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DECREASED DRUG ACCUMULATION IN A MITOXANTRONE-RESISTANT GASTRIC CARCINOMA CELL LINE IN THE ABSENCE OF P-GLYCOPROTEIN

Udo KELLNER[1]*, Lesley HUTCHINSON[2], André SEIDEL[1], Hermann LAGE[3], Mary K. DANKS[4], Manfred DIETEL[3] and Scott H. KAUFMANN[5]

[1]*Institute for General Pathology, Christian-Albrechts University, Kiel, Germany*
[2]*University College London Medical School, Department of Oncology, London, UK*
[3]*Institute for Pathology, Charité, Humold University, Berlin, Germany*
[4]*Department of Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN, USA*
[5]*Division of Oncology Research and Department of Pharmacology, Mayo Clinic, Rochester, MN, USA*

An established gastric-carcinoma cell line, EPG85-257P, is extremely sensitive to mitoxantrone (IC_{50}, 0.12 ng/ml). Stepwise selection with mitoxantrone for 3 years resulted in a cell line (EPG85-257RN) that is 7,056-fold resistant to mitoxantrone (IC_{50}, 846 ng/ml) and displays cross-resistance to the topoisomerase(topo)-II poisons ametantrone (411×), etoposide (112×) and teniposide (60×) as well as the topo-I poisons 7-ethyl-10-hydroxycamptothecin (331×) and topotecan (58×). We now show that this resistance is multifactorial. Western blotting revealed a 5-fold decrease in topo-IIα polypeptide in the mitoxantrone-resistant cells. Immunohistochemistry and Western blotting failed to demonstrate P-glycoprotein overexpression. Formation of trapped topo-II–DNA complexes in the resistant cells required higher mitoxantrone concentrations than in parental cells, even though nuclei isolated from the EPG85-257RN cells formed cleavage complexes normally. In agreement with these observations, which suggest the possibility of a defect in mitoxantrone accumulation, examination of mitoxantrone accumulation in both cell lines by confocal laser microscopy revealed that the EPG85-257RN cells accumulate less mitoxantrone at steady state. From these results, we propose that mitoxantrone accumulation, along with alterations in topo-IIα expression, contribute to the resistance to mitoxantrone observed in these cells. *Int. J. Cancer* 71:817–824, 1997.
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Mitoxantrone is a widely utilized anti-neoplastic agent of the anthracendione class (Lown and Hanstock, 1985). Like the less potent member of this family, ametantrone (Foye *et al.*, 1982), mitoxantrone is an intercalating agent (Kapuscinski and Darzynkiewicz, 1986) that inhibits topo-II (Crespi *et al.*, 1986) and induces DNA single- and double-strand breaks (reviewed in Durr, 1984). These multiple mechanisms of cytotoxicity presumably contribute to the usefulness of mitoxantrone in the clinic, where it is currently employed to treat leukemia, lymphoma, ovarian cancer and breast cancer. This widespread clinical use emphasizes the importance of elucidating the mechanisms of resistance to mitoxantrone.

Two of these mechanisms have been characterized. First, mitoxantrone can be transported by P-glycoprotein (Pgp). In cells that over-express Pgp, export of mitoxantrone is enhanced; and this enhanced export can be blocked by Pgp modulators such as verapamil and trifluoperazine. Interestingly, mitoxantrone does not appear to be affected in a similar fashion by the multidrug-resistance-associated protein (MRP). Transfection of the human MRP gene into HeLa cells results in moderate resistance to drugs such as doxorubicin, daunorubicin, epirubicin, vincristine and etoposide but not to mitoxantrone (Cole *et al.*, 1994). Similarly, MCF-7/VP cells that over-express MRP as part of multifactorial resistance remain almost as sensitive to mitoxantrone as parental cells (28.4-fold VP-16 resistance but only 2.8-fold mitoxantrone resistance) (Schneider *et al.*, 1994).

Alterations in topo-II appear to constitute a second mechanism that can contribute to mitoxantrone resistance. Harker *et al.* (1989) have described a mitoxantrone-resistant HL-60 line with reduced topo-IIα activity, diminished levels of the topo-IIβ isoform and increased amounts of a cross-reactive 160-kDa polypeptide. A study of 4 mitoxantrone-selected multidrug-resistant cell lines showed no over-expression of MRP, thus suggesting that MRP does not play a primary role in the resistance mechanisms (Futscher *et al.*, 1994). Sullivan *et al.* (1995) have described a mitoxantrone-resistant Chinese hamster ovary line in which topo-II content was identical to parental cells, but the complexes between DNA and topo-II polypeptide purified from the resistant line were not stabilized by mitoxantrone.

Various investigators also have described a number of mitoxantrone-resistant cell lines that do not fit the categories outlined above. Durr (1984) described a mitoxantrone-resistant WiDr colon-carcinoma line that exhibited decreased mitoxantrone uptake without a typical pattern of Pgp-mediated cross-resistance. Subsequent studies confirmed that this cell line did not express detectable Pgp. Membrane-active agents, including polysorbate 80 and amphotericin B, enhanced mitoxantrone uptake and cytotoxicity in the resistant WiDr cells but not in parental cells (Durr, 1984). In contrast, dinitrophenol treatment in the absence of glucose did not enhance mitoxantrone uptake (Durr, 1984). Wallace *et al.* (1987) described a mitoxantrone-resistant colon-carcinoma cell line with similar features, except that dinitrophenol enhanced mitoxantrone uptake in the resistant line and glucose diminished it. Nakagawa *et al.* (1992) described mitoxantrone-resistant MCF-7 lines, one of which was 4,000-fold resistant to mitoxantrone but strikingly less resistant (only 10-fold) to etoposide and daunorubicin. Although there was no evidence for Pgp or MRP over-expression, this line accumulated decreased amounts of mitoxantrone. Treatment with dinitrophenol and sodium azide increased mitoxantrone accumulation. Yang *et al.* (1995) indicate that this cell line is also cross-resistant to certain semi-synthetic derivatives of the topo-I inhibitor camptothecin but not to camptothecin itself.

In the present report, we describe a human gastric-carcinoma line that exhibits high-level resistance to mitoxantrone. Investigation of the mechanism of this resistance has provided evidence for altered topo-IIα expression as well as decreased mitoxantrone accumulation that is not explained by Pgp or MRP.

MATERIAL AND METHODS

Reagents

Daunorubicin and doxorubicin were obtained from Farmitalia Carlo Erba (Freiburg, Germany) and stored at −20°C. Mitoxantrone was provided by Lederle (Wolfratshausen, Germany). Ametantrone was donated by Dr. D. Cairns, Sunderland University, UK. Topotecan and 7-ethyl-10-hydroxycamptothecin (SN-38) were donated by SmithKline Beecham (King of Prussia, PA) and Pharmacia Upjohn (Kalamazoo, MI), respectively. Etoposide (VP-16) and teniposide (VM-26) were from Bristol-Myers (Toisdorf, Germany). Camptothecin, amsacrine (m-AMSA) and most other chemicals were obtained from Sigma (Deisenhofen, Germany).

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*Correspondence to: Institut für Allgemeine Pathologie und Pathologische Anatomie, Michaelisstrasse 11, D-24105 Kiel, Germany. Fax: (49) 431/597-3428. e-mail: ukellner@path.uni-kiel.de

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Two rabbit anti-topo-II anti-sera were used in this study. Rabbit anti-topo-IIα, recognizing an epitope at the C-terminal end of topo-IIα, was obtained from Cambridge Research Biochemicals (Northwich, UK). Rabbit anti-serum A10 (Kaufmann *et al.,* 1991), which recognized the topo-IIα and topo-IIβ isoforms, was kindly provided by Dr. L. Liu (Piscataway, NJ). Both anti-sera were diluted 1:500 in PBS containing 0.1% (v/w) BSA and 1.3 g/l sodium azide (PBS/BSA). Mouse monoclonal IgG anti-Pgp antibodies C219, obtained from cis-Isotopen (Dreieich, Germany), and JSB-1, donated by Dr. R.J. Scheper (Scheper *et al.,* 1988), were used at a dilution of 1:200 in PBS/BSA. Mouse monoclonal C21 anti-topo-I was kindly provided by Dr. Y.-C. Cheng (Chang *et al.,* 1992) and was used at a dilution of 1:1,000 in PBS/BSA.

Cell culture

EPG85-257P (257P) parental gastric-carcinoma cells, as well as mitoxantrone- and daunorubicin-resistant forms (EPG85-257NOV and EPG85-257DAU [257DAU], respectively) have been described elsewhere (Dietel *et al.,* 1990). EGP85-257RN (257RN) cells were derived subsequently from EPG85-257NOV cells by incubation for more than 2 years in Leibovitz's L-15 medium containing 0.2 µg/ml mitoxantrone. This resulted in substantially increased resistance to mitoxantrone (from 186× to 7056×). Cells were cultured subsequently in L-15 medium (GIBCO, Paisley, UK) containing 10% (v/v) heat-inactivated FCS (GIBCO), L-glutamine (2 mM) and 0.2 µg/ml mitoxantrone (257RN) or 2.5 µg/ml daunorubicin (257DAU). Under these conditions, the population doubling times were 16 hr for 257P cells and 30 hr for 257RN cells. For some investigations, cells were trypsinized with trypsin/PBS (GIBCO) for at least 5–10 min at 37°C. HL-60 leukemia cells were grown in RPMI-1640 medium with the same supplements as L-15 medium. All cells were screened once a month for *Mycoplasma* contamination, by means of 10 µM Hoechst 33258 (Frankfurt, Germany).

Sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid (XTT) and sulforhodamine B (SRB) assays

Four thousand cells were distributed into 96-well flat-bottomed plates (XTT and SRB assay) prior to testing. After 1 day (to allow cells to adhere and resume logarithmic growth), drug was added to triplicate wells. After 4–5 days' chronic incubation (XTT) or after 1-hr acute drug exposure followed by 5 days' incubation in drug-free medium (SRB), the assay was terminated. For XTT assays, the XTT components (Boehringer, Mannheim, Germany) were added to a final concentration of 0.3 mg/ml and the absorbance difference at 420–690 nm was measured after 3-hr incubation in the dark at 37°C. For SRB assays, the incubation was terminated by adding 50 µl 30% TCA followed by incubation at 4°C for 20 min. After samples were washed 3 times with tap water and air-dried, 50 µl 0.4% SRB (Sigma) in 1% acetic acid was added and samples were incubated at 20°C for 20 min. Absorbance was measured at 540 nm after drying and re-solubilization in 10 mM Tris-HCl. To determine the IC_{50}, the absorbance difference of control cells without drug was set to 100%. Linear regressions were plotted using the linear region of the curve, and IC_{50} was calculated. The mean ± standard error of the IC_{50} was calculated from multiple independent experiments (usually 3) for each drug and cell line.

RNA isolation and Northern blotting

Total cellular RNA was extracted by modified 1-step guanidinium-isothiocyanate-phenol-chloroform extraction. Total RNA (50 µg) was fractionated by agarose-gel electrophoresis and transferred to a Hybond-N membrane (Amersham, Aylesbury, UK). The membrane was pre-hybridized for 4 hr at 68°C in buffer consisting of 6× SSPE, 10% (w/v) dextran sulfate, 7% (w/v) SDS and 0.5% BLOTTO and hybridized overnight with a ^{32}P-labeled 1.2-kb MRP cDNA probe (kindly provided by Drs. S. Cole and R. Deeley, Queen's University, Kingston, Canada) labeled to a specific activity of $>5 \times 10^8$ dpm/µg using the Multiprime DNA Labeling kit (Amersham). The blot was washed at high stringency (0.1× SSC, 1% [w/v] SDS at 65°C). Autoradiography was performed at −80°C for 6 hr using intensifying screens. For the control hybridization, the membrane was erased 3 × 1 hr at 95°C with the high-stringency buffer and probed with a phosphoglycerate kinase (PGK) cDNA (kindly provided by Dr. K.J. Scanlon; Kashani-Sabet *et al.,* 1992).

Western blotting, nuclear extraction and cleavable complex formation

Semi-confluent cells were washed with ice-cold PBS buffer, solubilized in guanidine hydrochloride and prepared for SDS-PAGE as described (Kaufmann *et al.,* 1991). Alternatively, extracts were prepared from trypsinized cells: trypsinized cells were washed twice with PBS (pH 7.4) and once with buffer A (10% [w/v] sucrose, 100 mM NaCl, 15 mM KCl, 15 mM HEPES [pH 7.4], 0.5 mM EGTA, 0.15 mM spermine, 0.05 mM spermidine, 1 mM α-phenylmethylsulfonyl fluoride and 14 mM β-mercaptoethanol). All further steps were performed at 4°C. Cells were re-suspended in buffer A at a concentration of 2 to 5×10^8/ml. Triton X-100 was added to a final concentration of 0.5% (v/v). Following 10-min incubation, nuclei were recovered by centrifugation at 1,000 *g* for 10 min. Supernatants were used for Pgp Western-blot analysis. Nuclei were re-suspended in buffer B (100 mM NaCl, 5 mM potassium phosphate [pH 7.4], 1 mM PMSF and 14 mM β-mercaptoethanol; 5 M NaCl was added dropwise to a final concentration of 0.35 M). After 15-min incubation at 4°C, DNA was sedimented at 5,000 *g* for 20 min and discarded. Protein content in the supernatant was estimated. Nuclear extracts were used for topo-activity assays or diluted with an equal volume of SDS sample buffer (250 mM Tris-HCl [pH 6.8], 2% [w/v] SDS, 10 mM EDTA and 5% [v/v] β-mercaptoethanol) for Western-blot analysis.

Samples containing equal amounts of protein (confirmed by Coomassie-blue staining of SDS gels) were separated on an 8% (w/v) SDS-polyacrylamide gel and transferred to a PVDF membrane (Millipore, Eschborn, Germany) by semi-dry blotting. Blots were blocked for 1 hr in PBS containing 5% (w/v) low-fat powdered milk and incubated with the primary antibody for 2 hr. After washing 3 × 10 min with PBS containing 0.1% (w/v) BSA, blots were incubated for 1 hr with biotinylated secondary antibody (Dako, Hamburg, Germany), washed 3 times with PBS, incubated with streptavidin/alkaline-phosphatase complex (Sigma), washed and stained with NBT/X phosphate (Boehringer). Alternatively, peroxidase-coupled secondary antibodies and an enhanced chemiluminescence kit (Amersham) were utilized according to the supplier's instructions. Densitometry was performed using a Vilber Lourmat video imaging system and Bioprofil CV 4.6 software (Marne la Vallée, France).

For investigations of cleavable complex formation using a band-depletion assay (Hsiang and Liu, 1988), plated cells were incubated for 1 hr with different concentrations of mitoxantrone. To exclude the effect of cytoplasmic transport mechanisms, nuclei (prepared as described above) were incubated in 200 µl buffer B containing the indicated mitoxantrone concentration for 1 hr as described by Pommier *et al.* (1984). After incubation, cells or nuclei were washed with ice-cold PBS (cells) or buffer B (nuclei) and prepared for SDS-PAGE as described above.

Immunohistochemistry

Cells grown on slides for approximately 2 days in the conditions described above were fixed in 1:1 acetone:methanol for 10 min at −20°C, blocked in 5% (w/v) milk-PBS and incubated with primary antibody or normal mouse serum in 0.1% (w/v) BSA-PBS for 1 hr at room temperature. Samples were washed with 0.1% (w/v) BSA-PBS, reacted for 30 min with secondary biotin-labeled goat anti-mouse antibody (Dako), washed and detected by the alkaline-phosphatase/anti-alkaline-phosphatase method.

Assays of topo-II-mediated DNA relaxation, decatenation and DNA cleavage

Reactions (20 µl) containing normalized nuclear extracts (protein content was assessed by the Bradford assay) in 50 mM Tris-HCl (pH 8.0), 60 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 0.5 mM dithiotreitol (DTT), 0.5 mM EDTA, 30 µg/ml BSA and 0.5 µg pBR322 (Boehringer) for relaxation assays or 0.3 µg kDNA (TopoGen, Columbus, OH) for decatenation assays were incubated at 37°C for 30 min in the absence or presence of drug and then analyzed by agarose-gel electrophoresis on 1% (w/v) agarose gels in TBE buffer. For DNA cleavage and decatenation assays, reactions were terminated by the addition of 2 µl of stop buffer containing 5% (w/v) SDS and 5 mg/ml proteinase K (Sigma). Following an additional 30-min incubation at 37°C, samples were electrophoresed as described above. After electrophoresis, gels were stained with ethidium bromide and photographed under UV illumination.

Single-strand conformational polymorphism (SSCP) analysis

SSCP analysis was performed in the conditions described in detail by Danks *et al.* (1993). The primers used in these assays encompass all previously identified mutations in the topo-II cDNA. The conditions of the assay detect all known mutations in these regions.

Confocal laser scanning

For visualization of intracellular mitoxantrone, cells were grown on slides in drug-free medium for 2 days and then exposed to

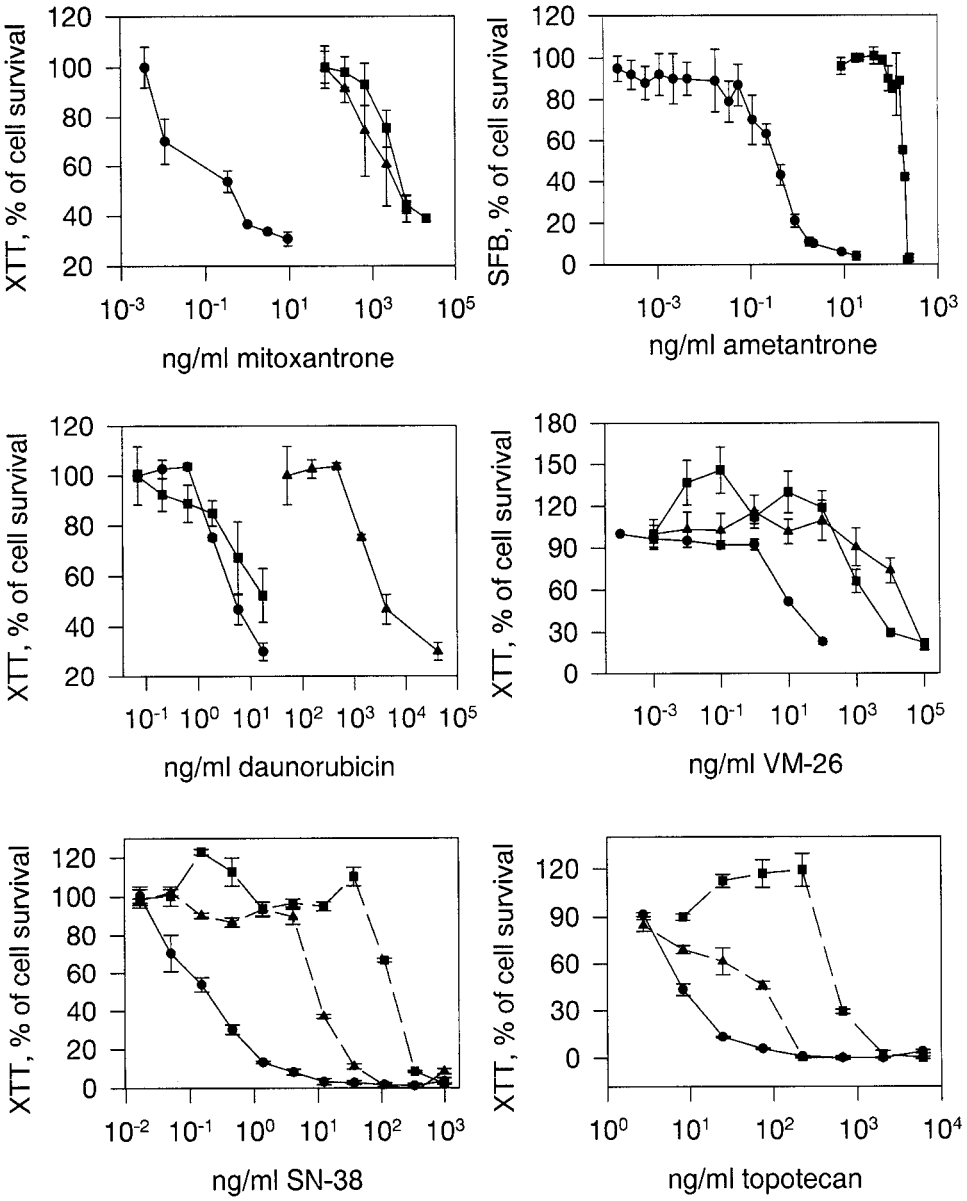

FIGURE 1 – Cytotoxicity studies. XTT and SRB assays were used to determine the cytotoxicity of mitoxantrone, ametantrone, daunorubicin, VM-26, SN-38 and topotecan in each cell line (top left to bottom right). Points represent the mean of values obtained from 3 separate wells. Bars represent standard deviation. Parental cells (●) exhibit high sensitivity to mitoxantrone (IC_{50}, 0.12 ng/ml) and ametantrone (IC_{50}, 0.4 ng/ml) and normal sensitivity to daunorubicin, VM-26, SN-38 and topotecan. Both resistant cell lines, 257RN (■) and 257DAU (▲), exhibit high-level resistance to mitoxantrone, whereas only 257DAU cells have a significant Pgp-like cross-resistance pattern. The calculated IC_{50} for these agents and additional drugs is given in Table I.

1 μg/ml mitoxantrone for 0–24 hr, as indicated. Following incubation, slides were rinsed 3 times with 37°C PBS, sealed with wax and viewed under a Zeiss LSM10 confocal microscope equipped with an Apoplan 40×/1.3 NA lens. The 514-nm line of a 25-mW Ar/Kr-laser was used for excitation, and images were recorded using a filter set designed for rhodamine emission. The sensitivity threshold of the photomultiplier was set to detect fluorescence in cells treated with mitoxantrone but not in cells incubated without the drug. Images of sensitive and resistant cells were recorded in identical conditions, stored on an optical disc and photographed from a high-resolution flat-screen monitor.

RESULTS

Cytotoxicity assays

The cytotoxicity of 10 drugs was examined in parental, mitoxantrone-selected and daunorubicin-selected EPG 85-257 human gastric-carcinoma cells using XTT-based and SRB-based assays. Figure 1*a–f* shows representative results obtained with these assays. Parental cells exhibit high sensitivity to mitoxantrone (Fig. 1*a*), whereas both drug-selected lines are highly resistant to mitoxantrone (257RN, 7,056-fold; 257DAU, 2,794-fold; Fig. 1; Table I). These results, obtained using an XTT assay after chronic mitoxantrone treatment, were confirmed using an acute-treatment protocol and an SRB assay (Table I). For mitoxantrone, the resistance factor of 257RN cells compared with 257P cells differs only 2-fold between these different cytotoxicity assays (7,056-fold using XTT and 14,000-fold using SRB). The 2 resistant lines differed, however, in their patterns of cross-resistance: 257DAU cells exhibited cross-resistance to doxorubicin (2,506-fold; Table I), daunorubicin (5,171-fold; Fig. 1), VM-26 (405-fold; Fig. 1) and VP-16 (353-fold; Fig. 1), a pattern consistent with the observation that this cell line over-expresses Pgp (Seidel *et al.*, 1995). In contrast, the resistance observed in 257RN cells was somewhat more selective for mitoxantrone. In particular, the cells showed a much lower degree of resistance to doxorubicin (11-fold; Table I) and daunorubicin (11-fold; Fig. 1*b*) than the 257DAU cell line, whereas there was cross-resistance to ametantrone (411-fold; Fig. 1*e*). Interestingly, 257RN cells also exhibited fairly strong cross-resistance to the topo-I inhibitors SN-38 and topotecan, with resistance factors of 332- and 58-fold, respectively, in XTT assays, even though the 257RN cells were only 2-fold resistant to the parent drug camptothecin.

The resistance of 257RN cells was stable. Long-term mitoxantrone deprivation (>20 passages) resulted in only a slight decrease in the resistance of 257RN cells to mitoxantrone (data not shown). Additional experiments were undertaken to determine the cause(s) of resistance in the 257RN cell line.

Expression of Pgp and MRP

Pgp levels were evaluated in 257P, 257RN and 257DAU cells and in 257DAU cells cultured 3 months without daunorubicin. Antibody C219 recognized a 170-kDa polypeptide in extracts from 257DAU cells (Fig. 2*a*, lane 3). In contrast, 257P, 257RN and 257DAU cultured in the absence of daunorubicin did not contain enough Pgp to yield a signal on Western blots (Fig. 2*a*, lanes 1, 2 and 4, respectively). Likewise, immunocytochemical studies using 2 antibodies, C219 and JSB-1, demonstrated Pgp on the surface of 257DAU cells (Fig. 3*c*) but not on the 257P or 257RN lines (Fig. 3*a*,*b*, respectively).

Northern blotting was utilized to examine levels of MRP mRNA in 257P, 257RN and 257DAU cells. RNA from the MRP-expressing ovarian-carcinoma cell line A2780 served as a positive control. A 2- to 3-fold increase in MRP mRNA in the 257RN and 257DAU cells (Fig. 2*b*, lanes 2 and 3) compared with the parental line (Fig. 2*b*, lane 1) was observed. Parental 257P cells themselves had an elevated basal MRP mRNA level in relation to the control fibrosarcoma cell line EPF 85-079, which shows very weak expression (Fig. 2*b*, lane 4).

Topo-II analysis

Because both selected cell lines showed some degree of cross-resistance to topo-II-directed agents, various aspects of topo-II-mediated drug action were examined. Analysis of decatenation activity in nuclear extracts indicated that topo-II catalytic activity was similar in the 257P and 257RN cell lines (Fig. 4*a*). In further experiments, Western blots were probed with polyclonal antiserum A10, which recognizes topo-IIα and -β (Fig. 5). Densitometric analysis of the resulting blots revealed that topo-IIα polypeptide content in 257RN cells was 5-fold lower than in 257P or 257DAU cells. This decreased topo-IIα expression in 257RN cells was accompanied by an increase in topo-I expression (Fig. 5). Topo IIβ, which could be readily detected in human HL-60 leukemia cells, was undetectable under these conditions in all 3 gastric-carcinoma cell lines (Fig. 5).

To examine the regulation of topo-II during mitoxantrone treatment, cells were treated with mildly toxic concentrations of mitoxantrone for 24 hr (257P, 20 ng/ml; 257RN and 257DAU, 20 μg/ml each). In all 3 cell lines, this treatment resulted in G_2 arrest as assessed by flow cytometry (data not shown). This G_2 arrest was accompanied by increases of topo-IIα in 257P and 257RN cells (Fig. 5, lanes 2 and 4) but not 257DAU cells (Fig. 5, lane 6). Interestingly, topo-I appeared to decrease simultaneously with this treatment (Fig. 5, lanes 3 and 4).

In addition to this semi-quantitative analysis, 2 methods were used to search for potential qualitative changes in topo-II. First, we

TABLE I – SENSITIVITY OF 257 GASTRIC-CARCINOMA CELL LINES TO CYTOSTATIC DRUGS

IC_{50} (ng/ml)	Assay	257P	257RN	257DAU	RN/P	DAU/P
Ametantrone	SRB	0.40 (3)	163 (3)	—	411	—
Camptothecin	XTT	5.20 (3)	12 (3)	5 (3)	2	1
Camptothecin	SRB	886.70 (3)	1,650 (3)	—	2	—
Daunorubicin	XTT	1.30 (3)	11 (3)	2,582 (5)	11	2,506
Doxorubicin	XTT	10.50 (3)	117 (3)	54,300 (4)	11	5,171
mAMSA	XTT	316.70 (3)	788 (4)	1,212 (3)	3	4
Mitoxantrone	XTT	0.12 (5)	846 (3)	335 (3)	7,056	2,794
Mitoxantrone	SRB	0.10 (3)	846 (3)	—	14,000	—
SN-38	XTT	0.30 (3)	104 (3)	9 (3)	332	30
SN-38	SRB	63.00 (3)	4,600 (3)	—	73	—
Topotecan	XTT	4.51 (3)	260 (3)	34 (4)	58	8
Topotecan	SRB	603.00 (3)	4,883 (3)	—	8	—
VM-26	XTT	5.30 (5)	266 (5)	2,133 (3)	50	405
VP-16	XTT	10.80 (3)	1,217 (3)	3,333 (3)	112	353

XTT and SRB assays, performed as described in ''Material and Methods,'' were used to determine the IC_{50} for indicated drugs (IC_{50} indicated as mean in ng/ml; number of independent experiments indicated in parentheses). RN/P and DAU/P represent ratio of IC_{50} values of 257RN *vs.* 257P or 257DAU *vs.* 257P, respectively.

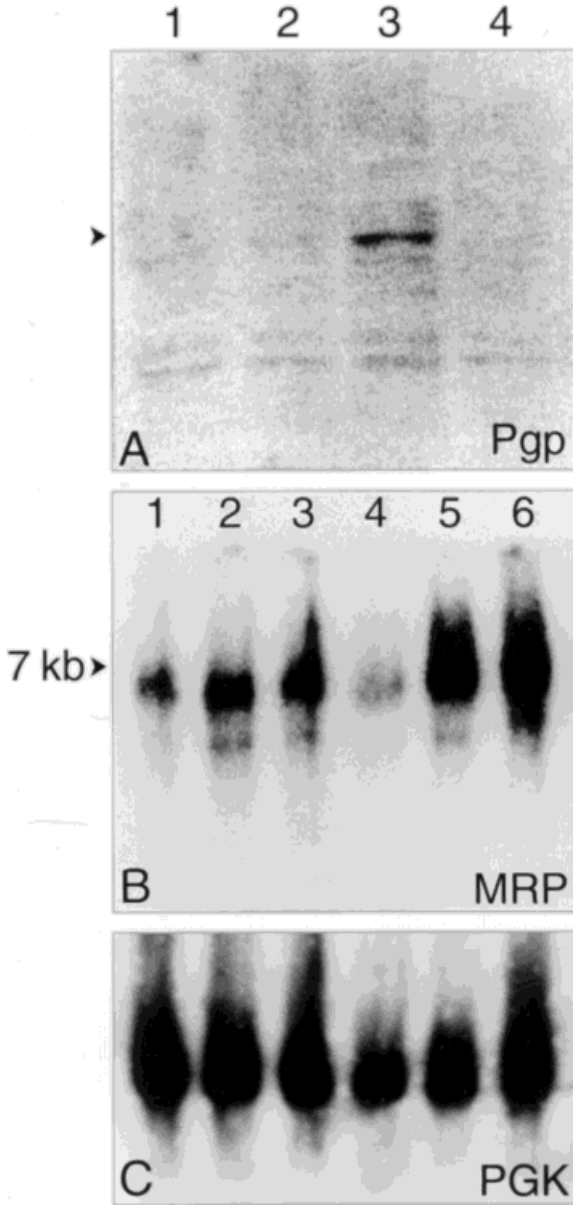

FIGURE 2 – Analysis of Pgp and MRP expression. *(a)* Western-blot analysis of Pgp expression using antibody C219. Lanes were loaded with 60 µg of cytosolic and membrane-associated proteins from 257P (lane 1), 257RN (lane 2), 257DAU grown in daunorubicin (lane 3) or 257DAU cells grown for approx. 3 months without daunorubicin (lane 4). An immunoreactive 170-kDa band (arrow) was detected only in extracts of 257DAU cells (lane 3) cultivated in the presence of daunorubicin. *(b)* Northern-blot analysis of MRP expression using a 1.2-kb MRP cDNA probe, as described in ''Material and Methods''. Lanes contain 50 µg total RNA from 257P cells (lane 1), 257DAU (lane 2), 257RN (lane 3), EPF 86-079 fibrosarcoma cells (lane 4), MCF-7 breast-carcinoma cells (lane 5) and A2780 ovarian-carcinoma cells (lane 6). Controls in this experiment included the A2780 cells (lane 6), which express MRP; the MCF-7 cell line (lane 5); and the fibrosarcoma cell line EPF 86-079, which shows very weak expression of MRP (lane 4). MRP mRNA levels were slightly increased in 257DAU (lane 2) and 257RN (lane 3) as compared with 257P (lane 1). *(c)* As a control, the blot in *(b)* was probed with a 1.8-kb cDNA probe of the PGK house-keeping gene (Kashani-Sabet *et al.*, 1992). Equal loading of RNA is shown in lanes 1–3 and 6 (257 cells and A2780 cells), whereas lanes 4 and 5 are slightly under-loaded (EPF 86-079 and MCF-7).

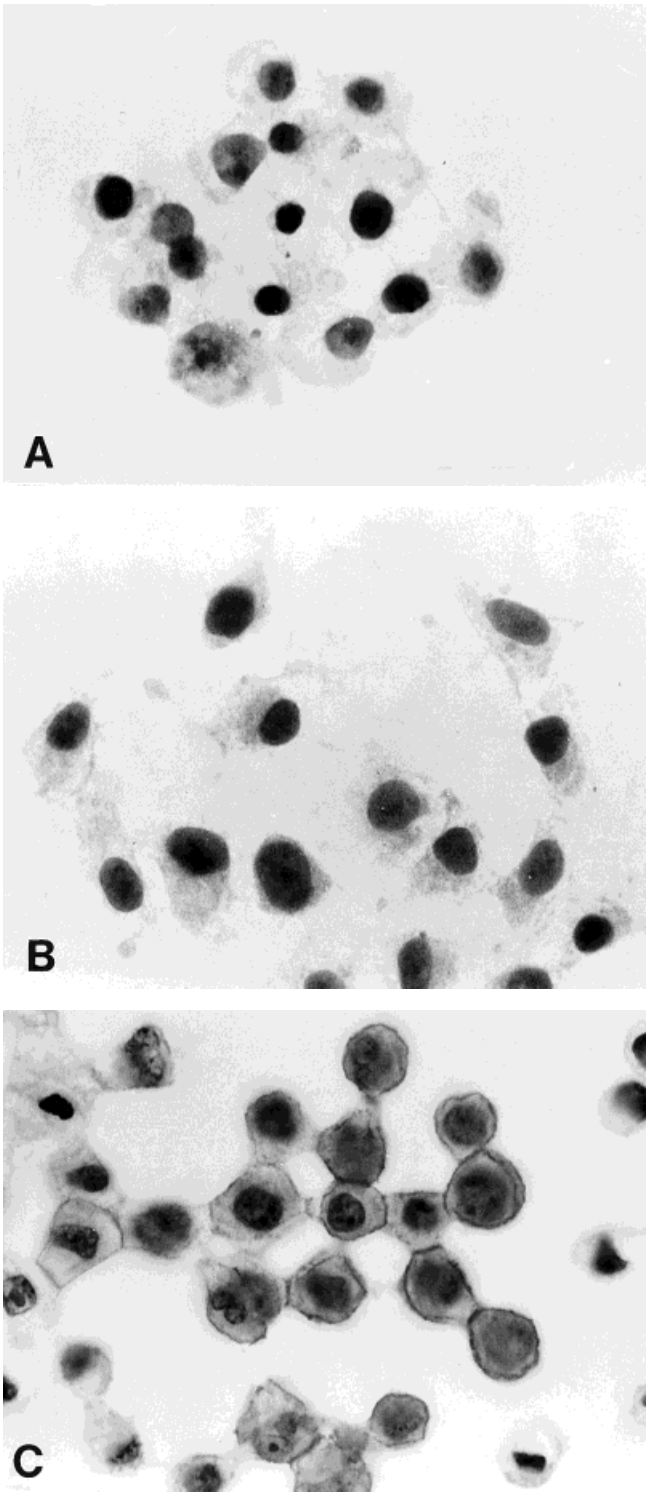

FIGURE 3 – Immunohistochemistry of Pgp. C219 antibody was used to detect cellular expression of Pgp in 257P *(a)*, 257RN *(b)* and 257DAU *(c)* as described in ''Material and Methods''. Pgp expression, a black membrane-associated stain, is seen only in 257DAU cells *(c)*.

used SSCP analysis to search for mutations in 6 regions of the topo-IIα sequence: 3 consensus nucleotide-binding sequences, one DNA-binding site and 2 regions thought to influence the sensitivity of topo-II to inhibitors (Danks *et al.*, 1993). No differences were observed between the parental and the drug-resistant cells (Fig. 4*c*).

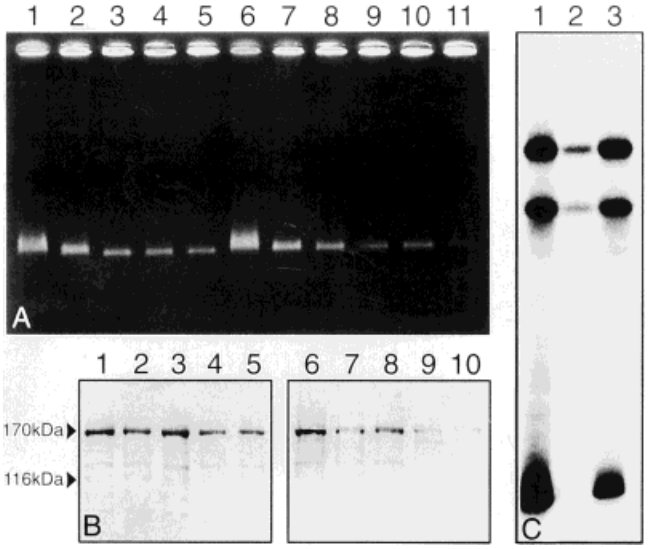

FIGURE 4 – Topo-II analysis. *(a)* Topo-II decatenation activity of nuclear extracts from 257P (lane 1, 3.2 µg; lane 2, 1.6 µg; lane 3, 0.8 µg; lane 4, 0.4 µg; lane 5, 0.2 µg protein) and 257RN cells (lane 6, 2 µg; lane 7; 1 µg; lane 8, 0.5 µg; lane 9, 0.25 µg; lane 10, 0.125 µg protein). A sample without nuclear protein was used as control (lane 11). Nuclear extracts of parental cells (lanes 1–5) and mitoxantrone-resistant cells (lane 5–10) had similar topo-II decatenation activity. *(b)* Band-depletion analysis with whole cells (lanes 1–5) and isolated nuclei (lanes 6–10); 200,000 257RN cells or nuclei were incubated for 1 hr with different concentrations of mitoxantrone (0 µg/ml, lanes 1 and 6; 0.02 µg/ml, lanes 2 and 7; 0.2 µg/ml, lanes 3 and 8; 2 µg/ml, lanes 4 and 9; and 20 µg/ml, lanes 5 and 10), as described in "Material and Methods". Very little induction of cleavable complexes is seen with whole cells (lanes 1–5). Increased mitoxantrone concentration induces the formation of cleavable complexes in isolated nuclei (lanes 6–10). *(c)* SSCP analysis performed using primers for topo-II region coding for motif B/DNBS (DNA-binding sequence). No band shift was detected in 257RN (lane 2) or 257DAU (lane 3) cells compared with 257P cells (lane 1). Similar results were observed with the other 5 primer sets (Danks *et al.*, 1993).

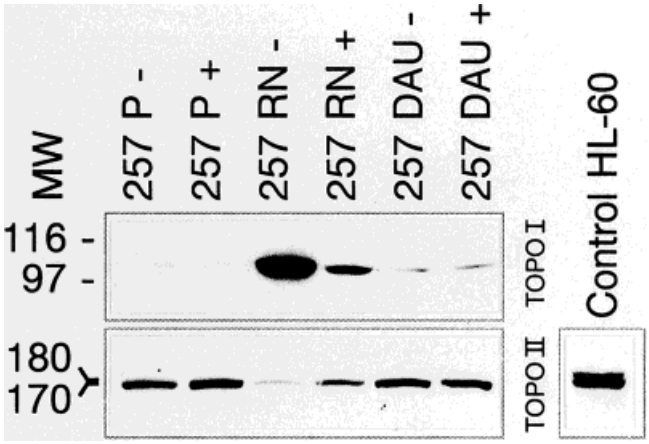

FIGURE 5 – Western-blot analysis of topoisomerase content. Duplicate gels were reacted with mouse monoclonal anti-topo-I (TOPO I) or polyclonal anti-serum A10 recognizing topo-IIα and topo-IIβ (TOPO II). Lanes were loaded with 30 µg protein from 257P, 257RN or 257DAU cells: + indicates that cells were treated for 24 hr with mitoxantrone at concentrations of 20 ng/ml (257P) or 20 µg/ml (257RN, 257DAU) prior to harvest, − indicates untreated cells. Molecular weight is indicated in kDa on the left. A Western blot with protein from HL-60 cells reacted with the same polyclonal anti-topo-IIα and -β is shown on the right. Compared with the 257P cells, untreated 257RN cells had diminished topo-IIα and increased topo-I. Mitoxantrone treatment resulted in increased topo-IIα in 257P and 257RN but not 257DAU.

From these experiments, we consider it unlikely that the mRNA encoding topo-IIα contains a mutation in the regions analyzed.

In a complementary series of experiments, the formation of drug-stabilized topo-II–DNA adducts was examined in band-depletion assays. When isolated nuclei from 257RN cells were incubated for 1 hr with increasing concentrations of mitoxantrone, the signal for topo-II on Western blots started to disappear at drug concentrations higher than 0.2 µg/ml (Fig. 4*b*, lanes 6–10). This result is again consistent with the view that topo-II from the 257RN cell line is able to form topo-II–DNA adducts. However, this disappearance was not detectable when whole cells were assayed (Fig. 4*b*, lanes 1–5). This is in contrast to 257P cells (data not shown). Even though the 257RN cells did not over-express Pgp, these results raised the possibility that 257RN cells might have a defect in mitoxantrone accumulation.

Fluorescence microscopy

To search for altered drug accumulation, the cellular distribution of mitoxantrone was examined in 257P and 257RN cells by fluorescence microscopy using mitoxantrone's intrinsic fluorescence. All pictures were taken with a confocal microscope using identical exposure times. Cells examined prior to mitoxantrone treatment displayed weak fluorescence in a punctuate vesicle-like pattern (Fig. 6*a*). In sensitive cells exposed to mitoxantrone for 6 or 24 hr, enhanced fluorescence was observed, primarily in the perinuclear region (Fig. 6*b,c*). Nuclear fluorescence was localized at the nuclear membrane and showed a patchy distribution within the nucleus (Fig. 6*c*) similar to the pattern described by Smith *et al.* (1992). In resistant cells, the level of fluorescence was much lower (Fig. 6*e,f*). Even after 24-hr drug exposure, fluorescence in the resistant cells was limited to the cytoplasm in a vesicular pattern (Fig. 6*f*).

DISCUSSION

In the present study we characterized 257RN, a gastric-carcinoma cell line that exhibits high-level, relatively selective resistance to mitoxantrone. The parental line 257P exhibits normal sensitivity to most anti-cancer drugs but a degree of sensitivity to mitoxantrone that is unusual in gastric-carcinoma cell lines (Dietel *et al.*, 1990). Compared with this parental line, 257RN cells exhibit multiple changes that might contribute to their resistance.

First, 257RN cells have a doubling time twice as long as that of the parental and the 257DAU cells. This cell-cycle prolongation could provide more time for the repair of mitoxantrone-induced DNA damage but is unlikely to account for more than low-level resistance. As indicated in Table I, the resistance to topo-II-directed agents varies from 3-fold (m-AMSA) to 7,000-fold (mitoxantrone). The cell-cycle prolongation and the concomitant enhanced opportunity for repair might contribute to the 3-fold resistance to m-AMSA, but it is difficult to envisage how the same cell-cycle prolongation could result in much higher resistance to mitoxantrone.

Alterations in topo-II also appear to contribute to resistance in 257RN cells. Harker *et al.* (1989) have described a mitoxantrone-selected cell line in which alterations in topo-IIβ were thought to contribute to anthracenedione resistance. In our case, topo-IIβ polypeptide was undetectable in all 3 gastric sub-lines, whereas immunoblot analysis using the same antibody could detect a distinct topo-IIβ band using extracts from other cell lines (Fig. 5; Kaufmann *et al.*, 1991; Sullivan *et al.*, 1995). Although we cannot rule out a subtle difference in sensitivity based on undetected

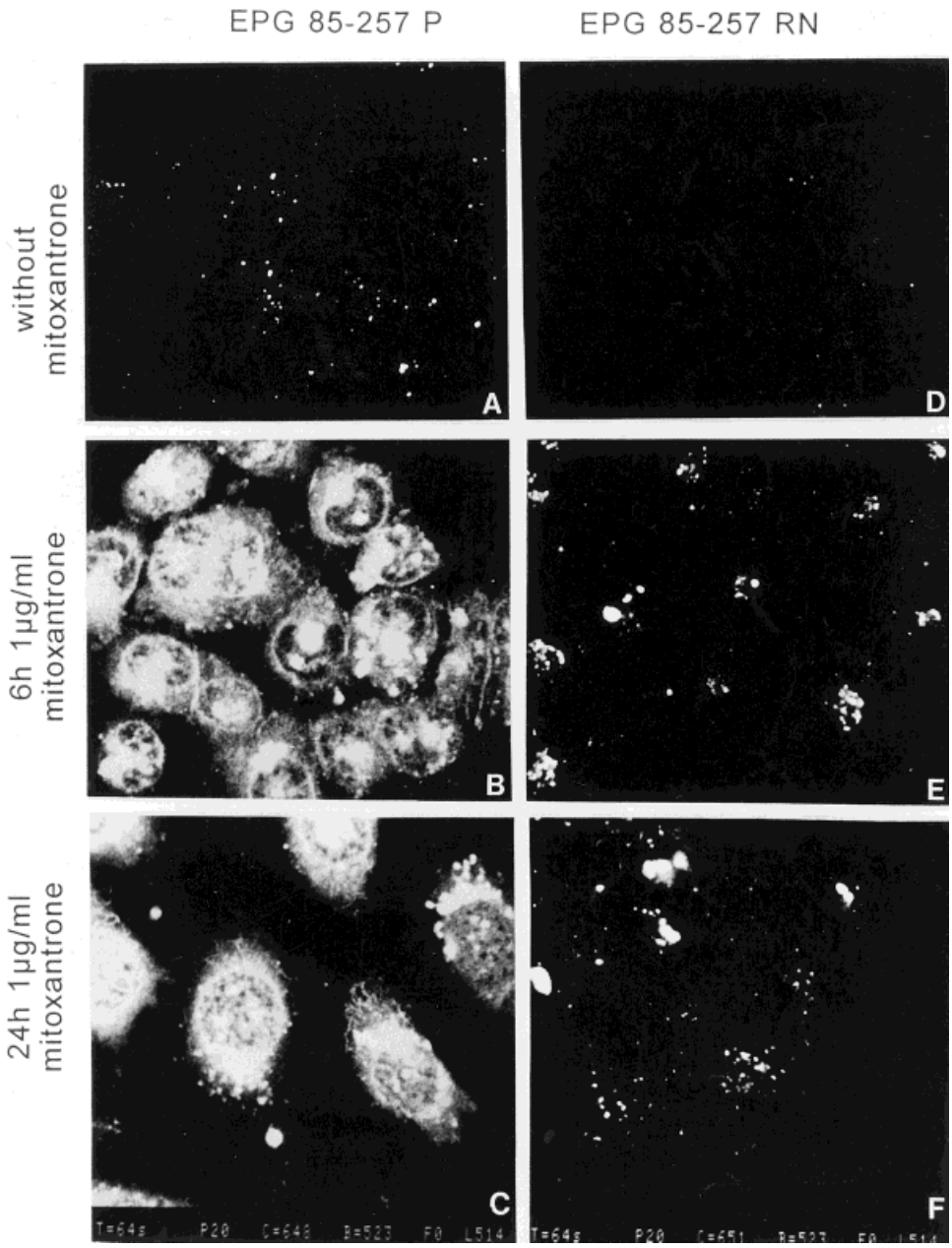

FIGURE 6 – Mitoxantrone fluorescence pattern detected by confocal microscopy. *(a–c)* 257P cells, *(d–f)*, 257RN cells. Images were collected before mitoxantrone treatment *(a, d)* and after incubation with 1 µg/ml mitoxantrone for 6 hr *(b, e)* or 24 hr *(c, f)*. A faint vesicular pattern of background fluorescence is present in the absence of mitoxantrone *(a, d)*. Mitoxantrone is readily detectable in parental cells *(b, c)* but markedly diminished in resistant cells *(e, f)*.

differences in topo-IIβ polypeptide content, the present data provide no evidence that changes in topo-IIβ play a major role in the resistance observed in 257RN cells. In contrast, the 5-fold decrease in topo-IIα in the 257RN cells compared with the 257P and 257DAU cells might well contribute to the observed resistance of 257RN cells to mitoxantrone as well as to other topo-II poisons.

This decrease in topo-IIα was accompanied by an increase in topo-I content in 257RN cells, as observed in another cell line selected for resistance to topo-II-directed agents (Lefevre *et al.*, 1991). Surprisingly, over-expression of topo-I did not result in increased sensitivity to topo-I poisons such as camptothecin, topotecan and SN38 (Fig. 1; Table I). Instead, 257RN cells display 332-fold resistance to SN-38, 58-fold resistance to topotecan and 2-fold resistance to camptothecin. A similar dissociation between topo-I content and camptothecin sensitivity has been described in other cell lines (Yang *et al.*, 1995).

The decreased proliferation rate and diminished topo-IIα content described above do not appear to be sufficient to account for the high-level resistance to mitoxantrone. These observations prompted us to search for additional mechanisms of resistance. Although mutations in the gene for topo-IIα have been reported in cell lines selected for high-level resistance to anthracyclines and epipodophyllotoxins, band-depletion assays performed in membrane-free condi-

tions and SSCP analysis failed to provide any evidence for qualitative alterations in topo-IIα from 257RN cells.

The observation that mitoxantrone stabilizes topo-II–DNA adducts in isolated nuclei but not in whole cells (Fig. 4*b*) suggests a membrane-associated mechanism of resistance. Consistent with this hypothesis, confocal microscopy indicates that steady-state accumulation of mitoxantrone in 257RN cells was diminished (Fig. 6). This decreased accumulation does not appear to be mediated by over-expression of Pgp. The lack of expression of Pgp in 257RN cells (Figs. 2*a*, 3*b*) confirms the results of Dietel *et al.* (1990) in the somewhat less resistant EPG-257NOV cells. Also, it is unlikely that MRP is the carrier involved in this mitoxantrone efflux. Experiments by Futscher *et al.* (1994) indicate that MRP over-expression is rarely seen in mitoxantrone-selected cells. Even though MRP expression was about 2- or 3-fold higher in 257RN cells than in 257P (Fig. 2*b*), the data of Cole *et al.* (1994) and Schneider *et al.* (1994) suggest that MRP over-expression confers, at most, low-level resistance to mitoxantrone. These considerations suggest that the mitoxantrone efflux is mediated by another, as yet unidentified, transporter that is increased in 257RN cells. In view of the cross-resistance pattern, especially cross-resistance to the topo-I poisons SN-38 and topotecan (Table I), we suggest that this transporter is similar or identical to the postulated mitoxantrone/topotecan transporter that appears to account for resistance in mitoxantrone-selected MCF-7 cells (Yang *et al.*, 1995).

In summary, the present results suggest that mitoxantrone resistance in the 257RN gastric-carcinoma line is associated with a decrease in mitoxantrone accumulation relative to parental 257P cells. This transport change, along with a reduction in proliferative rate and decrease in the content of topo-IIα, appears to contribute to a pattern of resistance that includes multiple topo-II- and topo-I-directed agents (Table I) but is somewhat selective for mitoxantrone.

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