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*Advances in Brief*

# Overexpression of the *BCRP/MXR/ABCP* Gene in a Topotecan-selected Ovarian Tumor Cell Line[1]

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## Abstract

**Topotecan- or mitoxantrone-selected cell lines (T8 and MX3, respectively), derived from the human IGROV1 ovarian cancer cell line, were resistant to the topoisomerase I inhibitors topotecan, SN-38 (the active metabolite of irinotecan), and 9-aminocamptothecin, as well as to the topoisomerase II drug mitoxantrone. In both resistant cell lines, decreased accumulation of topotecan and mitoxantrone was observed, caused by enhanced energy-dependent efflux of the drugs involved. In both cell lines, we found that the *breast cancer resistance protein/mitoxantrone resistance/placenta-specific ATP binding cassette* (*BCRP/MXR/ABCP*) gene was overexpressed. Furthermore, *BCRP/MXR/ABCP* expression levels in various partially revertant T8 cells correlated with the levels of resistance to topotecan, SN-38, and mitoxantrone, strongly suggesting *BCRP/MXR/ABCP* to be the transporter responsible for the enhanced efflux. Pharmacodynamic analysis demonstrated that BCRP/MXR/ABCP is a very efficient transporter of topotecan; *in vitro*, 70% of the intracellular topotecan pool was transported out of the T8 or MX3 cells within 30 s. In conclusion, we report for the first time that *BCRP/MXR/ABCP* can also be upregulated upon exposure of tumor cells to the clinically important drug topotecan, and that BCRP-mediated efflux of topotecan is very efficient. This highly efficient efflux of topotecan by BCRP/MXR/ABCP may have clinical relevance for patients being treated with topotecan.**

## Introduction

Resistance to chemotherapeutic agents is a major clinical complication in cancer therapy, in many cases resulting in treatment failure. Various members of the ABC[3] protein family, *i.e.*, P-gp and MRP1, have been described to be capable of transporting drugs out of the cells and are involved in *in vitro* resistance (1, 2). More recently, other MRP analogues, *i.e.*, MRP2–MRP6, have been described (3). MRP2 is also referred to as cMOAT, and is involved in biliary excretion of irinotecan (CPT-11) and its active metabolite SN-38 (4). The functions of the other MRPs are unclear at this moment.

Drug transporters, other than P-gp or the MRP family, may be important for resistance. Recently, the *BCRP/MXR/ABCP* gene, a member of the ABC transporter family, has been described in breast, colon, gastric cancer, and fibrosarcoma cell lines (5–8). Overexpression of *BCRP/MXR/ABCP*, caused by exposure of the cells to MX or doxorubicin/verapamil, resulted in a resistance pattern that was different from that generally observed in the cases of P-gp or MRP1 overexpression. Cells were resistant to MX, doxorubicin, and daunorubicin but lacked resistance to paclitaxel and vincristine (5). Overexpression of *BCRP/MXR/ABCP* has now been demonstrated in a number of MX-selected tumor cell lines (6, 7). In some MX-selected *BCRP/MXR/ABCP*-overexpressing cell lines, cross-resistance to TPT has been reported (6, 9).

In this report, we demonstrate for the first time that overexpression of *BCRP/MXR/ABCP* in human tumor cells cannot only be invoked by exposure to MX but also by exposure to the clinically important drug TPT. Cells selected with TPT or MX from the human IGROV1 ovarian cancer cell line were resistant to TPT, as well as to the analogues SN-38 and 9-AC, with concomitant overexpression of *BCRP/MXR/ABCP*. Furthermore, *BCRP/MXR/ABCP* expression levels correlated with the levels of resistance in various partially revertant cell lines. BCRP/MXR/ABCP appears to be a highly efficient, energy-dependent transporter of TPT and MX in these resistant cells.

## Materials and Methods

**Development of T8 and MX3 Cell Lines.** The TPT-resistant T8 and MX-resistant MX3 cell lines were developed from the IGROV1 human ovarian carcinoma cell line (10). All cell lines were cultured in RPMI 1640 supplemented with 25 mM HEPES, 10% BCS, 110 IU/ml penicillin, and 100 $\mu$g/ml streptomycin at 37°C in a humidified atmosphere with 5% $CO_2$. The T8 cell line was developed by continuous exposure to stepwise increased TPT concentrations, ranging from 24 to 240 nM. After treatment at this concentration, one colony was picked. These cells were further exposed to stepwise increased concentrations of TPT, up to 950 nM TPT. From this time point on, the cells were exposed to this concentration of drug weekly for 1 h. Under these conditions, resistance in the T8 remained unchanged for at least 25 weeks. A MX-resistant cell line was developed by exposing IGROV1 cells to 170 nM MX for 72 h. After this treatment, a small number of colonies survived. One of the colonies that was picked and subsequently cultured was denoted MX3. This cell line is exposed weekly to 340 nM MX for 1 h and is stable for at least 25 weeks under these conditions. The relative cell volume of the IGROV1:T8:MX3 cells was 1.00:1.29:1.11.

**Cross-Resistance Pattern.** Cytotoxicity of antitumor drugs was assessed using the sulforhodamine B assay (11). The number of cells plated was chosen in such a way that cells did not reach confluence during the time of assay. Each experiment was carried out in quadruplicate and repeated at least three times.

**topo I and II Catalytic Activity.** topo I catalytic activities were determined using the relaxation of pBR322 DNA by nuclear extracts (serial dilutions from 47 to 1.4 ng) added to the reaction mixture (12). For this purpose, nuclear extracts were isolated from cells in logarithmic growth, as described by others (13). topo II catalytic activity was determined by assessing the decatenation of kinetoplast DNA by nuclear extracts of the cells (14).

**Protein and Expression Levels of topo I, II$\alpha$, II$\beta$, P-gp, and MRP1 to MRP6.** topo I and II$\alpha$ protein levels were detected with a human polyclonal topo I antibody and a mouse monoclonal antibody against topo II$\alpha$, respectively (Topogen, Columbus, Ohio). topo II$\beta$ was detected with a rabbit polyclonal antibody against topo II$\beta$ (Biotrend Chemikalien, Cologne, Germany). P-gp was detected using the C219 antibody (Centocor, Leiden, the Netherlands), MRP1 was hybridized with MRPr1 monoclonal antibody, and

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[3] The abbreviations used are: ABC, ATP-binding cassette; ABCP, placenta-specific ABC transporter; 9-AC, 9-aminocamptothecin; BCRP, breast cancer resistance protein; $IC_{50}$, 50% inhibitory concentration; MRP, multidrug resistance protein; MX, mitoxantrone; MXR, mitoxantrone resistance; P-gp, P-glycoprotein; TPT, topotecan; topo, topoisomerase.

Table 1 *$IC_{50}$s of a panel of antitumor drugs in the IGROV1, T8, and MX3 cell lines, as assessed using the sulforhodamine B method* [a]

| | | T8 | | | MX3 | | |
|---|---|---|---|---|---|---|---|
| | IGROV1 $IC_{50}$ (nM) | $IC_{50}$ (nM) | Rf[b] | *P* | $IC_{50}$ (nM) | Rf | *P* |
| Topotecan | 12.6 | 659 | 52 | <0.0005 | 171 | 14 | <0.0005 |
| 9-AC | 2.64 | 207 | 79 | <0.0005 | 42.1 | 16 | <0.0005 |
| SN-38 | 1.95 | 344 | 176 | <0.0005 | 86.2 | 44 | <0.0005 |
| Camptothecin | 3.15 | 13.4 | 4.3 | <0.0005 | 5.88 | 1.9 | 0.06 |
| Mitoxantrone | 30.4 | 319 | 11 | <0.0005 | 322 | 11 | <0.0005 |
| Cisplatin | 547 | 776 | 1.4 | 0.2 | 374 | 0.7 | 0.2 |
| 5-Fluorouracil | 1754 | 3199 | 1.8 | 0.2 | 1668 | 1.0 | 0.9 |
| Paclitaxel | 1.32 | 2.41 | 1.8 | 0.06 | 1.92 | 1.5 | 0.2 |
| Doxorubicin | 88.2 | 46.6 | 0.5 | 0.2 | 78.7 | 0.9 | 0.7 |

[a] $IC_{50}$s shown are means from at least three independent experiments.
[b] Rf, resistance factor.

MRP2 was hybridized with a mouse monoclonal antibody $M_2$III-6 (15). After applying the appropriate secondary peroxidase-linked secondary antibodies, proteins were visualized with the ECL blotting detection reagents (Amersham Life Science, 's Hertogenbosch, the Netherlands). Expression levels of MRP2 to MRP6 were determined using RNase protection, as described by others (3).

**Accumulation and Efflux of Topotecan.** Accumulation of TPT in the IGROV1, T8, and MX3 cells was monitored using a sensitive HPLC assay as described by Rosing *et al.* (16). Exponentially growing cells were exposed to 0, 0.95, 1.90, 19.0, or 95.0 μM TPT for 30 min at 37°C. After this incubation period, flasks were processed, and intracellular TPT levels were determined as described previously (17). Protein concentrations were determined using the Bradford method. For efflux studies, IGROV1, T8, and MX3 cells were loaded with 1.90, 5.70, and 5.70 nM TPT, respectively, for 30 min at 37°C to obtain approximately equal intracellular concentrations of TPT. After loading the cells, medium was removed and replaced by fresh medium. Directly after incubation and at several time points after ending incubation, intracellular concentrations of TPT were determined. Accumulation and efflux of TPT were determined in at least three independent experiments. To investigate accumulation under energy-deprived conditions, the medium of cells in exponential growth was changed with RPMI 1640, in which glucose was replaced by 2-deoxy-D-glucose, and to which 10 mM sodium azide was added (18), 15 min before loading the cells with TPT. Intracellular ATP levels, which were measured using the luciferase/luciferine assay (19), were decreased to ~10% after this 15-min exposure to glucose-free medium and decreased to ~5% in the next 30 min (data not shown). Under energy-deprived conditions, IGROV1, T8, and MX3 cells were exposed to 1.90 μM TPT for 30 min to obtain approximately equal intracellular concentrations of TPT in all cell lines. After loading the cells, medium was replaced by fresh energy-deprived medium.

**Accumulation and Efflux of Mitoxantrone.** MX accumulation in the IGROV1, T8, and MX3 cells was monitored by flow cytometry, using an excitation wavelength of 633 nm, whereas emission was measured using a

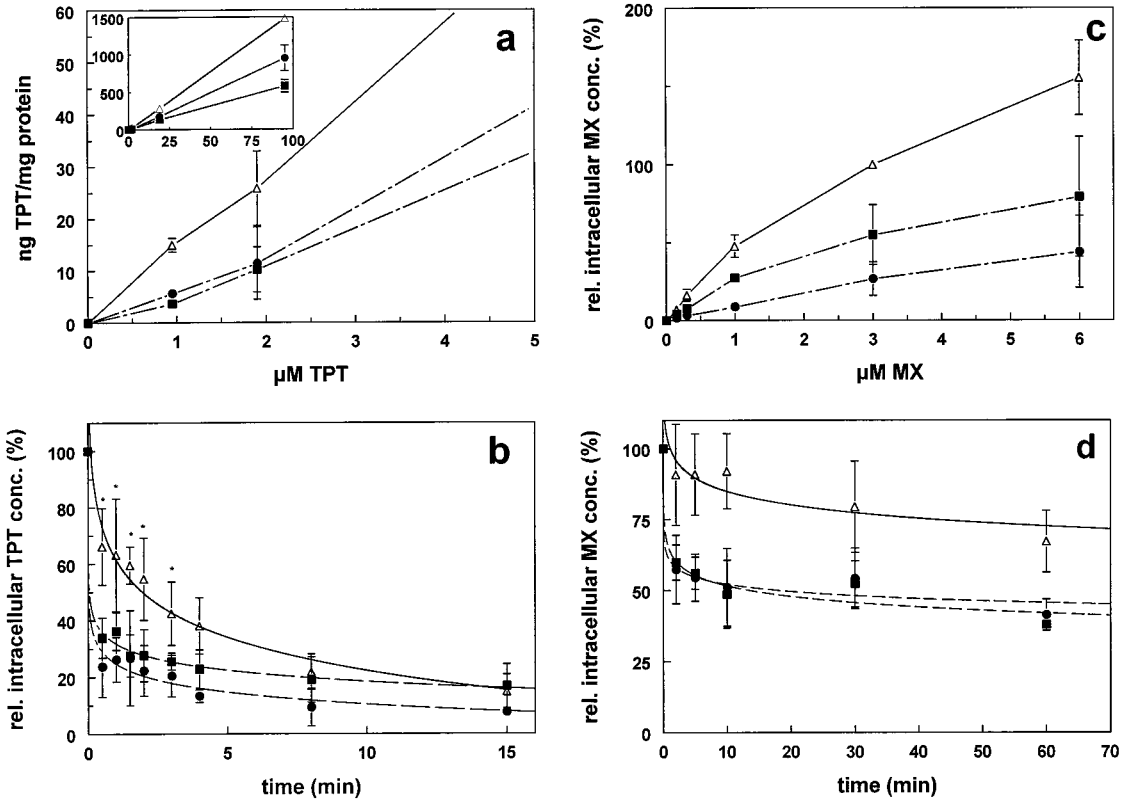


Fig. 1. *a*, accumulation of TPT in IGROV1 (△), T8 (■), and MX3 (●) cells after loading of the cells for 30 min using 0, 0.95, 1.90, 19.0, or 95.0 (*inset*) μM TPT for 30 min at 37°C. Data are means from at least three experiments; *bars*, SD. *b*, efflux of TPT from IGROV1, T8, and MX3 cells after loading of the cells using 1.90, 5.70, and 5.70 μM TPT, respectively, for 30 min at 37°C. Data shown are means from at least four experiments for the time points up to 3 min and at least three for time points from 4 to 15 min; *bars*, SD. Efflux data are fitted plotted logarithmically. ∗, $P < 0.01$ (Student's *t* test). *c*, relative accumulation of MX in IGROV1, T8, and MX3 cells after loading of the cells with 0.15, 0.3, 1.0, 3.0, and 6.0 μM MX for 60 min at 37°C. After processing the samples, intracellular concentrations of MX were determined using flow cytometry. Accumulation in IGROV1 cells, loaded with 3.0 μM MX, was arbitrarily taken as 100%; *bars*, SD. *d*, efflux of MX from IGROV1, T8, and MX3 cells after loading of the cells using 3.0, 6.0, and 6.0 μM MX, respectively, for 60 min at 37°C. Data shown are means from at least three experiments; *bars*, SD. Efflux data are fitted plotted logarithmically.

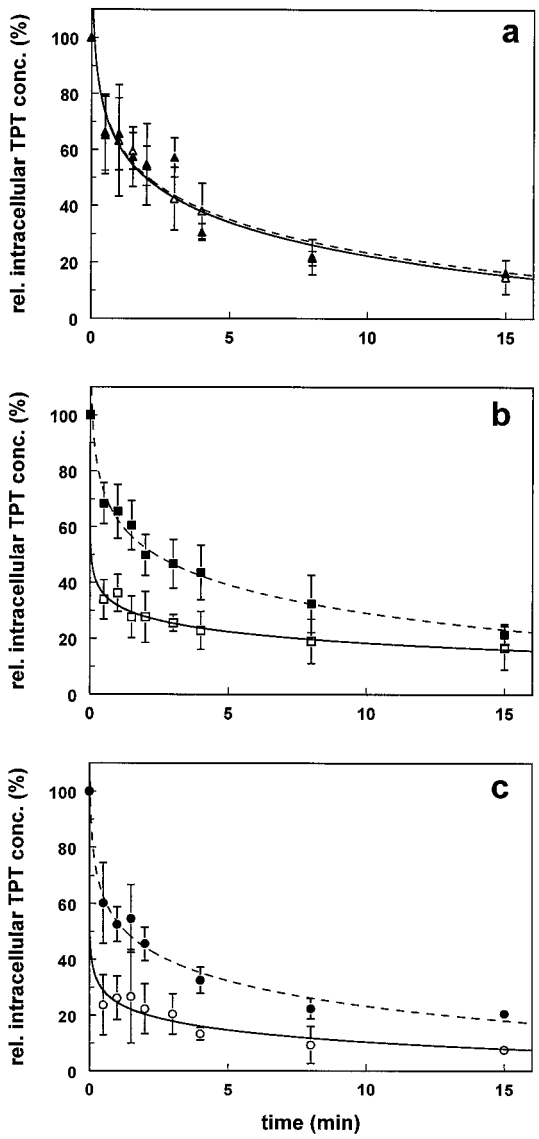


Fig. 2. Efflux of TPT under energy-proficient (△, □, ○) or energy-deprived (▲, ■, ●) conditions. Cells were loaded with 1.90 μM TPT for 30 min at 37°C. Subsequently, efflux was determined as described in "Materials and Methods." Efflux data are fitted logarithmically. *a*, IGROV1 cells (△, ▲); *b*, T8 cells (□, ■); *c*, MX3 cells (○, ●). Data shown are means from at least three experiments; *bars*, SD.

661-nm filter. In these experiments, exponentially growing cells were scraped and resuspended in RPMI 1640 ($2 \times 10^6$ cells/tube). These cells were loaded with 0, 0.15, 0.3, 1.0, 3.0, or 6.0 μM MX for 60 min at 37°C. After this incubation, cell suspensions were put on ice, cells were spun down (1100 rpm for 5 min at 4°C), washed with ice-cold PBS, and put on ice until measurement of intracellular MX concentrations. To examine efflux of MX from the IGROV1, T8, and MX3 lines, cells were loaded with 3.0, 6.0, and 6.0 μM MX, resulting in approximately equal intracellular concentrations of MX. After replacing medium, cells were incubated for another 15, 30, or 60 min in drug-free medium at 37°C. At these time points, tubes were put on ice, and samples were processed as described above for the accumulation experiments.

***BCRP/MXR/ABCP* Probe.** cDNA was generated from poly(A)$^+$ RNA obtained from a human testis/colon biopsy. A probe for *BCRP/MXR/ABCP* was generated by PCR amplification of this cDNA. The following primers were used: 5′-AGACTTATGTTCCACGGGCC-3′ (forward primer); and 5′-CAAGGCCACGTGATTCTTCC-3′ (backward primer). The expected PCR product was 1113 bp in length. PCR was performed, starting by heating the sample at 95°C for 3 min, followed by 35 cycles of 30 s at 94°C, 1 min at 60°C, and 2 min at 72°C. The probe was purified by agarose gel electrophoresis and labeled with [$^{32}$P]dCTP by random labeling (Rediprime II; Amersham Pharmacia Biotech).

**Northern Blotting *BCRP/MXR/ABCP*.** Total RNA was prepared using Trizol reagent, according to the manufacturer's instructions. Twenty μg of total RNA were fractionated on a 1% agarose-formaldehyde gel and subsequently transferred to nitrocellulose Hybond-N$^+$. Blots were prehybridized for 1 h at 42°C in 5× SSC (1× SSC = 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0), 5× Denhardt's solution, 0.2% SDS, 100 μg/ml salmon sperm DNA, and 50% deionized formamide. Subsequently, the blots were probed using 25 ng of the $^{32}$P-labeled *BCRP/MXR/ABCP* probe at 42°C overnight. After washing in 1× SSC/0.1% SDS for 20 min at room temperature and three times with a 10-min wash with 0.2× SSC/0.1% SDS at 65°C, blots were analyzed using a phosphor imaging system (Fujix Bas 2000).

## Results

The T8 and MX3 ovarian cancer cell lines were selected by exposure to TPT or MX, respectively. Both cell lines displayed a pronounced resistance against the topoisomerase I drugs TPT, SN-38, and 9-AC (Table 1). However, resistance to the parent topo I inhibitor camptothecin was much less pronounced. A marked cross-resistance to MX was observed in both cell lines. The T8 and MX3 cell lines were not or hardly cross-resistant to cisplatin, 5-fluorouracil, paclitaxel, and doxorubicin.

topo I and II activities in the IGROV1, T8, and MX3 cells were equal, and no differences in topo I protein levels were observed. topo IIα and IIβ protein levels in the IGROV1 and T8 cell lines were equal, whereas in the MX3 cells, levels of both isoforms were slightly decreased by ~25% (not shown). No significant overexpression of multidrug resistance-associated pumps P-gp and MRP1, nor of the putative transporters MRP3, MRP4, MRP5, or MRP6, was observed in the T8 and MX3 cells (not shown). MRP2 protein and mRNA levels were increased 1.5- and 3-fold in the T8 and MX3 cell lines,

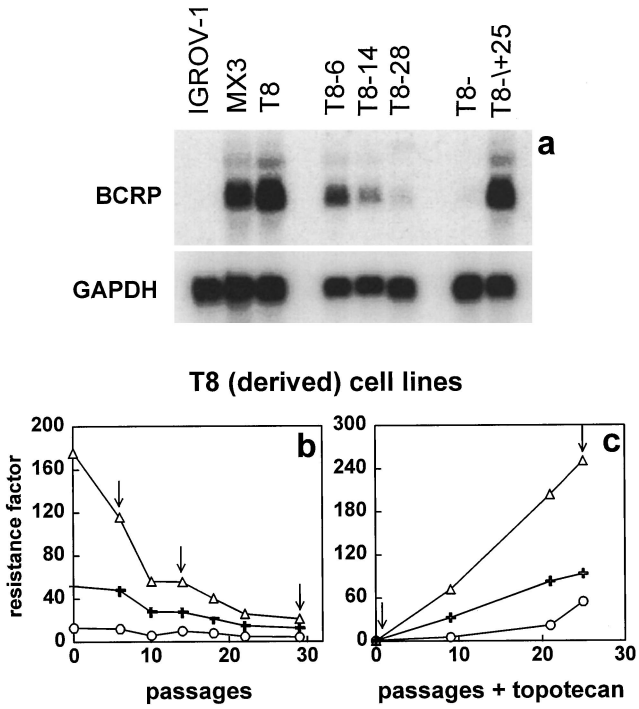


Fig. 3. *a*, Northern blot hybridization of *BCRP/MXR/ABCP* mRNA in the IGROV1, T8, and MX3 cell lines. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. Also shown are hybridizations with mRNA from T8 revertant cell lines. *b*, levels of resistance to SN-38 (△), TPT (✚), and MX (○) in the T8 and revertants, derived from this cell line. *c*, levels of resistance to SN-38 (△), TPT (✚), and MX (○) in a re-exposed complete revertant T8. The cells were (re-)exposed to 950 nM TPT for 1 h/week. mRNA was isolated and probed from passages indicated with an *arrow*.

respectively. However, cellular localization of MRP2, as detected by immunocytochemical staining, showed that MRP2 was located diffusely throughout the cell, whereas using confocal laser scanning microscopy, no vacuolar localization of MX or TPT was observed in the T8 and MX3 cells (not shown).

A 4–5-fold reduction of TPT accumulation was observed in the T8 and MX3 cells (Fig. 1*a*). The accumulation of TPT could not be saturated, even when using high doses (95 $\mu$M; Fig. 1*a*, *inset*). A significantly increased initial efflux rate of TPT was observed in the T8 and MX3 lines, as compared with the efflux rates in IGROV1 (Fig. 1*b*). This initial efflux of TPT in the T8 and MX3 cell line appeared to be very efficient: within 30 s, 70–80% of the intracellular TPT content was transported out of the cell. MX accumulation was also decreased in the T8 and MX3 cells (Fig. 1*c*); this was caused by enhanced efflux of MX from the resistant cells (Fig. 1*d*).

The efflux rate of TPT in the IGROV1 cells under energy-proficient conditions was not different from that under energy-deficient conditions, whereas in the T8 and MX3 cells, initial efflux rates of TPT decreased markedly under energy-deficient conditions (Fig. 2). Notably, accumulation of TPT was not affected by known inhibitors of P-gp or MRP1, *i.e.*, verapamil, D, L-buthionine-5, R-sulfoximine (BSO), and cyclosporin A, nor by the $Na^+/K^+$ ATPase inhibitor ouabain (not shown). The efflux of MX in the T8 and MX3 cell lines was also energy dependent (not shown).

Northern blotting revealed that *BCRP/MXR/ABCP* mRNA was undetectable in the IGROV1 cells, whereas very substantial levels were observed in the T8 and MX3 cells (Fig. 3*a*). *BCRP/MXR/ABCP* mRNA levels in the T8 cells were ~2–3-fold higher than in the MX3 cells. Furthermore, expression levels of *BCRP/MXR/ABCP* in various revertant T8 cells were qualitatively correlated to the level of resistance (Fig. 3*b*), and a re-exposed complete revertant T8, with again increased resistance (Fig. 3*c*), showed increased *BCRP/MXR/ABCP* mRNA levels (Fig. 3*a*).

## Discussion

Resistance to cytostatic agents is a major clinical problem. The involvement of multidrug transporters like P-gp and MRP1 in development of resistance is well documented *in vitro* (1, 2). Recently, *BCRP/MXR/ABCP*, another member of the ABC family of transporter proteins, has been reported to be important in the development of resistance. It was overexpressed in a number of cell lines, which were selected by exposure to the topo II inhibitor MX (6). The MX3 cell line described in this report was also developed using MX exposure. Cross-resistance to TPT in MX-selected *BCRP/MXR/ABCP*-overexpressing cell lines has been reported (6, 9). However, in this report, we demonstrate that *BCRP/MXR/ABCP* overexpression and related resistance can also be caused directly by exposure of tumor cells to TPT, as in the TPT-exposed T8 cell line, pronounced overexpression of *BCRP/MXR/ABCP* was observed.

The T8 and MX3 cell lines display a similar pattern of resistance, *i.e.*, to topo I inhibitors TPT, SN-38, and 9-AC, and to the topo II inhibitor MX. However, resistance to the parent topo I inhibitor camptothecin is limited or absent in the T8 and MX3 cells, respectively. Resistance in the T8 and MX3 cells could not be explained by topo I- or II-related factors. The probable cause of resistance in the T8 and MX3 cell lines is the markedly decreased accumulation of the drugs involved. Reduced accumulation appeared to be caused by enhanced efflux, which occurred by an energy-dependent process. This enhanced efflux was not caused by any of the (putative) multidrug transporters P-gp or MRP1 to MRP6. Although MRP2 was overexpressed in T8 and MX3, this protein is not believed to be important, because it is not localized in the membrane, whereas vacuolar localization of TPT and MX in the resistant cells does not occur.

*BCRP/MXR/ABCP* expression is, therefore, likely to be responsible for this enhanced efflux. The involvement of *BCRP/MXR/ABCP* is strongly supported by two findings: (*a*) *BCRP/MXR/ABCP* is overexpressed both in the T8 and MX3 cells; and (*b*) *BCRP/MXR/ABCP* expression levels in various partially revertant T8 cells correlate with the level of resistance to TPT, SN-38 and MX. In this report, we demonstrated that BCRP/MXR/ABCP is a very efficient transporter of TPT. Under our experimental conditions, ~70% of the intracellular TPT was transported out of the cell within the first 30 s.

Notably, in contrast to other *BCRP/MXR/ABCP*-overexpressing cell lines (6, 7), in the T8 and MX3 cells no resistance to doxorubicin was observed. This may be attributable to cell type-specific features. Interestingly, BCRP/MXR/ABCP is a half-transporter that may form homo- or heterodimers. If heterodimers are important, cell type-specific differences in the levels of the partner proteins will probably affect specific transport. We cannot formally rule out the possibility that a protein, highly related to BCRP/MXR/ABCP, is overexpressed in the T8 and MX3 cell lines and responsible for the observed resistance pattern. Expressed sequence tags for *bcrp2*, related to *bcrp1*, are present in mice databases, but homology is probably too low to cause cross-hybridization on a Northern blot under our stringent washing conditions. Nevertheless, the importance of a human homologue of this *bcrp2* gene, as well as possible partners for *BCRP/MXR/ABCP*, is presently under investigation in our laboratory.

In conclusion, *BCRP/MXR/ABCP* mRNA levels in the human IGROV1 ovarian cancer cell line can be up-regulated by exposure of the cells to TPT. Resistance to topo I inhibitors and MX in the T8 and MX3 cell lines is caused by enhanced energy-dependent efflux, mediated by BCRP/MXR/ABCP. BCRP/MXR/ABCP appears to be a very efficient transporter of TPT, and levels of expression may therefore have pronounced effects on sensitivity of tumors. Because of the efficient transport of TPT by BCRP/MXR/ABCP, this finding may be clinically relevant in development of drug resistance, and investigations aimed at this clinical relevance of *BCRP/MXR/ABCP* for TPT-treated patients have been initiated at our laboratory.

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