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Reduced levels of topoisomerase IIα and IIβ in a multidrug-resistant lung-cancer cell line

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Abstract. We have previously shown that the doxorubicin-selected multidrug-resistant small-cell lung-cancer cell line H69AR is resistant to VP-16-induced single-strand DNA breaks as compared with its parental H69 cell line. Levels of immunoreactive topoisomerase IIα are also reduced in H69AR cells. In the present study, we found that cleaved complex formation in the presence of VP-16 was decreased in H69AR cells as compared with H69 cells. In addition, the resistant cells contained lower levels of both topoisomerase IIα and topoisomerase IIβ protein and mRNA. However, these changes were not accompanied by a decrease in the P4-unknotting (strand-passing) activity of 0.67 *M* NaCl nuclear extracts of H69AR cells, nor was there any difference in VP-16 inhibition of unknotting activity in the H69 and H69AR nuclear extracts. These data suggest that reduced levels of topoisomerase IIα and IIβ may contribute to the resistance of H69AR cells to VP-16 and other drugs that target these isoenzymes.

Introduction

Small-cell lung cancer (SCLC) accounts for 20%–25% of all lung cancers, and although up to 90% of patients will initially respond well to chemotherapy, the majority of afflicted individuals will ultimately relapse with multidrug-resistant disease. Although some advances have been made in treatment, acquired drug resistance in SCLC remains a major obstacle to cure and long-term survival remains low at only 10% [45].

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The doxorubicin-selected H69AR SCLC cell line derived in this laboratory displays a typical multidrug-resistance phenotype but does not overexpress P-glycoprotein [11, 30]. This finding is consistent with a number of clinical studies that have not shown a correlation between the elevated expression of P-glycoprotein and multidrug resistance in SCLC [4, 28, 31]. The cross-resistance pattern of H69AR includes many natural products that exert their cytotoxicity through multiple subcellular targets [9, 10, 30]. In addition to the vinca alkaloids that target microtubules, H69AR is resistant to agents such as VP-16 and doxorubicin that exert their cytotoxic action, at least in part, by interacting with topoisomerase II (topo II, EC 5.99.1.3). This enzyme interconverts DNA topoisomers by introducing a double-strand break in a DNA segment and allowing the passage of another DNA helix through the break followed by religation [33]. Drugs that target topo II appear to act by preventing religation of the DNA, thus stabilizing the double-strand break and the topo II-DNA covalent complex [2, 32]. Decreases in cleaved-complex formation and double-strand breaks have been correlated with decreases in cytotoxicity, although the events that follow cleaved-complex formation and lead to cell death remain to be fully elucidated [2, 3, 47, 48]. Drug resistance that is associated with topo II may result from any process that reduces the formation of cleaved complexes [17]. This can occur via a decrease in the amount of topo II or an alteration in the enzyme such that the interaction of topo II with either drug or DNA is altered.

There are two highly homologous isoforms of human topo II that have been designated topo IIα (170 kDa) and topo IIβ (180 kDa) [8, 20, 27]. They are encoded by two comigrating 6.5-kb mRNAs [8], but their cognate genes are located on different chromosomes [41, 43]. Some investigators have found that topo IIα activity is more sensitive to inhibition by several drugs, including VP-16 [21], and there is some evidence that the two isoforms may be differentially regulated during changes in cell cycle and proliferation [21, 38, 46]. These and other differences strongly suggest that the topo IIα and topo IIβ isoenzymes

carry out different cellular functions and, therefore, that the role of each isoform in drug resistance may differ [34].

In a previous study, we observed that H69AR cells and isolated nuclei were resistant to VP-16-induced single-strand DNA breaks as compared with parental H69 cells [11]. We also found that there was less immunoreactive topo IIα in the H69AR cells. These observations suggested that changes in topo II function might contribute to the drug resistance in H69AR cells. In the present study, we extended our earlier investigations by comparing cleaved-complex formation and the topo II strand-passing activity in the two cell lines and determining the relative levels of topo IIα and IIβ protein and mRNA using isoform-specific antibodies and cDNA probes.

Materials and methods

Drugs and chemicals. VP-16 was obtained from the Kingston Regional Cancer Centre pharmacy and the vehicle control was obtained from Bristol-Myers (Syracuse, N.Y.). [methyl-^{3}H]-Thymidine (sp. act., 6.7 Ci/mmol), [^{14}C(U)]-leucine (sp. act., 325 mCi/mmol), and [α-^{32}P]-deoxycytidine triphosphate (dCTP; sp. act., 3,000 Ci/mmol) were obtained from NEN-Dupont (Markham, Ont.). Herring testes (HT) DNA sodium salt (type XIV), ethylene glycol tetraacetic acid (EGTA), adenosine triphosphate (ATP), aprotinin, and 3-(cyclohexylamino-1-propanesulfonate) (CAPS) were obtained from Sigma Chemical Co. (St. Louis, Mo.). The protease inhibitors pepstatin, benzamidine, antipain dihydrochloride, and leupeptin were obtained from Boehringer Mannheim (Lavel, Que.). Phenylmethylsulfonyl fluoride (PMSF) was obtained from Aldrich (Milwaukee, Wis.), and DNase I and RNase A were obtained from Pharmacia (Baie d'Urfe, Que.). Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Zymed Laboratories Inc. (San Francisco, Calif.), and the enhanced chemiluminescence (ECL) detection system was obtained from Amersham (Oakville, Ont.). The BRL random primers DNA Labelling System was obtained from Gibco-BRL (Burlington, Ont.). Guanidine HCl was supplied by ICN Biomedicals Inc. (Cleveland, Ohio), and formamide was obtained from BDH Inc. (Toronto, Ont.). All other chemicals were of reagent grade and were purchased from common commercial suppliers.

Cell culture. The human SCLC cell line H69 was obtained from Drs. A. Gazdar and J. Minna (NCI, Bethesda, Md.). H69AR was derived in this laboratory by stepwise selection of H69 in doxorubicin [30]. All cell lines were routinely cultured in 125-ml glass bottles in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 5% defined/supplemented bovine calf serum (Hyclone Laboratories, Logan, Utah) and 4 m*M* L-glutamine and were maintained at 37° C in a 95% air/5% CO_2 atmosphere without antibiotics. The H69AR cells were challenged monthly with 0.8 μ*M* doxorubicin and drug was removed at least 48 h prior to each experiment. Cell viability was determined by trypan blue exclusion.

Sodium dodecyl sulfate-KCl assay of topo II cleavage activity. The sodium dodecyl sulfate (SDS)-KCl precipitation assay was performed according to the method of Trask and Muller [42] as modified by Zwelling et al. [49]. Exponentially growing cells were incubated with [^{14}C]-leucine (0.2 μCi/ml) and [methyl-^{3}H]-thymidine (0.6 μCi/ml) for 18–20 h. After removal of the medium and resuspension in phosphate-buffered saline (PBS), aliquots of cells were incubated in various concentrations of drug or appropriately diluted vehicle for 30 min at 37° C. To each tube, warm (37° C) stop solution [2.5% SDS, 10 m*M* ethylenediaminetetraacetic acid (EDTA), and 0.8 mg HT DNA/ml] was added. The lysate was passed through a 22-gauge needle ten times and then heated to 65° C for 15 min. To precipitate the protein, 1 *M* KCl was added and the precipitate was washed three times with wash solution (10 m*M* TRIS-HCl, pH 8.0; 100 m*M* KCl, 1 m*M* EDTA, 0.1 mg HT DNA/ml). At each washing step the pellet was dissolved at 65° C and precipitated on ice. The washed pellet was dissolved in water at 65° C and briefly centrifuged to sediment any particulate material. The supernatant was transferred to a scintillation vial and the radioactivity was determined. The ratio of ^{3}H to ^{14}C (which reflects the ratio of precipitated DNA to precipitated protein) was calculated and the data were expressed as the *X*-fold increase in ^{3}H:^{14}C ratios of drug-treated cells relative to vehicle-treated cells.

Immunoblots of topo IIα and IIβ protein. Exponentially growing cells were incubated at 4° C for 60 min in 0.25% SDS, 0.5 mg DNase I/ml, 0.25 mg RNase A/ml, 10 m*M* $MgCl_2$, and 50 m*M* TRIS (pH 7.4) with the following protease inhibitors: antipain dihydrochloride, 50 μg/ml; aprotinin, 2 μg/ml; EDTA, 200 μg/ml; benzamidine, 200 μg/ml; leupeptin, 0.5 μg/ml; pepstatin, 1 μg/ml; and PMSF, 2 m*M*. Proteins were solubilized in 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.6 *M* TRIS (pH 7.5) with protease inhibitors at 68° C for 5 min. The solubilized whole-cell lysates were electrophoresed on 7% polyacrylamide gels and electroblotted onto Immobilon-P membrane (Millipore, Mississauga, Ont.) in 50 m*M* CAPS (pH 11). Topo IIα was detected with a rabbit antisera raised against the recombinant 70-kDa C-terminus of HeLa topo IIα (kindly provided by Drs. W. Ross and D. Sullivan) [39]. Topo IIβ was detected with affinity-purified rabbit antisera (FHD21) raised against a synthetic peptide corresponding to a region of the topo IIβ cDNA sequence unique to the topo IIβ isoform (kindly provided by Dr. F. Drake) [8]. Antibody binding was visualized using an ECL detection system. The resulting X-ray films were scanned by densitometry to estimate the relative levels of topo IIα and IIβ protein present in each cell line. Immunoblots were carried out on three independent whole-cell lysates.

Northern-blot analyses of topo IIα and IIβ mRNA. Total RNA was isolated from exponentially growing H69 and H69AR cells using the guanidine-HCl/sodium acetate method [18]. RNA (approximately 10 μg) from each cell line was electrophoresed on 1%-agarose/0.66-*M* formaldehyde gels and capillary-blotted onto nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) with 20X SSC (150 m*M* NaCl, 15 m*M* sodium citrate, pH 7.0) [36]. Blots were baked under vacuum at 80° C and then prehybridized at 42° C in hybridization buffer [50% formamide; 4X Denhardt's solution (1% bovine serum albumin, BSA; 1% polyvinylpyrrolidine; 1% ficoll), 5X SSPE (150 m*M* NaCl, 10 m*M* NaH_2PO_4, 1 m*M* EDTA, pH 7.4), 100 μg HT DNA/ml, 1% SDS] at 2 ml/cm^2 for 4–6 h. Topo IIα mRNA was detected with the *Eco*RI/*Sca*I 3.1-kb cDNA insert of SP-1, and topo IIβ mRNA was detected with the *Eco*RI/*Pst*I 1.8-kb cDNA insert of SP-12 [8] (kindly provided by Dr. K. B. Tan). Sample loading was quantitated using the β-actin-specific cDNA pBV2.1 [44]. All cDNA probes were used at 2–4×10^6 cpm/ml after labeling with [α-^{32}P]-dCTP to a specific activity of 1×10^8 cpm/μg. Blots were hybridized with the individual probes at 42° C for 18–20 h. Blots were washed (1X SSC, 0.1% SDS) for 15 min at room temperature, followed by three washes (0.1X SSC, 0.1% SDS) for 30 min at 52° C, and were then used to expose Kodak X-Omat AR film at –70° C. The relative intensities of the H69 versus H69AR bands on the autoradiograms were determined by densitometry and corrected for RNA loading according to the intensity of the β-actin band. Three independent RNA preparations were assessed for topo IIα and IIβ mRNA levels.

P4-unknotting assay of topo II catalytic activity. Unknotting activity was measured as the ability of nuclear extracts to unknot double-stranded knotted P4 DNA [14, 29]. The P4 DNA substrate was prepared from the P4 bacteriophage (kindly provided by Dr. Y. C. Cheng) according to the method of Lui et al. [29] as modified by Danks et al. [14]. Nuclear extracts were prepared in 0.67 *M* NaCl from exponentially growing cells according to the method of Sullivan et al. [38] as modified by Danks et al. [14].

To compare the unknotting activity of H69 and H69AR nuclear extracts, various concentrations of nuclear extract protein were added to knotted P4 DNA (0.3 μg) and reaction buffer (50 m*M* TRIS-HCl, pH 7.7; 100 m*M* KCl; 10 m*M* $MgCl_2$; 0.5 m*M* dithiothreitol; 0.5 m*M* EDTA; 30 μg BSA/ml; and 1 m*M* ATP). In a second set of experi-

ments, the ability of VP-16 to inhibit the unknotting activity of the nuclear extracts was examined. Various concentrations of VP-16 were added to nuclear extract, P4 DNA (0.3 μg), and reaction buffer. The reaction mixtures were incubated at 37° C for 30 min, after which reactions were terminated by a 2.5-h incubation in 1% SDS, 150 μg proteinase K/ml, and 10 m*M* EDTA at 37° C. Samples were brought to 10% glycerol and 0.02% bromophenol blue and electrophoresed on a 0.7% agarose gel in 1X TBE buffer (90 m*M* TRIS-borate, 2 m*M* EDTA). Gels were stained with ethidium bromide (1 μg/ml), and photographs were taken under UV light. The amount of unknotted DNA was estimated by densitometry of photographic negatives.

Results

Cleaved-complex formation in H69 and H69AR cells

The effect of increasing concentrations of VP-16 on cleaved-complex formation in intact H69 and H69AR cells was examined by the SDS-KCl precipitation assay. Incubation of the cells with VP-16 for 30 min resulted in a significantly greater increase in complex formation in H69 cells as compared with H69AR cells at each drug concentration tested (Fig. 1). In the H69 cells, the complex formation increased linearly as the VP-16 concentration increased from 2 to 25 μ*M* (r^2 = 0.89), whereas no significant increase was observed in H69AR cells over the same drug concentration range. No further increase in complex formation in H69 cells was observed at VP-16 concentrations above 25 μ*M*. At 25 μ*M* VP-16, the increase in complex formation occurring in treated cells relative to untreated cells amounted to a factor of 7 in H69 cells as compared with a factor of 1.5 in H69AR cells.

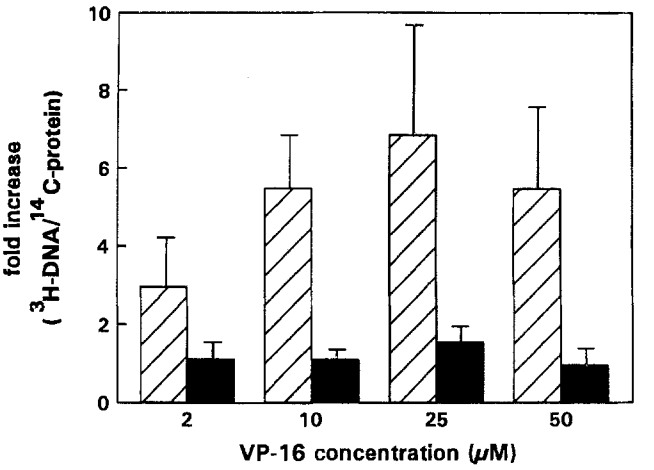


Fig. 1. Effect of VP-16 on cleaved-complex formation in H69 (*hatched bars*) and H69AR (*solid bars*) cells. Cells were labeled overnight with [^{3}H]-thymidine and [^{14}C]-leucine and then incubated with VP-16 for 30 min. DNA-protein covalent cleaved complexes were precipitated and the ratios of ^{3}H-labeled DNA to ^{14}C-labeled protein were determined. Results are expressed as the *X*-fold increase in the ^{3}H: ^{14}C ratio of drug-treated cells relative to that obtained with cells receiving vehicle. Bars represent the mean values (± SD) of results obtained in 4 independent experiments. Complex formation in H69 cells was significantly greater than that in H69AR cells at all drug concentrations tested (Student's unpaired *t*-test; $P < 0.05$)

Levels of topo IIα and IIβ protein and mRNA

When whole-cell lysates of H69 and H69AR were probed with topo II isoform-specific antibodies, approximately 3–4 times less topo IIα protein (Fig. 2, left panel) and approximately 2–3 times less topo IIβ protein (Fig. 2, right panel) was detected in the H69AR cells as compared with the H69 cells. Similar quantitative differences were observed in immunoblots of nuclear extracts (data not shown) and were consistent with subsequent Northern-blot analyses of topo IIα and IIβ mRNA. An approximately 3- to 4-fold decrease in the amount of the 6.1-kb topo IIα mRNA (Fig. 3, left panel) and a slightly smaller (2-fold) decrease in the amount of the 6.1-kb topo IIβ mRNA (Fig. 3, right panel) was observed in the H69AR cells. Two additional bands at 7.5 and 4.5 kb were observed in the topo IIα blot and are of unknown origin. The 7.5-kb band has previously been reported in the H69 cell line [24], and our results indicate that it is also present in the H69AR cell line. The 4.5-kb band may be the result of nonspecific hybridization with ribosomal RNA.

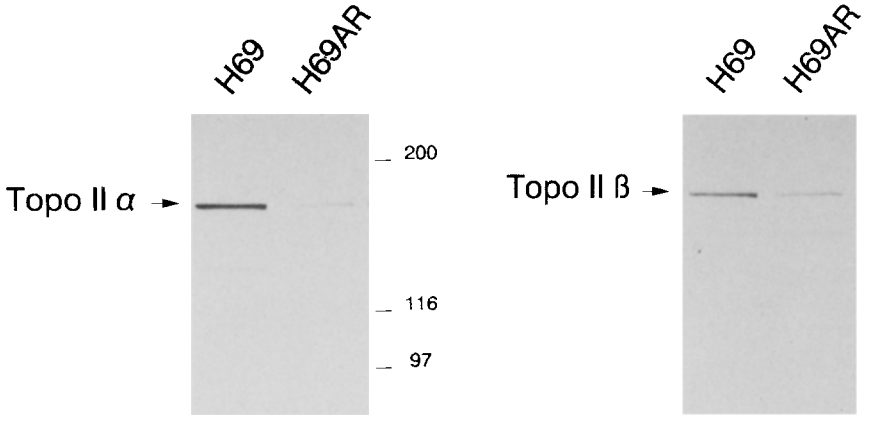


Fig. 2. Immunoblots of topo IIα and IIβ protein in whole-cell lysates of exponentially growing H69 and H69AR cells. Whole-cell lysates (25 μg protein/lane) were subjected to electrophoresis and electrotransferred to Immobilon-P membrane. The blot was incubated with topo II isoform-specific antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG, and antibody binding was determined by ECL detection. *Left panel*, Topo IIα was detected with a rabbit antiserum raised against the recombinant 70-kDa COOH-terminus of HeLa topo IIα. *Right panel*, Topo IIβ was detected with antisera FHD21 raised against a synthetic peptide corresponding to a region of the topo IIβ cDNA sequence that is not shared with the topo IIα sequence

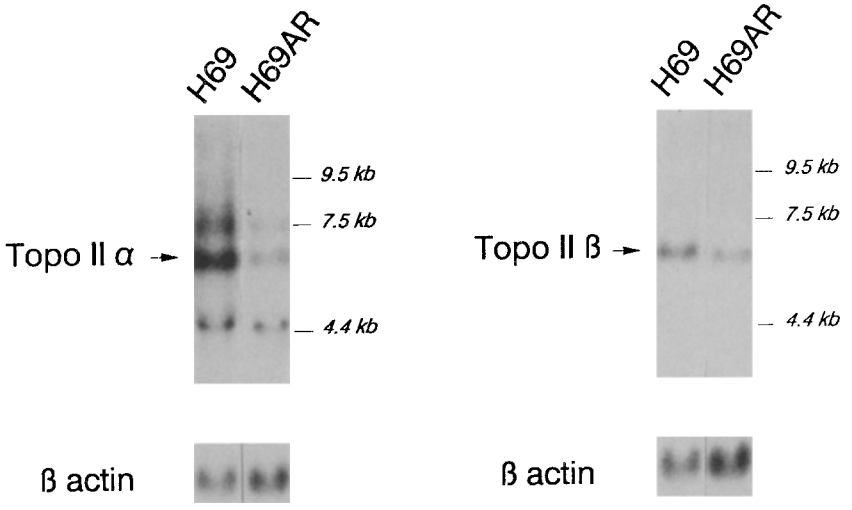
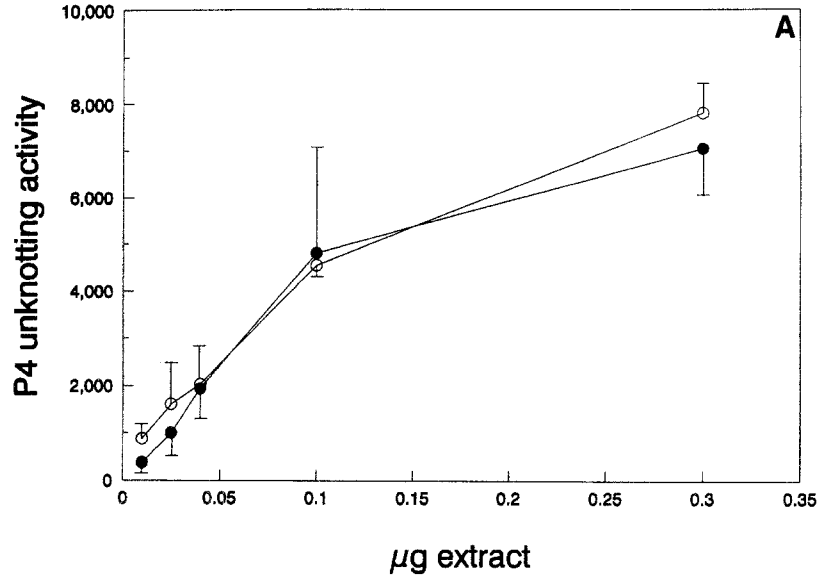


Fig. 3. Northern-blot analysis of topo IIα and IIβ mRNAs in H69 and H69AR cells. Total RNA (10 μg) was electrophoresed through a formaldehyde/agarose gel and blotted onto nitrocellulose membrane. The blots were probed first with topo II isoform-specific ^{32}P-labeled cDNAs SP-1 or SP-12, which detect topo IIα (*left panel*) and IIβ (*right panel*) mRNAs, respectively, and then with β-actin cDNA to allow correction for sample loading



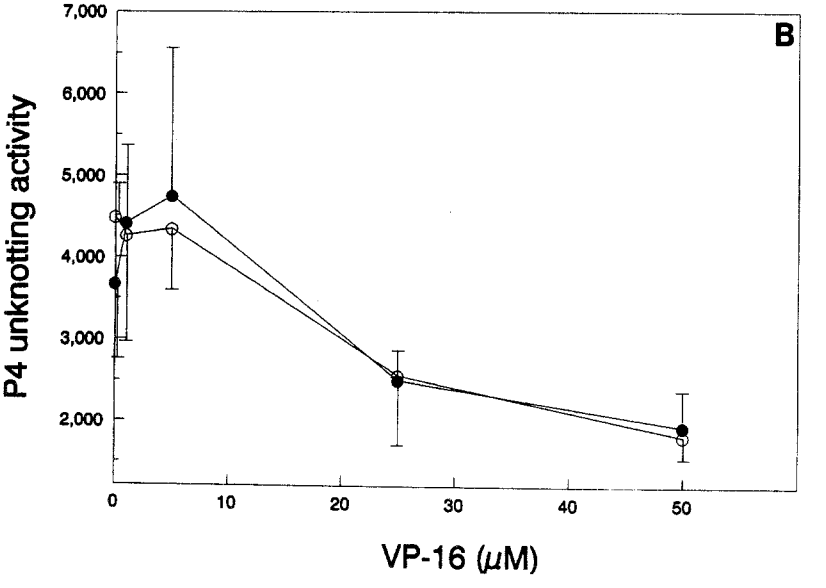


Fig. 4. A Unknotting activity of 0.67 *M* NaCl nuclear extracts from H69 and H69AR cells. Nuclear extracts were incubated with 0.3 μg of P4-knotted DNA for 30 min. The samples were treated with proteinase K, SDS, and EDTA and electrophoresed on a 0.7% agarose gel. Unknotting activity is expressed in arbitrary densitometry units. Points represent the mean (± SD) of values obtained with 3 independent nuclear extract preparations. **B** Effect of VP-16 on the unknotting activity of 0.67 *M* NaCl nuclear extracts from H69 and H69AR cells. Nuclear extracts (0.1 μg protein) were incubated with 0.3 μg of P4 knotted DNA and VP-16 for 30 min and treated as described in **A**. Unknotting activity is expressed in arbitrary densitometry units. Points represent the mean (± SD) of values obtained with 3 independent nuclear extract preparations

Topo II strand-passing activity in nuclear extracts.

To assess topo II strand-passing activity in H69 and H69AR cells, P4-unknotting activity was measured in nuclear extracts. Possible variations in topo II levels due to cell-cycle changes were minimized by ensuring that both cell lines were in exponential growth. The relationship of unknotting activity to protein concentration was examined and no significant difference in the unknotting activity per microgram of protein was observed between the H69 and the H69AR nuclear extracts (Fig. 4A). Unknotting activity increased linearly in both the H69 and the H69AR nuclear extracts up to 0.1 μg protein ($r^2 = 0.99$). As expected, no topo II activity was detectable in the absence of ATP (results not shown). In a second set of experiments, the ability of VP-16 to inhibit an equivalent amount of topo II unknotting activity from nuclear extracts of the two cell lines (0.1 μg protein) was compared. No significant difference in inhibition of the activity by VP-16 was observed between H69 and H69AR nuclear extracts (Fig. 4B).

Discussion

By definition, the H69AR cell line is multidrug-resistant because it is resistant to a variety of natural-product drugs with multiple subcellular targets, including the vinca alkaloids, the epipodophyllotoxins, and the anthracyclines [9, 10, 30]. Although this cell line does not overexpress P-glycoprotein [11, 30], we have recently reported the molecular cloning of a new member of the ATP-binding cassette superfamily of transporter genes, designated multidrug-resistance-associated protein (MRP), that is highly overexpressed in H69AR [12] and can confer a multidrug-resistance phenotype when transfected into drug-sensitive cells [25]. We have previously shown that H69AR is resistant to VP-16-induced single-strand breaks and that levels of immunoreactive topo IIα are reduced in this cell line [11]. These data suggested that alterations in topo II could also contribute to the resistance of H69AR cells to anthracyclines and epipodophyllotoxins [11], but they cannot explain their resistance to the vinca alkaloids and colchicine, which do not have this enzyme as their target.

Drug-resistant cell lines have been described that appear to be resistant primarily due to a decrease in the amount of topo IIα protein present [23, 37, 50]. Other resistant cell lines have been characterized in which the amount of immunoreactive topo IIα protein is unchanged but the biochemical properties of the enzymes are altered [15, 39, 49]. In the latter cell lines, several mutations, including ones in the ATP-binding sequences or the active-site tyrosine region, have been identified [5, 14, 15]. The majority of studies to date have described alterations in the topo IIα isoform, whereas the involvement of topo IIβ in drug resistance has not been widely evaluated.

The decreased formation of protein-associated DNA cleaved complexes in the presence of VP-16 in H69AR cells as compared with H69 cells (Fig. 1) could be explained, at least in part, by the decreases in levels of topo IIα and IIβ protein and mRNA observed in the resistant cell line (Figs. 2, 3). Reduced levels of both human topo II isoforms in the same cell line have only rarely been described [26]. However, since many investigators have just measured levels of topo IIα, reduced levels of topo IIβ in drug-resistant cell lines may occur more frequently than is presently known.

Although topo IIα and IIβ protein and mRNA levels were reduced in H69AR cells, this decrease was not accompanied by measurable differences in P4-unknotting (strand-passing) activity (Fig. 4A). Similar findings have been reported in other drug-resistant cell lines [37, 40] and it has been suggested that topo II may be modified in some resistant cells such that a decreased amount of enzyme does not result in a concomitant decrease in topo II activity. Topo II is phosphorylated and casein kinase II appears to be the major kinase responsible in vivo [1, 7]. Phosphorylation of topo II has been reported to result in a 2- to 3-fold increase in the relaxing or unknotting activities of the enzyme [19]. Takano et al. [40] found that the relative specific phosphorylation of topo II was approximately 14–18 times higher in resistant human KB cells as compared with sensitive KB cells. They suggested that this increase in phosphorylation elevated the specific activity of the enzyme such that a reduced level of topo II in the resistant cell line was capable of carrying out an equivalent amount of strand-passing activity as compared with the topo II in the parental cell line. It is possible that a similar increase in phosphorylation may have occurred in H69AR cells. In this way, the topo II in nuclear extracts from the H69AR cells could maintain strand-passing activity similar to that observed in the H69 cells, despite the apparent decrease in the quantity of enzyme present. DeVore et al. [19] have also demonstrated that phosphorylation of purified topo II from *Drosophila melanogaster* increases the rate of DNA ligation in the presence of VP-16. If topo II in H69AR cells is indeed hyperphosphorylated, then it is possible that cleaved complexes in this cell line are less stable, contributing at least partially to the very low levels of VP-16-induced cleaved complexes observed (Fig. 1).

Inhibition of the strand-passing activity by VP-16 was similar in H69 and H69AR nuclear extracts (Fig. 4B), suggesting that the H69AR cells contain a drug-sensitive form of topo II. The presence of a drug-sensitive enzyme in nuclear extracts has been reported in other model systems and, surprisingly, has been associated with a marked resistance to drug-induced cleaved-complex formation in intact cells in some instances [6, 35, 50]. It has been suggested that nuclear extracts of these cell lines may lack the cytosolic or membranous factors present in intact cells that modulate the interaction of topo II with DNA and drug [6, 35, 50]. A comparison of the biochemical and pharmacological properties of the purified topo II isoenzymes will be required to determine if this is the case in H69AR cells. Such studies will reveal whether the topo II isoenzymes are intrinsically resistant to drug-induced cleavage and will allow an assessment of the relative contribution of the α and β isoforms to drug resistance in these cells.

At present our data would suggest that a quantitative rather than qualitative alteration in topo II contributes to drug resistance in H69AR cells. However, the relatively modest (2- to 5-fold) decreases in topo IIα and IIβ are probably insufficient to account for the 35- and 50-fold

resistance of H69AR cells to VP-16 and doxorubicin, respectively. They also cannot be responsible for the resistance of H69AR cells to non-topo II-targeting drugs. Thus, as in other non-P-glycoprotein multidrug-resistant cell lines [22], a second resistance mechanism must be involved. In H69AR cells, the overexpression of the novel transporter gene MRP is strongly implicated [12, 13].

The results of the present study taken together with work from other laboratories could have important implications with respect to testing human material for the involvement of topo II in clinical drug resistance. An examination of topo IIα protein or mRNA levels alone may not provide sufficient information to ascertain whether or not this enzyme is involved in the resistance of a particular tumor. Reduced levels of topo IIβ may also affect drug sensitivity [26], and the potential contribution of this topo II isoform to drug resistance should not be overlooked. Finally, investigations of clinical samples should ideally take into account the observation that topo II-drug interactions can be modulated by biochemical [6, 16, 19, 50] and molecular alterations [15] that may not affect the apparent levels of topo II protein or mRNA.

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