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Topoisomerase II Activity Involved in Cleaving DNA into Topological Domains Is Altered in a Multiple Drug-Resistant Chinese Hamster Ovary Cell Line

DANIEL M. SULLIVAN, LOU ANN ESKILDSEN, KATHLEEN R. GROOM, CHARLES D. WEBB, MICHAEL D. LATHAM, ALVIN W. MARTIN, SAMUEL R. WELLHAUSEN, PAUL E. KROEGER, and THOMAS C. ROWE

James Graham Brown Cancer Center, University of Louisville, Louisville, Kentucky 40292 (D.M.S., L.A.E., K.R.G., C.D.W., M.D.L., A.W.M., S.R.W.), and Department of Pharmacology and Therapeutics, University of Florida, Gainesville, Florida 32610 (P.E.K., T.C.R.)

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SUMMARY

Drug resistance to inhibitors of DNA topoisomerase II can result from qualitative or quantitative alterations in the target enzyme, topoisomerase II, or from perturbations in drug transport that may or may not involve P-glycoprotein. In the present study, a drug-resistant Chinese hamster ovary cell line, SMR_{16}, was selected in the presence of an epipodophyllotoxin (VP-16) and was found to be cross-resistant to all classes of topoisomerase II inhibitors (3–35-fold). The 3-fold level of resistance of these cells to vincristine is likely due to diminished uptake of this drug, and this is not mediated by overexpression of P-glycoprotein. No alteration in transport of VP-16 was observed. Immunoblotting with several polyclonal anti-topoisomerase II antibodies demonstrated that the resistant cells contain approximately two-thirds of the parental enzyme amount. The topoisomerase II catalytic activity present in 0.35 M NaCl nuclear extracts paralleled this decrease. VP-16- and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide-induced DNA damage, mediated by topoisomerase II, was found to be decreased 10–12-fold in both intact SMR_{16} cells and nuclei isolated from these cells, when measured by alkaline filter elution. However, the VP-16-induced DNA cleavage activity present in 0.35 M NaCl nuclear extracts of the resistant cells was attenuated only 2-fold, relative to wild-type cells. Homogeneous preparations of the enzyme obtained from resistant cells demonstrated the same cleavage and catalytic activity as purified wild-type topoisomerase II. Analysis by pulse-field gel electrophoresis of the DNA isolated from VM-26- and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide-treated sensitive and resistant cells demonstrated significantly less conversion of SMR_{16} chromosomal DNA into 50–150-kilobase DNA fragments. Chinese hamster ovary SMR_{16} cells are apparently resistant to topoisomerase II poisons because the topoisomerase II that defines the DNA topological domains is either decreased in amount or insensitive to drug action.

Several clinically important classes of antineoplastic agents have as their major mechanism of cytotoxicity the inhibition of DNA topo II (EC 5.99.1.3). Specific antitumor drugs that inhibit this enzyme include doxorubicin, VP-16, VM-26, *m*-AMSA, and mitoxantrone. These drugs act by inhibiting the DNA religation activity of topo II, thus resulting in an apparent increase in DNA scission. Resistance to the cytotoxic effects of these agents may be an intrinsic property of the cell or may be an acquired characteristic and often results from classic multidrug resistance (overexpression of P-glycoprotein) or alterations in DNA topo II. Mechanisms of resistance to the aforementioned drugs, which involve quantitative or qualitative changes in topo II, have been recently defined by several investigators (reviewed in Ref. 1), working both with established cell culture models selected for resistance to a particular agent and with fresh tumor samples, and include (i) mutations or alterations in DNA topo II that result either in an enzyme with altered catalytic activity (2) or in an enzyme that is resistant to drug-induced DNA cleavage (2–5), (ii) the presence of different isoforms of topo II (p170 and p180), which exist in different ratios in various cell lines and have different biochemical properties and drug sensitivities (6, 7), (iii) altered binding of ATP (necessary for strand passage) by topo II (8), (iv) a quantitative change in the drug target (topo II) resulting from inactivation of a normal topo II allele (9), a decrease in topo II mRNA (10), or a differential subcellular partitioning of the enzyme (11), (v) overexpression of P-glycoprotein in associa-

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ABBREVIATIONS: topo II, DNA topoisomerase II; VP-16, 9-(4,6-*O*-ethylidene-β-D-glucopyranosyl)-4′-demethylepipodophyllotoxin; VM-26, 4′-demethyl-9-[4,6-*O*-(2-thenylidene-β-D-glucopyranosyl)oxy]epipodophyllotoxin; *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; CHO, Chinese hamster ovary; kb, kilobase(s); SDS, sodium dodecyl sulfate; kDNA, kinetoplast DNA; FPLC, fast protein liquid chromatography; WT, wild-type; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

tion with alterations in topo II activity (12), (vi) perturbations in the processing of drug-induced topo II-mediated DNA strand breaks (13), and (vii) changes in drug transport or subcellular distribution of topo II inhibitors, which may not involve the enzyme molecule itself but result in cellular resistance because the drug does not reach the target (14). A final potential mechanism of resistance to topo II inhibitors, which is as yet poorly defined, may involve the presence of intracellular factors that modulate the activity of the enzyme. Phosphorylation and poly(ADP)-ribosylation have been shown to increase and decrease, respectively, the activity of DNA topo II (15–19). Recently described VP-16-resistant human epidermoid carcinoma KB cells, which demonstrate a 10-fold decrease in topo II by immunoblotting, were found to have parental topo II catalytic activity; this was attributed to hyperphosphorylation of topo II (20). Finally, it is more common for a drug-resistant cell line to have combinations of two or more of the aforementioned mechanisms in operation; rarely is the resistance of a cell line uniquely defined by a single process.

In addition to its role in various intracellular processes that require the topological isomerization of DNA, topo II has been shown to have a functional and structural role as a major component of the nuclear matrix (21, 22). Topo II has been found to integrate into DNA at matrix-attachment regions, such that scission of DNA in the presence of topo II inhibitors results in fragments that are entire DNA loops or domains (23–27). Matrix topo II may be the critical target of topo II-active agents (28, 29). Indeed, a human leukemia cell line resistant to VM-26 and *m*-AMSA has been shown by immunoblotting to have parental cellular content of topo II with a 3-fold decrease in nuclear matrix enzyme (11). In this paper we report the characterization of a multiple drug-resistant CHO cell line, CHO-SMR_{16}, which likely has an alteration in the topo II population that cleaves chromosomal DNA into discrete 50–150-kb fragments in the presence of topo II inhibitors.

Experimental Procedures

Materials

The WT and drug-resistant CHO cell lines were grown in monolayer and suspension cultures at 37° in α minimum essential medium (GIBCO Laboratories, Grand Island, NY) with 5% fetal bovine serum (GIBCO), in the presence of 5% CO_2. AuxB1 and CH^RC5 CHO cells (generously provided by Dr. Victor Ling, Ontario Cancer Institute, Toronto, Canada) were also grown in α minimum essential medium to which was added 10% fetal bovine serum and 10 mg/liter concentrations of both adenosine and thymidine. Penicillin (100 IU/ml) and streptomycin (100 μg/ml) were added to all culture media.

[^{3}H]Vincristine sulfate (6.46 Ci/mmol) and the enhanced chemiluminescence Western blotting detection system were supplied by Amersham (Arlington Heights, IL). [^{3}H]VP-16 (400 mCi/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA) and [α-^{32}P]dCTP (3000 Ci/mmol) from NEN Products (Boston, MA). Sigma Chemical Co. (St. Louis, MO) supplied ethyl methanesulfonate, daunomycin HCl, actinomycin D, *cis*-platinum(II)diammine dichloride, cytosine arabinoside, camptothecin, verapamil HCl, vincristine sulfate, Nonidet P-40, EGTA, RNase A, DNase I, aprotinin, leupeptin, pepstatin A, chymostatin, and antipain HCl. VP-16 and mitoxantrone were generously provided by Bristol-Myers Co. (Wallingford, CT) and Lederle Laboratories (Pearl River, NY), respectively. The Drug Synthesis and Chemistry Branch of the National Cancer Institute kindly provided *m*-AMSA and VM-26. Doxorubicin was obtained from Adria Laboratories (Columbus, OH) and phenylmethylsulfonyl fluoride from Bethesda Research Laboratories (Gaithersburg, MD). The horse and goat sera used in the flow cytometry analysis were obtained from Vector Laboratories (Burlingame, CA). C219 murine monoclonal antibody to P-glycoprotein was supplied by Centocor (Malvern, PA). Mouse IgG1, fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin, and the FACScan instrument are all products of Becton Dickinson (San Jose, CA).

Isolation of the CHO-SMR_{16} Cell Line

This drug-resistant cell line was generated and isolated using previously described methods (10). Briefly, CHO cells were treated with ethylmethane sulfonate and drug-resistant colonies were selected in the presence of 1 μg/ml VP-16. The resistant cell line was cloned from a single cell to ensure a homogeneous population of cells. All of the following experiments were performed with this cloned cell line.

Cytotoxicity Assays

Drug sensitivity was determined by colony-forming assays as recently described (10). The duration of drug treatment and solvents used for dissolution of drugs not previously reported are as follows: daunomycin and actinomycin D, 4 hr, H_2O; cisplatin, 4 hr, 0.85% NaCl; and cytosine arabinoside, 18 hr, H_2O. Controls were treated with the appropriate amount of solvent alone, and when verapamil was included it was added concurrently with either VP-16 or vincristine. The degree of resistance was calculated as the ratio of the resistant cell IC_{50} drug concentration to the WT IC_{50}.

Alkaline Elution

Whole cells. Drug-induced DNA damage in intact cells was quantified as DNA single-strand breaks by the alkaline elution technique described by Kohn (30). [^{14}C]Thymidine-labeled logarithmic phase CHO cells were treated with various concentrations of doxorubicin, *m*-AMSA, mitoxantrone, or VP-16 for 1 hr at 37°. The cells were then washed two times with cold buffer A (150 mM NaCl, 5 mM potassium phosphate, pH 7.4), removed from the flasks (either by a 30-sec trypsinization or by scraping), resuspended in cold medium, and finally assayed for DNA single-strand breaks. [^{3}H]Thymidine-labeled L1210 cells were included as an internal standard. The DNA damage is expressed as rad equivalents, obtained by converting the drug-induced DNA breakage into the equivalent radiation dose that would result in that degree of strand scission (a radiation dose-response curve for logarithmic phase CHO cells was used).

Isolated nuclei. VP-16-induced DNA single-strand breaks were also measured in isolated nuclei by the following method. [^{14}C]Thymidine-labeled logarithmic phase monolayer CHO cells in 25-cm^2 flasks (2×10^6 to 4×10^6 total cells) were washed two times with 10 ml of cold buffer B (1.0 mM KH_2PO_4, pH 6.4, 5 mM $MgCl_2$, 150 mM NaCl, 10 mM EGTA, 4 mM dithiothreitol) and then overlaid at 4° for 10 min with 2 ml of buffer B containing 0.5% (v/v) Nonidet P-40. The flasks were then struck sharply against the hand five to seven times to release the nuclei. The presence of intact nuclei was confirmed by fluorescence microscopy after staining with either propidium iodide or acridine orange. Each flask was rinsed twice with 10 ml of cold buffer B, and the pooled contents were diluted to a final volume of 50 ml with cold buffer B in a 50-ml conical polypropylene centrifuge tube. The nuclei were pelleted by centrifugation at $2000 \times g$ for 10 min at 4°, resuspended in 5 ml of cold buffer B, and counted with a hemacytometer. Equivalent numbers of nuclei ($1–2 \times 10^6$) were incubated with VP-16 (50 μM) for 30 min in a 37° water bath. Immediately after drug treatment, the nuclei were deposited on a prewetted elution filter and the VP-16 was removed by washing the nuclei with 20–30 ml of cold buffer A under vacuum. [^{3}H]Thymidine-labeled internal standard cells were then deposited on the filter and the usual low sensitivity alkaline elution procedure to detect DNA single-strand breaks was then followed.

DNA Topo II Assays

Detection by Western blot electrophoresis. Both intact CHO cells and nuclear extracts (see below) were assayed for immunoreactive

DNA topo II using our polyclonal rabbit anti-recombinant HeLa topo II antibody (3), as well as polyclonal rabbit antibodies raised against synthetic peptides from human topo II sequences for p170 and p180 forms of the enzyme (kindly provided by Dr. Fred Drake, SmithKline Beecham Pharmaceuticals). Optimal immunoblotting of whole cells was obtained when 1–2 × 10^6 logarithmic phase cells were pelleted at 13,000 × *g* for 5 min at 4° and then treated with 90 μl of DNase/RNase solution (0.5 mg/ml DNase I, 0.25 mg/ml RNase A, 10 mM $MgCl_2$, 50 mM Tris, pH 7.4, 2 mM phenylmethylsulfonyl fluoride, and 20 μg/ml concentrations of antipain, aprotinin, chymostatin, leupeptin, and pepstatin A) and 10 μl of 2.5% SDS on ice for 60 min. To this solution were then added 60 μl of SDS solution (8% SDS, 0.4 M dithiothreitol, 40% glycerol, and the aforementioned protease inhibitors), and the solution was finally boiled in a water bath for 4–5 min. Nuclear extracts (20–100 μg of protein) were diluted with 1/3 volume of SDS solution and then boiled for 4–5 min. All samples were then electrophoresed on SDS-polyacrylamide gels (7.5–10% resolving gels), transferred to nitrocellulose at 0.75 A overnight at 4°, and incubated with the aforementioned anti-topo II antibodies. DNA topo II was detected either with the Bio-Rad immunoblot assay (which uses goat anti-rabbit alkaline phosphatase conjugates) or with the Amersham enhanced chemiluminescence detection system (which uses a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody) according to the manufacturer's instructions. The topo II signals were quantified by densitometry.

Nuclear extracts. DNA topo II was extracted from the nuclei of CHO cells as described previously (3), with the following modifications. Nuclei collected from the sucrose step gradient were extracted with either 0.35 M NaCl or 1.0 M NaCl (buffer F containing 0.7 M NaCl or 2.0 M NaCl, respectively) for 60 min. The DNA was not precipitated with polyethylene glycol. The supernatant from the 60-min 100,000 × *g* centrifugation step was used for assays of decatenation activity, covalent enzyme-DNA complex formation, and immunoreactive topo II and for two-dimensional gel electrophoresis.

Purification of DNA topo II. Topo II was purified to homogeneity from 1.0 M NaCl nuclear extracts (precipitated with polyethylene glycol) of WT and SMR_{16} cells by sequential chromatography on a hydroxylapatite column, a Mono Q anion exchange column, and a Mono S cation exchange column. The FPLC-based procedure developed earlier was followed (3). Topo II purified by this technique was used in assays of enzyme catalytic activity, cleavage activity, and thermal stability.

Catalytic activity. Topo II catalytic activity was quantified from both nuclear extracts and homogeneous preparations of the enzyme by decatenation of kDNA networks isolated from *Crithidia fasciculata* as described previously (31). Agarose gel electrophoresis of released minicircles was used to determine topo II activity during FPLC enzyme purification, as well as the activity remaining in purified preparations after thermal inactivation at 37° for various time periods. The catalytic activity of purified topo II from WT and SMR_{16} cells was quantified by following the release of [^{3}H]thymidine-labeled minicircles from kDNA networks (3).

Quantitative precipitation of the covalent topo II-DNA complex. The ability of purified WT and SMR_{16} topo II enzymes to form covalent complexes with 3′-^{32}P-labeled pUC 18 DNA, as well as the cleavage activity present in nuclear extracts from these cells, was quantified by precipitating the complex with K/SDS as previously described (32). Covalent complex formation with nuclear extracts was quantified with both extract amount (μg of protein) and drug concentration (VP-16) as variables.

Detection of P-Glycoprotein

Northern blot analysis for *mdr1*. *Escherichia coli* containing the pGEM4 plasmid with cDNA probe 5A for the human *mdr1* gene subcloned into the *Eco*RI site (33, 34) was generously provided by Dr. Michael Gottesman, National Cancer Institute (Bethesda, MD). The plasmid was isolated by alkaline lysis and was purified using a CsCl density gradient (35). After *Eco*RI digestion, the 5A insert was purified by electroelution from a 1.2% agarose gel. Nick translation of this probe with the BRL nick translation system (Gaithersburg, MD) using [α-^{32}P]dCTP resulted in a specific activity of 1.5 × 10^8 cpm/μg of DNA. Total cellular RNA was obtained from the CHO cell lines by the guanidinium lysis method, followed by a CsCl step gradient (36). The RNA was electrophoresed on an agarose/formaldehyde gel and transferred to nitrocellulose using the LKB Vacugene blotting pump. Hybridization with ^{32}P-labeled probe 5A was performed overnight at 42° in 5× standard saline citrate (750 mM NaCl, 75 mM sodium citrate, pH 7.0), 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 50 μg/ml salmon sperm DNA, 50% formamide, 10% dextran sulfate (37). Total RNA from the CHO cell lines AuxB1 and CH^RC5 was isolated as negative and positive controls, respectively, for overexpression of *mdr1* mRNA (38).

P-Glycoprotein expression determined by flow cytometry and immunohistochemistry. Logarithmic phase cells (1 × 10^6) were centrifuged at 1000 × *g* for 3 min and washed once with 1 ml of buffer A. The cells were fixed in 1 ml of 70% methanol for 10 min at −20°, pelleted as described above, washed with buffer A and with 1 ml of 10% horse serum, and finally centrifuged at 1000 × *g*. To the pellet were added 5 μl of goat serum, 10 μl of C219 monoclonal antibody (100 μg/ml of buffer A), and 10 μl of buffer A. The control sample received, in addition to the goat serum, 20 μl of mouse IgG1 (50 μg/ml of buffer A). After 45 min at room temperature, the cells were washed twice with 1 ml of 10% horse serum and to the final pellet were added 5 μl of goat serum and 5 μl of a 1/5 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (250 μg/ml of buffer A). After 20 min at room temperature, the cells were washed sequentially with 1 ml of 10% horse serum and 1 ml of buffer A. They were then analyzed with a FACScan instrument for the expression of P-glycoprotein. CH^RC5 cells were used as a positive control for the overexpression of P-glycoprotein. DNA histograms for cell cycle distribution were generated for logarithmic phase WT and resistant CHO cells by flow analysis as described previously (39).

Cultured resistant and sensitive CHO cells were assayed for P-glycoprotein by immunohistochemistry as follows. Cells were fixed in 100% acetone for 10 min at room temperature and the slide was then washed with Automation buffer (Biomeda Corp., Foster City, CA) for 5 min. After incubation with 10% horse serum for 30 min at room temperature, the cells were treated with 50 μl of a 1/200 dilution of the murine C219 monoclonal antibody (in Automation buffer) for 1 hr at 37°. After washing, the slides were incubated with a 1/200 dilution of biotinylated horse anti-mouse immunoglobulin (Vector) for 30 min at room temperature, washed again, and incubated with avidin-biotin complex conjugated to peroxidase (Vector) for 30 min at room temperature. The slides were finally washed, counterstained with hematoxylin, dehydrated through graded alcohols to xylene, and coverslipped with Permount.

Pulse-Field Analysis of *m*-AMSA- and VM-26-Treated WT and SMR_{16} CHO Cells

Logarithmically growing CHO cells (1 × 10^6) were treated with either *m*-AMSA or VM-26 for 30 min at 37°, washed twice with buffer A, and then harvested. The DNA isolated from these cells (5 μg/assay) was analyzed by pulse-field electrophoresis using a modification of a previously reported procedure (40). Electrophoresis was at 150 V for 16 hr with the following pulse times: forward, 0.9 sec; reverse, 0.3 sec; with a ramp of 5.0.

Uptake of [^{3}H]VP-16 and [^{3}H]Vincristine

Equilibrium drug concentrations were determined for logarithmic phase WT and SMR_{16} cells incubated with either [^{3}H]VP-16 (10 or 50 μM) or [^{3}H]vincristine (0.05, 0.1, or 0.5 μM) for 1 hr as described previously (31). The results are expressed as cpm/mg of dry weight of cells.

One- and Two-Dimensional Polyacrylamide Gel Electrophoresis

One-dimensional SDS-polyacrylamide gels, used for immunoblotting or to follow enzyme purification, were run as described by Laemmli (41) and were stained with either Coomassie Brilliant Blue or silver. Protein concentrations were determined by the Bio-Rad protein assay. The presence (or absence) of a nuclear protein that potentially modulates topo II activity was examined by two-dimensional gel electrophoresis. Nuclear extracts (0.35 M and 1.0 M NaCl) of logarithmic phase WT and SMR_{16} cells were analyzed by either isoelectric focusing (pH 4–8) or nonequilibrium pH gradient electrophoresis (pH 5–10) in the first dimension, followed by gradient (7.5–15%) SDS-polyacrylamide gel electrophoresis in the second dimension (42, 43). These gels were stained with either Coomassie Blue or silver. Logarithmic phase sensitive and resistant cells were also labeled for 2 hr with Tran[^{35}S]-label (1000 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA) in methionine-free medium, at 50 μCi/ml of medium. Nuclear extracts of these cells were analyzed by the two-dimensional techniques described above and labeled proteins were visualized by autoradiography. WT and SMR_{16} protein patterns from all gels were directly compared by visual inspection as well as by densitometry localization of the separated proteins.

Presentation of Data

All data are the mean of three to seven experiments and, unless otherwise noted, had an error of <10%. Immunoblots, Northern blots, and agarose gels are representative of five to 10 separate experiments. Topo II was purified to homogeneity from WT and resistant cells on three separate occasions.

Results

Isolation, Growth Characteristics, and Drug Uptake of Resistant CHO-SMR_{16} Cells

Several VP-16-resistant CHO cell lines were generated utilizing the methods described above. Initial cytotoxicity screening experiments demonstrated that the SMR_{16} cell line was 35–40-fold resistant to VP-16; because of this relatively high level of resistance this cell line was chosen for further investigation. This cell line was first cloned by limiting dilution in a 96-well plate and five clones were re-examined for resistance to the cytotoxic and DNA-damaging effects of VP-16. All subsequent experiments have been performed with a cloned population of cells.

The growth characteristics of SMR_{16} cells, relative to WT cells, were first examined (Table 1). The drug-resistant cell line has a doubling time in monolayer culture of 18 hr (compared with 15 hr for parental cells). The SMR_{16} and WT cell lines plateau at cell densities of $\sim 1 \times 10^7$/25-cm^2 flask and $\sim 6 \times 10^6$/25-cm^2 flask, respectively. Quiescence here is defined as >95% viability (by trypan blue dye exclusion) with no net gain in cell number. There is no significant difference in the cell cycle distribution of logarithmic phase cells, as determined by flow cytometry.

Drug uptake studies demonstrated that the equilibrium concentrations of VP-16 in logarithmic phase WT and resistant cells were the same in both monolayer and suspension culture after a 1-hr exposure to this drug (Table 1). The VP-16 drug uptake studies performed with spinner cultures resulted in 4–5-fold more drug uptake than the monolayer cultures for both sensitive and resistant cells. Equilibrium concentrations resulting from a 1-hr exposure to [^{3}H]vincristine were found to be reproducibly diminished by 20–30% in logarithmic phase SMR_{16} cells in suspension, compared with WT cells, when incubated with 0.05 μM, 0.1 μM, and 0.5 μM vincristine.

TABLE 1

Growth and drug uptake characteristics of WT and CHO-SMR_{16} cells

	WT	SMR_{16}
Doubling Time (hr)[a]	14.7 ± 1.23	17.8 ± 1.98
Plateau density ($\times 10^6$)[a, b]	9.67 ± 0.47	5.75 ± 0.83
Cell cycle distribution (%)[c]		
G_1	56.4 ± 0.93	55.2 ± 1.63
S	21.6 ± 3.79	18.9 ± 1.63
G_2/M	22.0 ± 2.54	25.9 ± 1.73
Uptake of [^{3}H]VP-16 (cpm/mg)[a, d]		
10 μM[e]	208	196 (94.2)[f]
50 μM	1,037	989 (95.4)
50 μM[g]	4,631	4,767 (102.9)
Uptake of [^{3}H]vincristine (cpm/mg)[d, g]		
0.05 μM	2,644	1,889 (71.4)
0.1 μM	5,319	3,633 (68.3)
0.5 μM	42,312	33,499 (79.2)

[a] Cells grown in monolayer culture.
[b] Total cell number in 25-cm^2 flask with 10 ml of medium; cells were not refed from initial seeding.
[c] By flow cytometry of logarithmic phase cells.
[d] Results expressed as cpm/mg of dry weight of cells, with a total weight of 3–10 mg; experiments were performed three to five times at each drug concentration, with <10% error.
[e] Extracellular drug concentration.
[f] Percentage of WT uptake.
[g] Cells grown in suspension culture.

TABLE 2

Drug resistance phenotype of CHO-SMR_{16} cells

Antitumor agent	WT IC_{50}	Resistance[a]
	μM	
VP-16	4.00	35.3
VP-16[b]	0.70	31.4
VM-26	0.25	20.8
Doxorubicin	0.15	16.0
m-AMSA	0.08	11.2
Mitoxantrone	0.004	8.8
Daunomycin	0.10	6.7
Vincristine	0.021	3.6
Vincristine[b]	0.003	3.0
Actinomycin D	0.17	2.9
Cisplatin	3.50	1.1
Cytosine arabinoside	0.90	1.0
Camptothecin	0.075	1.0

[a] Fold resistance is the ratio of SMR_{16} cell to WT cell IC_{50} values obtained from colony-forming assays.
[b] In the presence of 10 μM verapamil.

Drug Resistance of SMR_{16} Cells

Colony-forming cytotoxicity assays demonstrated that SMR_{16} cells are 35.3- and 20.8-fold resistant to the cytotoxic effects of the epipodophyllotoxins VP-16 (the antineoplastic agent of selection) and VM-26, respectively (Table 2). This cell line is also cross-resistant to several other classes of DNA topo II inhibitors. It demonstrates 16- and 6.7-fold resistance, respectively, to the anthracyclines doxorubicin and daunomycin. SMR_{16} cells are 11.2-fold resistant to the acridine *m*-AMSA, 8.8-fold resistant to mitoxantrone (an anthracenedione), and 2.9-fold resistant to actinomycin D. This resistant cell line demonstrated no cross-resistance or collateral sensitivity to cisplatin, cytosine arabinoside, or camptothecin (an inhibitor of DNA topoisomerase I). Interestingly, this cell line is 3.6-fold resistant to vincristine, which may be explained at least in part by the decreased uptake of this *Vinca* alkaloid.

The drug resistance phenotype of SMR_{16} cells is characteristic of classic multidrug resistance, which involves overexpression of the efflux pump, P-glycoprotein. Verapamil is known to reverse P-glycoprotein-mediated multidrug resistance; there-

fore, the effects on cytotoxicity of coincubation with 10 μM verapamil (which was not cytotoxic by itself to resistant or WT CHO cells) were examined for VP-16- and vincristine-treated cells (Table 2). Verapamil did lower the IC_{50} for both WT and SMR_{16} cells, but the level of resistance of the SMR_{16} cells was unaffected, suggesting that sensitization of WT and resistant cells to the cytotoxic effects of VP-16 and vincristine by verapamil was equivalent. Data presented below demonstrate that both the parental and drug-resistant cell lines do have a basal level of P-glycoprotein expression but that SMR_{16} cells do not overexpress, relative to WT cells, this efflux pump.

The CHO-SMR_{16} cell line has been maintained in monolayer culture in the absence of selecting agent for >1 year. Monthly colony-forming cytotoxicity assays and alkaline elution studies demonstrated that the resistance to VP-16-induced cytotoxicity and DNA damage remained unaltered during this time period. Less frequent colony-forming assays also demonstrated that the resistance to other topo II inhibitors, as well as the resistance to vincristine, was stable during this same 1-year period.

Role of P-Glycoprotein in the Multiple Drug Resistance of SMR_{16} Cells

The results of the cytotoxicity assays described above (with or without verapamil) suggested that the multiple drug resistance of the SMR_{16} cell line was not likely due to overexpression of the *mdr1* gene. However, the reduction in uptake of vincristine, as well as the pattern of cross-resistance, was more compatible with this classic efflux mechanism. To resolve this issue, more definitive experiments were undertaken to examine the expression of the *mdr1* gene and its product. WT, control, and drug-resistant CHO cells were first examined for the expression of *mdr1* mRNA by Northern blot analysis of total RNA (Fig. 1). The P-glycoprotein-positive CHO cell line CH^RC5 was found to overexpress *mdr1* RNA, whereas the SMR_{16} cells had no increased expression above that seen in WT and AuxB1 control CHO cells. However, all three of these CHO cell lines did have a minimal expression of *mdr1* mRNA.

The expression of P-glycoprotein was also examined in these same cell lines by both flow cytometry and immunohistochemistry with the murine monoclonal antibody C219 (data not shown). The results paralleled the Northern analysis, i.e., a significant overexpression of P-glycoprotein in CH^RC5 cells was observed, whereas minimal but equivalent amounts of P-glycoprotein were found in WT and SMR_{16} cells. Therefore, it is likely that the basal production of P-glycoprotein in both WT and SMR_{16} cells is responsible for the similar augmentation of cytotoxicity by both VP-16 and vincristine in the presence of verapamil. However, the multidrug resistance of SMR_{16} cells is not explained by an abundance of this efflux pump.

Assays of DNA Topo II Activity

The results of the experiments described above suggested that the drug resistance of the CHO-SMR_{16} cell line might be due to either a quantitative or qualitative alteration in nuclear DNA topo II. The following experiments were undertaken to examine this possibility.

Immunoreactive DNA topo II in SMR_{16} cells. To determine whether a reduction in nuclear topo II contributed to the drug resistance of SMR_{16} cells, immunoblots of nuclear extracts and whole cells with various anti-topo II antibodies were prepared. Western blots of 0.35 M and 1.0 M NaCl nuclear extracts from WT and SMR_{16} cells with either our polyclonal mouse anti-HeLa cell topo II antibody (39) or polyclonal rabbit anti-recombinant HeLa topo II antibody (3) reproducibly demonstrated 1.5–1.8-fold more DNA topo II in the WT cell extracts (Fig. 2). Immunoblots of logarithmic phase whole cells (1×10^6) confirmed that the sensitive cells contain 1.5-fold more topo II, compared with the drug-resistant cells, and, therefore, that the alteration seen in nuclear extracts was not an artifact of the extraction procedure (Fig. 2). The enhanced chemiluminescence method for enzyme detection was found to be superior, in that it is more sensitive than the alkaline phosphatase assay and allows more precise quantitation of enzyme amount (by densitometric scanning of the exposed transparent film). Immunoblots of whole cells with polyclonal antibodies directed against the p170 and p180 forms of the enzyme (6) resulted in the same findings as described above for the p170 form. However, logarithmic phase sensitive and resistant cells contain <5% of total topo II as the p180 form (data not shown).

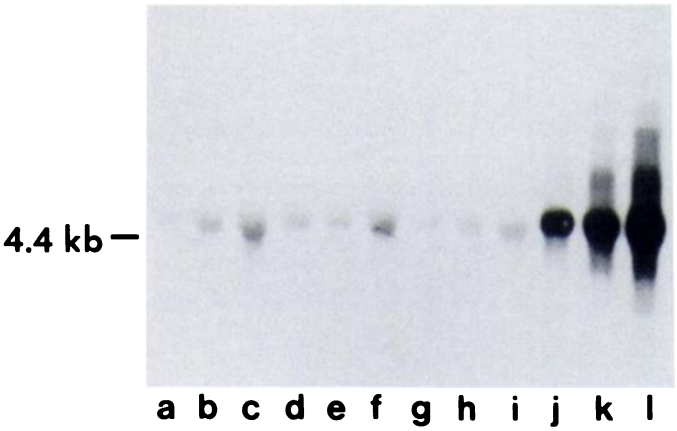

Fig. 1. Northern blot analysis of total RNA from sensitive and resistant CHO cells for overexpression of *mdr1* mRNA. Total RNA was extracted from WT (*lanes a-c*), SMR_{16} (*lanes d-f*), AuxB1 (*lanes g-i*), and CH^RC5 (*lanes j-l*) CHO cells and probed with ^{32}P-labeled probe 5A for *mdr1* mRNA. The amount of RNA loaded on the gel was 5 μg (*lanes a, d, g,* and *j*), 10 μg (*lanes b, e, h,* and *k*), or 15 μg (*lanes c, f, i,* and *l*). The P-glycoprotein-positive CH^RC5 cells demonstrate a significant increase in the *mdr1* message.

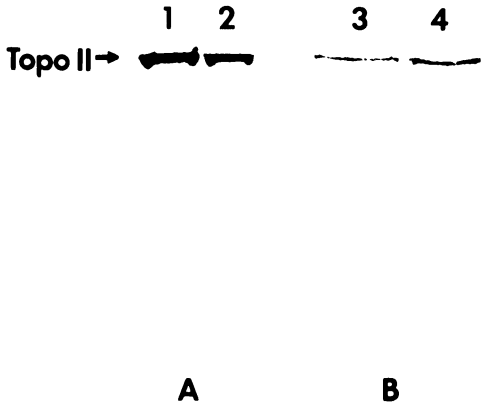

Fig. 2. Western blot electrophoresis of drug-resistant and drug-sensitive whole cells and nuclear extracts. One million logarithmic phase whole cells (A) or 100 μg of protein from 0.35 M NaCl nuclear extracts (B) were immunoblotted with a rabbit polyclonal anti-topo II antibody. WT whole cells and nuclear extracts (*lanes 1* and *4*) were found to have ~1.5-fold greater topo II content, compared with SMR_{16} cells (*lanes 2* and *3*). Topo II was quantified by densitometric scanning of immunoblots developed with either the enhanced chemiluminescence (A) or alkaline phosphatase (B) method.

Drug-induced DNA damage in whole cells and isolated nuclei. Immunoblots of whole cells and nuclear extracts demonstrated that SMR_{16} cells contain approximately two-thirds of the WT concentration of topo II. To determine whether topo II activity paralleled this attenuation of enzyme content, drug-induced DNA scission was measured *in situ* in whole cells and isolated nuclei. DNA damage was quantified as single-strand DNA breaks in logarithmic phase sensitive and resistant cells and nuclei by the technique of alkaline elution. Cells and nuclei were treated with 50 μM VP-16 for 1 hr and 30 min, respectively, and the amount of DNA damage induced by this topo II inhibitor was quantified as rad equivalents (Table 3). SMR_{16} cells and nuclei were found to be 10- and 12-fold resistant, respectively, to the DNA-damaging effects of VP-16, compared with WT cells and nuclei. Similar results were also obtained when the resistant cells were treated with *m*-AMSA (0.05–1.0 μM for 30 min), mitoxantrone (1–10 nM for 1 hr), or doxorubicin (0.01–1.0 μM for 1 hr); again, a marked reduction in DNA damage was observed. These elution studies demonstrate that the resistance of SMR_{16} cells to the DNA-damaging effects of topo II inhibitors is not directly proportional to the decrease in topo II observed by immunoblotting. In addition, the drug resistance of this cell line appears to reside in the nucleus of SMR_{16} cells.

Topo II catalytic and cleavage activity in nuclear extracts. The decatenation activity present in 0.35 M NaCl nuclear extracts obtained from WT and drug-resistant SMR_{16} cells was assayed by agarose gel electrophoresis of minicircles released from kDNA networks (Fig. 3). These results parallel those obtained with immunoblotting; there was a <2-fold difference in catalytic activity. Approximately 500 ng of nuclear extract protein from the SMR_{16} cell line were required to completely decatenate 1 μg of kDNA, whereas only 250 ng of WT protein were needed to decatenate the same amount of kDNA. DNA topo I activity present in nuclear extracts was also measured by relaxation of supercoiled pUC 18 DNA in the absence of ATP. The resistant and WT extracts were found to have equivalent topo I catalytic activity (data not shown), which correlates with their sensitivity to the cytotoxic effects of camptothecin (Table 2).

The formation of covalent complexes between the DNA topo II present in 0.35 M NaCl nuclear extracts and 3′-^{32}P-labeled DNA, in the presence of VP-16, was also determined for sensitive and resistant cell nuclear extracts (Fig. 4). When complex formation was assayed as a function of nuclear extract amount, in the presence of 100 μM VP-16, it was found to be maximal for both resistant and sensitive 0.35 M NaCl nuclear extracts at 0.5–1 μg of protein. This was also observed for 1.0 M NaCl nuclear extracts (data not shown). The covalent DNA-enzyme complex formation observed when SMR_{16} nuclear extracts were the source of topo II was ~2-fold less at all extract concentrations (compared with WT extracts), which roughly agrees with both the immunoblotting (Fig. 2) and decatenation (Fig. 3) data. Covalent DNA-topo II association was also measured with a fixed amount of nuclear extract (1.0 μg), with the concentration of VP-16 being varied from 0 to 200 μM (Fig. 4, *inset*). Again, ~2-fold more complex was precipitated from WT extracts.

TABLE 3

VP-16-induced DNA damage in WT and CHO-SMR_{16} cells and isolated nuclei

DNA scission was induced by 50 μM VP-16 and measured as single-strand breaks by alkaline elution. DNA damage is expressed in rad equivalents (cGy) and the values are the average of three separate experiments, with <10% error.

	DNA damage	
	WT	CHO-SMR_{16}
	rad equivalents	
Whole cells[a]	2780	280 (9.9)[b]
Nuclei	1310	108 (12.1)

[a] Whole cells were incubated with VP-16 for 1 hr; intact nuclei were treated with VP-16 for 30 min.

[b] Fold resistance of CHO-SMR_{16} cells to VP-16-induced DNA damage.

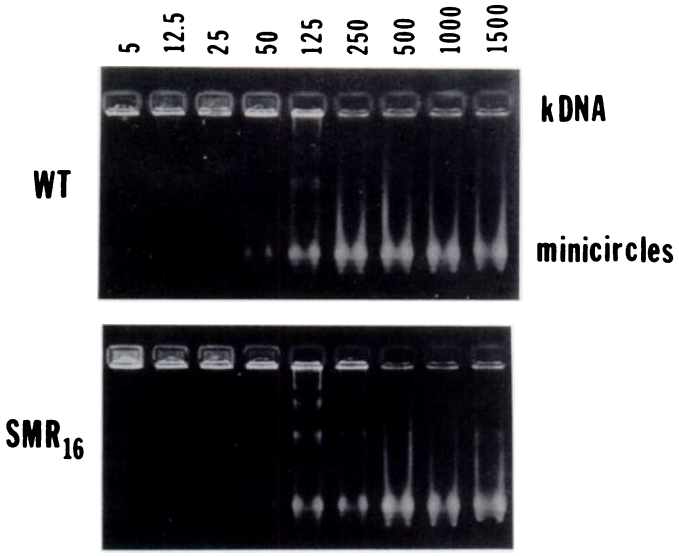

Fig. 3. DNA topo II catalytic activity present in 0.35 M NaCl nuclear extracts from logarithmic phase WT and SMR_{16} cells. The ability of nuclear extracts from sensitive and resistant cells to decatenate 1 μg of kDNA was measured by agarose gel electrophoresis of the released minicircles. *Numbers at the top of the gel*, amount of nuclear extract protein (ng) used in the assay.

Properties of purified SMR_{16} topo II. To determine whether the DNA topo II present in the resistant cells was qualitatively different from the WT enzyme, it was purified to homogeneity from 1.0 M NaCl nuclear extracts of resistant and WT cells and the physical and enzymatic properties of these enzymes were compared. Topo II from the resistant and sensitive cell lines was purified by an FPLC procedure that uses sequential hydroxylapatite, anion exchange, and cation exchange column chromatography; this method has been described in detail elsewhere (3). The two enzymes were purified to >98% homogeneity, as determined by densitometric scanning of SDS-polyacrylamide gels stained with silver (data not shown).

The enzymes from the resistant and sensitive cell lines were found to have the same monomer molecular mass of 170 kDa. The catalytic activity of the purified WT and SMR_{16} enzymes was measured by decatenation of [^{3}H]kDNA networks (Fig. 5). The enzymes were found to have the same specific activity in this assay. The thermal stability of the purified enzymes was also examined by preincubating topo II at 37° for 0–120 min and then performing a standard decatenation assay. The WT and SMR_{16} enzymes were similar, in that they both lost total catalytic activity after a 60-min preincubation (data not shown). Drug-induced covalent complex formation between purified topo II and 3′-^{32}P-labeled DNA was also quantified

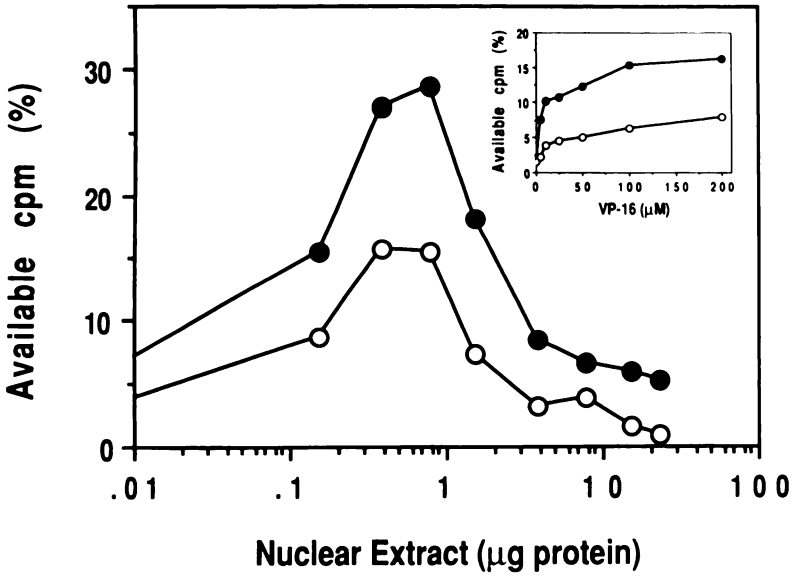

Fig. 4. Formation of covalent complexes between the topo II present in 0.35 M NaCl nuclear extracts of WT and resistant cells and 3′-^{32}P-labeled pUC 18 DNA. Complex formation was assayed at a fixed concentration of VP-16 (100 μM), with the amount of nuclear extract from WT (●) and SMR$_{16}$ (○) cells being varied from 0.1 to 25 μg of protein. *Inset*, covalent association between enzyme and DNA at a fixed amount of nuclear extract (1 μg for both cell lines) with VP-16 concentrations of 2.5–200 μM.

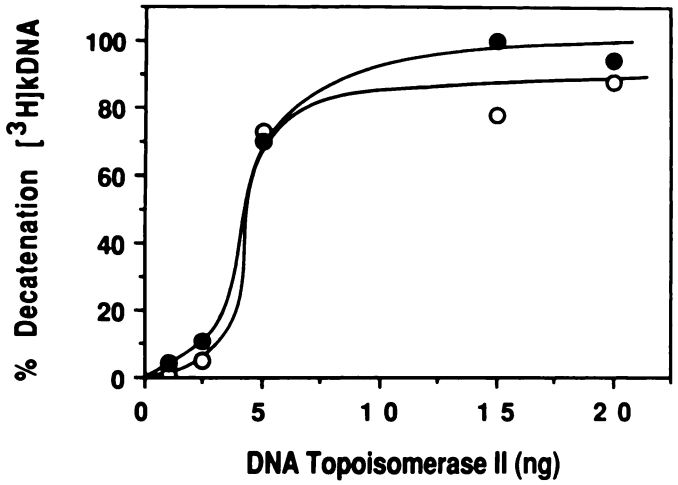

Fig. 5. Catalytic activity of homogeneous WT and SMR$_{16}$ DNA topo II. Topo II was purified from resistant (○) and parental (●) CHO cells and assayed for its ability to decatenate [^{3}H]kDNA networks. The two purified enzymes were found to have the same specific activity.

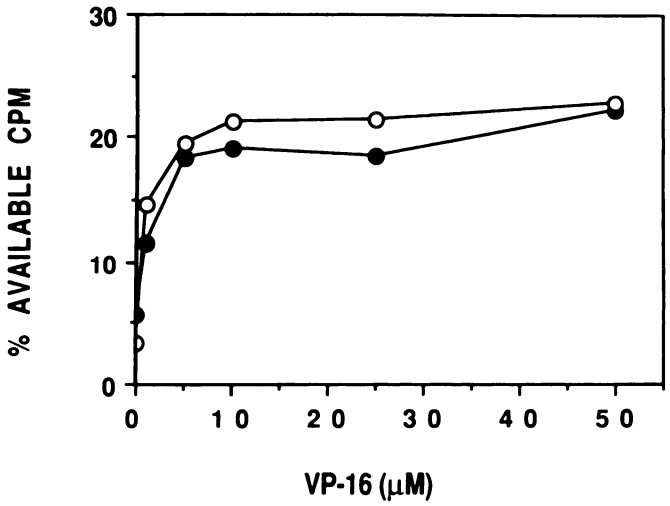

Fig. 6. Formation of covalent complexes between DNA and purified WT and SMR$_{16}$ topo II. The cleavage activity of homogeneous WT (●) and resistant (○) topo II was measured as covalent complex formation by the K/SDS assay, as described in Experimental Procedures. The amount of purified enzyme used in this assay was 35 ng for both the sensitive and resistant cell lines.

using the K/SDS method (Fig. 6). Both enzymes (35 ng of purified protein each) demonstrated the same maximal covalent complex formation, which occured at concentrations of VP-16 of ≥10 μM. In short, the DNA topo II purified from 1.0 M NaCl nuclear extracts of drug-resistant SMR$_{16}$ cells appears to have the same properties as homogeneous WT topo II.

Analysis of Drug-Induced DNA Damage by Pulse-Field Electrophoresis

The assays of DNA topo II activity described above for nuclear extracts and homogeneous preparations of the enzyme from CHO-SMR$_{16}$ cells did not explain the resistance of this cell line to either the cytotoxic effects of VP-16 (35-fold resistant) or the DNA-damaging effects of VP-16 seen by alkaline elution (10–12-fold resistant). To explore the possibility that a subpopulation of topo II (perhaps not extracted with 0.35 or 1.0 M NaCl) is involved in the drug resistance of SMR$_{16}$ cells, logarithmic phase WT and resistant cells were treated with either VM-26 or *m*-AMSA and the cleaved chromosomal DNA was subsequently analyzed by pulse-field gel electrophoresis (Fig. 7). These drugs were found to result in cleavage of WT CHO chromosomal DNA into 50–150-kb fragments. These fragments likely represent topological domains in chromosomes that are bound by topo II. When the DNA from SMR$_{16}$ cells was analyzed, essentially no 50–150-kb fragments were observed after treatment of the drug-resistant cells with either 25 μM VM-26 or 25 μM *m*-AMSA. Similar results were obtained when WT and SMR$_{16}$ nuclei, isolated by Dounce homogenization (3), were treated with 25–200 μM VP-16 (data not shown). These experiments demonstrated a correlation between the dose of VP-16 and production of 50–150-kb fragments in both WT and SMR$_{16}$ nuclei. Ten-fold more VP-16 was required by SMR$_{16}$ nuclei to give the same level of cleavage as in WT nuclei. These data suggest that the population of topo II responsible for creating the 50–150-kb DNA fragments is resistant to drug action in the SMR$_{16}$ cell line (see Discussion).

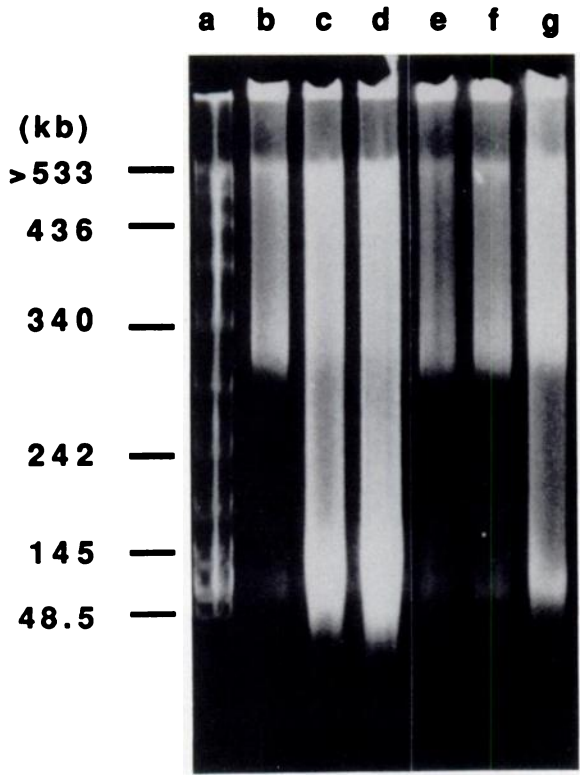

Fig. 7. Pulse-field analysis of DNA from drug-treated WT (*lanes b-d*) and SMR_{16} (*lanes e-g*) CHO cells. Logarithmic phase cells were treated with solvent alone (*lanes b* and *e*), with 25 μM VM-26 (*lanes c* and *f*), or with 25 μM *m*-AMSA (*lanes d* and *g*) and the DNA was isolated and analyzed as described in Experimental Procedures. *Lane a*, λ ladder marker DNA.

Discussion

The reaction mechanism of DNA topo II has been shown to involve the following five steps (44, 45): (i) recognition and binding of the enzyme to its DNA substrate, (ii) cleavage of double-stranded DNA, resulting in covalent phosphotyrosyl bonds between the 5′ end of each strand and a topo II monomer, (iii) passage of a second DNA duplex through the break site, (iv) religation of the cleaved DNA, and (v) ATP-dependent enzyme turnover. Topo II inhibitors have been shown to block the religation step, resulting in an increase in covalent enzyme-DNA complexes, which are measured as DNA strand breaks (46). Studies suggest that drug-augmented covalent complex formation prevents replication fork progression and that this is the basis for the cytotoxicity exhibited by these agents (47).

Resistance to topo II inhibitors can involve several different mechanisms (1) but broadly includes either a quantitative or a qualitative alteration in the enzyme target, topo II. Perturbations in enzyme amount have been shown to involve decreased topo II production [e.g., inactivation of a normal topo II allele (9)], increased degradation of topo II (48), and quantitative changes that are cell cycle or proliferation dependent (39, 49). A direct correlation between the degree of drug resistance to topo II poisons and the intracellular content/activity of topo II has recently been reported (10). However, the 35-fold resistance of SMR_{16} cells to VP-16 is not explained solely by the modest decrease (<2-fold) in topo II content/activity seen in whole cells and nuclear extracts, although the attenuation of a certain population of enzyme molecules might result in a higher level of resistance (see below).

Alterations in the topo II enzyme itself that result in a multiple drug-resistant phenotype have also been reported. For example, the apparent resistance of homogeneous CHO Vpm^R-5 topo II to VP-16-induced DNA cleavage likely results from enzyme insensitivity to drug inhibition of DNA religation (3). The resistance of HL-60/AMSA topo II to *m*-AMSA-induced cleavage appears to be due to a mutation (arginine to lysine at amino acid 486) in the resistant topo II gene (4, 5), and the altered cleavage and catalytic activity of the topo II present in drug-resistant CEM/VM-1–5 cells appear to be the result of a similar mutation (arginine to glutamine at amino acid 449) (2). In the present study, homogeneous CHO-SMR_{16} topo II was found to have the same molecular mass, the same decatenation and drug-induced cleavage activities, and similar thermal stability as purified WT enzyme. These results suggest that the "resistant" enzyme, or at least the topo II purified from 1.0 M NaCl nuclear extracts, is unaltered and has the same enzymatic properties as WT topo II.

The experiments described herein also demonstrate that the resistance of SMR_{16} cells to VP-16 is not due to perturbations in transport of this drug. The low level of resistance to vincristine may, however, be due to decreased uptake of this *Vinca* alkaloid in the resistant cells. The cytotoxicity experiments with vincristine and VP-16, each in the presence of 10 μM verapamil, suggest that the resistance of SMR_{16} cells is not the result of an overabundance of the drug efflux pump, P-glycoprotein. This was confirmed by both flow cytometry and immunohistochemistry analyses for the protein, as well as by Northern blots that failed to demonstrate overexpression of *mdr1* mRNA. Finally, immunoblots with rabbit polyclonal antibodies to the p170 and p180 isoforms of topo II demonstrated the same results for the 170-kDa monomer as our rabbit polyclonal antibody, but very little p180 was detected in either the resistant or sensitive cell line. It is unlikely that an altered ratio of topo II isoforms contributes significantly to the resistance of SMR_{16} cells, but it is possible that the antibodies used in these experiments did not recognize a resistant topo II species from these cells.

The mechanism of resistance of CHO-SMR_{16} cells may involve an alteration in a specific population of DNA topo II molecules that are involved in the formation or packaging of chromosomes. Recent investigations have shown that loops of DNA are anchored into the nuclear matrix by proteins, most likely DNA topo II (25–27, 50). DNA fragments are formed because topo II molecules specifically integrate into DNA at matrix-attachment regions; these fragments are thought to represent entire DNA loops of 50–300 kb. Indeed, when primary thymocyte cultures were treated with *m*-AMSA and VM-26, DNA fragments of 50 and 300 kb, respectively, were observed by pulse-field gel electrophoresis (25). Investigators have also proposed that the antiproliferative effects of topo II inhibitors may be localized to the nuclear matrix (11, 28). The drug-resistant CEM/VM-1 cell line has been shown to have the same 1.0 M NaCl-extractable (nonmatrix) topo II as parental cells but 3-fold less matrix topo II by immunoblotting, as well as a 6–7-fold decrease in matrix topo II catalytic activity.

In the present study, WT CHO cells and nuclei treated with VM-26, VP-16, or *m*-AMSA resulted in DNA fragments of 50–150 kb (Fig. 7), which probably represents an entire DNA domain. In contrast, CHO-SMR_{16} cells and nuclei were found to be very resistant to DNA fragmentation by these same topo

II inhibitors. Virtually no 50–150-kb fragments were seen. This may well be the basis for the multiple drug resistance of this cell line, because these findings agree with the relatively high levels of resistance to VP-16 seen in both cytotoxicity experiments and alkaline elution assays of intact cells and isolated nuclei.

When all of the present experimental results are considered, three possible explanations for the drug resistance of SMR$_{16}$ cells are suggested. First, there is the possibility that, in addition to the modest decrement in nuclear topo II content, there is a factor extrinsic to topo II that modulates its cleavage activity and contributes to the resistance of this cell line. Two recent sets of experiments make this the least likely model. First, an exhaustive search for the absence or presence of a protein in both 0.35 M and 1.0 M NaCl nuclear extracts by two-dimensional gel electrophoresis failed to reveal a difference between resistant and sensitive cell extracts (data not shown). Separate gels of each extract molarity were run with both isoelectric focusing and nonequilibrium pH gradient electrophoresis as the first dimension and were analyzed by inspection of gels after both silver and Coomassie blue staining, as well as by inspection of autoradiograms generated from extracts labeled with ^{35}S. The second set of experiments examined the inhibition of homogeneous WT topo II DNA cleavage activity (in the presence of VP-16) by 1.0 M NaCl nuclear extracts of both resistant and sensitive cells. The cleavage of 3′-^{32}P-labeled pUC 18 DNA by purified WT topo II was not inhibited by either extract; rather, the total cleavage was the expected additive result (data not shown). Thus, it is unlikely that a protein factor modulates topo II activity in SMR$_{16}$ cells; however, we have not excluded an alteration in phosphorylation of the enzyme as a mechanism of control. The second possibility is that the topo II responsible for DNA cleavage (at the nuclear matrix) is resistant to drug action such that DNA loops are not generated by treatment of the cells with VM-26 or *m*-AMSA. Certainly the topo II purified from SMR$_{16}$ cells is not resistant to drug-induced cleavage (Fig. 6), nor is the topo II present in nuclear extracts resistant to VP-16-induced cleavage beyond the resistance accounted for by the attenuation of topo II content and activity (Figs. 2 and 4). If the enzyme is resistant to drug-induced cleavage, it is either a population of topo II that is firmly bound to the nonextractable matrix or a salt-extractable population that is drug resistant by virtue of its association with DNA in the matrix. Finally, it is possible that the topo II that fragments DNA into discrete domains in the WT nucleus is simply not present in the nuclear matrix of the drug-resistant cells. Perhaps the one third reduction in topo II seen in the SMR$_{16}$ cells is accounted for, in part, by an attenuation of matrix topo II. Initial experiments with nuclear matrices, isolated from WT and SMR$_{16}$ cells as described previously (11, 28), suggest that the amount of topo II (by immunoblot) is decreased in the matrices of the drug-resistant nuclei (data not shown).

The CHO-SMR$_{16}$ cell line is unique in that its multiple drug resistance is the result of an alteration in a specific population of DNA topo II, that subset of target molecules intimately associated with the nuclear matrix that cleave DNA into topological domains in the presence of topo II-active drugs. Further studies of this topo II population, including its interaction with topo II inhibitors, will likely determine which model described above is involved in the drug resistance of these cells.

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Send reprint requests to: Daniel M. Sullivan, James Graham Brown Cancer Center, 529 South Jackson Street, University of Louisville, Louisville, KY 40292.