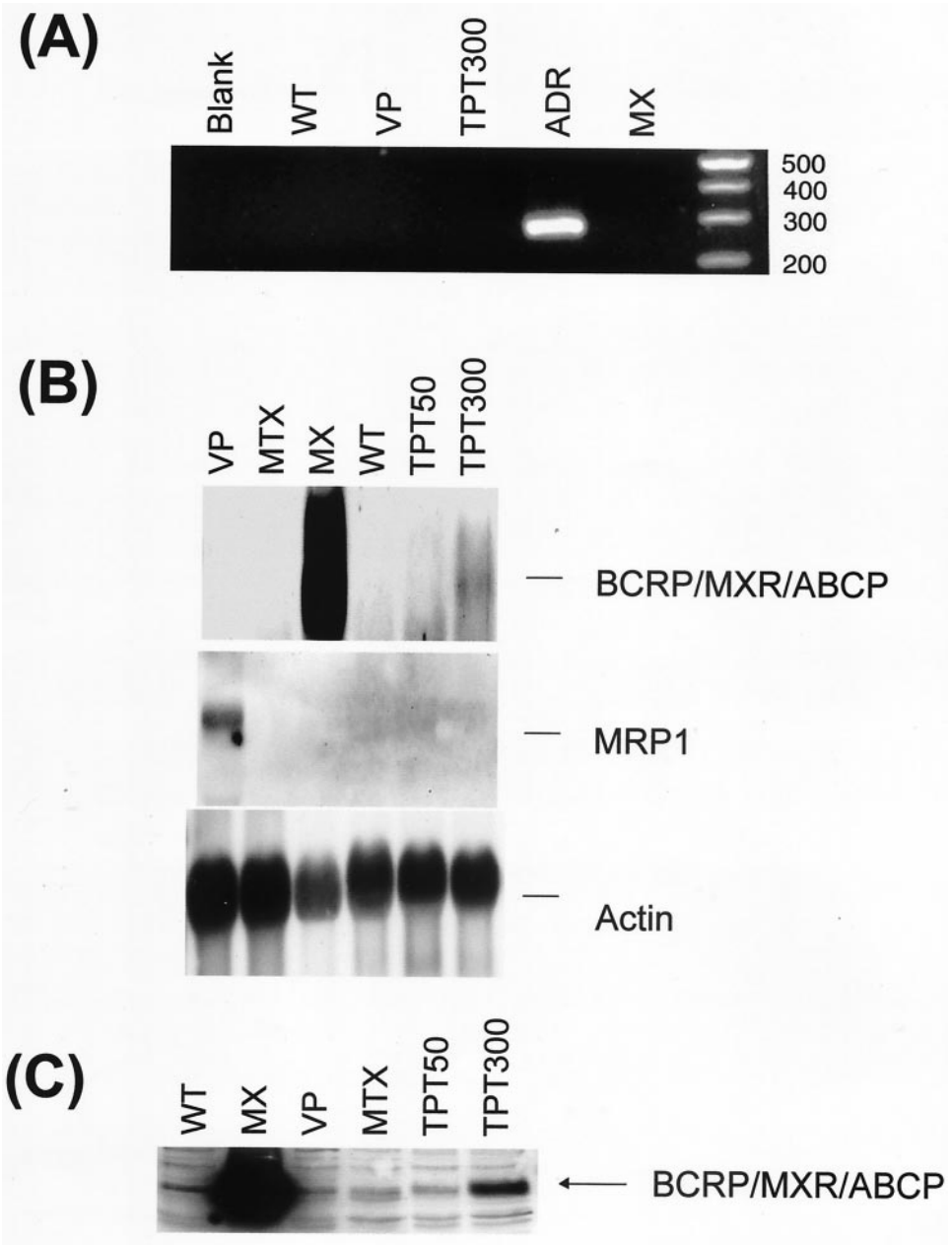

FIG. 2. (A) RT–PCR for MDR-1 mRNA expression in drug-sensitive and -resistant MCF7 cells. The size of the polymerase chain reaction (PCR) product was 286 bp. (B) Northern blots of BCRP/MXR/ABCP, MRP1, and actin control in drug-sensitive and -resistant MCF7 cells. (C) Western blot of BCRP/MXR/ABCP in drug-sensitive and -resistant MCF7 cells. Experiments were performed at least three times.

cells. These results suggested that an energy-dependent enhanced topotecan efflux is one of the main mechanisms of topotecan resistance in MCF7/TPT300 cells. However, this mechanism is not due to P-glycoprotein or MRP1 because neither of these genes/proteins is overexpressed in MCF7/TPT300 cells.

Several non-P-glycoprotein-expressing multidrug-resistant cells exhibit phenotypes similar to MCF7/TPT300 cells. For example, EPG85–257RNOV cells were selected from parental EPG85–257P gastric cancer cells in mitoxantrone. These cells were 7056-fold resistant to mitoxantrone, 58-fold resistant to topotecan, and 332-fold resistant to SN-38, but only 2-fold resistant to camptothecin. EPG85–257RNOV cells accumulated less mitoxantrone than their parental cells [14]. MCF7/AdVp cells, selected from MCF7 cells in doxorubicin and the P-glycoprotein reversing agent verapamil, were highly cross-resistant to mitoxantrone (100,000-fold) [15].

Recently, the breast cancer resistance protein (BCRP, also called MXR or ABCP) was described independently by three groups [16–18]. BCRP/MXR/ABCP is a half-size ATP-binding cassette protein that confers resistance to mitoxantrone and anthracyclines. Although BCRP/MXR/ABCP was overexpressed in some mitoxantrone-resistant cell lines that were cross-resistant to topotecan (MCF7/MX, MCF7/AdVp, and EPG85–257RNOV), resistance to topotecan in BCRP/MXR/ABCP-transfected cells has not been demonstrated [19]. Two topotecan-selected cancer cell lines were found to overexpress BCRP/MXR/ABCP. The mouse equivalent of BCRP/MXR/ABCP was overex-

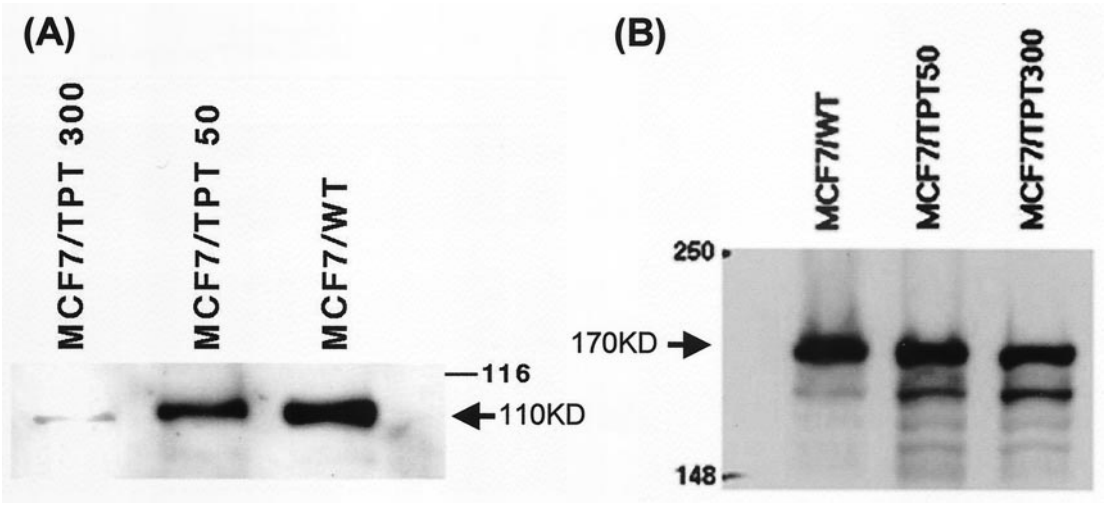

FIG. 3. Western blot of topoisomerase I (A) and II (B) in drug-sensitive and -resistant MCF7 cells. Numbers denote size of markers in kDa. Experiments were repeated three times.

pressed in topotecan-selected mouse fibroblast cells that lack functional *Mdr1a*, *Mdr1b*, and *Mrp1* genes [20]. Overexpression of BCRP/MXR/ABCP was also noted in a topotecan-selected human ovarian cell line, IGROV1 [21].

Despite the similarity of the cross-resistance phenotype between MCF7/TPT300 cells and other mitoxantrone-selected cells, MCF7/TPT300 cells only weakly overexpressed BCRP/MXR/ABCP, and MCF7/TPT50 cells did not express it at all. However, unlike the situation in MCF7/MX cells [6], topoisomerase I was down-regulated in MCF7/TPT300 cells. This may contribute to their topotecan resistance and also explain the 4.1-fold cross-resistance to camptothecin. We have shown clearly that topotecan accumulation and efflux were altered markedly in MCF7/TPT300 cells. The increased topotecan efflux in MCF7/TPT300 cells was energy-dependent. Therefore, it is possible that the relatively small amount of BCRP/MXR/ABCP overexpression in MCF7/TPT300 cells is sufficient to confer topotecan resistance. However, other possibilities should be considered. Since BCRP/MXR/ABCP is only half the size of a classical ATP-binding protein, containing only one ATP binding region, an undescribed partner to form a heterodimer has been suggested [19]. Since we were able to detect reduced topotecan efflux in MCF7/TPT50 cells despite a BCRP/MXR/ABCP level that was no different from that of MCF7/WT cells, it is possible that MCF7/TPT cells contain an additional efflux protein that may interact with BCRP/MXR/ABCP and contribute to topotecan efflux.

In summary, we demonstrated that chronic exposure to increasing concentrations of topotecan may have induced energy-dependent enhanced topotecan efflux in human breast cancer cells. This defect may contribute to acquired topotecan resistance. Exposure to topotecan was associated with cross-resistance to CPT-11 and mitoxantrone, but not to camptothecin. It is unclear at present whether the small overexpression of the recently described BCRP/MXR/ABCP completely accounts for topotecan resistance in MCF7/TPT300 cells. Understanding the mechanism of topotecan resistance and its expression in cancer and normal cells may be useful in designing clinical trials using reversing agents to augment the cytotoxic effects of topotecan, CPT-11, or mitoxantrone.

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