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Direct Correlation between DNA Topoisomerase II Activity and Cytotoxicity in Adriamycin-sensitive and -resistant P388 Leukemia Cell Lines[1]

Abdul M. Deffie, Janendra K. Batra, and Gerald J. Goldenberg[2]

The Manitoba Institute of Cell Biology [A. M. D., J. K. B., G. J. G.] and the Department of Medicine, University of Manitoba [G. J. G.], Winnipeg, Manitoba, Canada R3E 0V9

ABSTRACT

The relationship between DNA topoisomerase II activity and drug resistance was studied in cloned cell lines of Adriamycin (ADR)-sensitive and -resistant P388 leukemia; drug resistant P388/ADR/3 (clone 3) and P388/ADR/7 (clone 7) cells are 5- and 10-fold more resistant to ADR than the sensitive cell line P388/4 (Cancer Res., *46:* 2978, 1986). Topoisomerase II catalytic activity in crude nuclear extracts was reduced in drug-resistant cells as determined qualitatively by decatenation of kDNA. Using the centrifugal method of quantitative analysis, topoisomerase II catalytic activity (mean ± SE) was 81 ± 10 units/mg total nuclear protein in sensitive cells, 29 ± 2 units/mg total nuclear protein in resistant clone 3 cells, and 16 ± 2 units/mg total nuclear protein in resistant clone 7 cells; these differences were highly significant ($P < 0.005$). Similarly, quantitative analysis of DNA cleavage activity using 3′ ^{32}P-end-labeled pBR322 restriction fragments showed that drug-stimulated topoisomerase II cleavage activity in nuclear extracts from sensitive cells was approximately 1.7- and 2.9-fold greater than that from resistant clone 3 and 7 cells, respectively. Western blot analysis of nuclear extracts from the three cell lines using antibody against the C-terminal half of recombinant-prepared human topoisomerase II polypeptide revealed reduced immunoreactivity of topoisomerase II protein in the drug-resistant cells. These data suggest that reduced topoisomerase II activity in resistant cells, which may represent quantitative reduction of the enzyme, may be another property contributing to multifactorial drug resistance in these cells.

INTRODUCTION

Mammalian DNA topoisomerase types I and II are nuclear enzymes that have been proposed to function in a variety of genetic processes and induce topological changes of DNA (for reviews, see References 1–4). The type II topoisomerases are ATP-dependent enzymes that catalyze the topological passing of two double-stranded DNA segments, presumably by introducing a transient enzyme-linked double strand break in one of the passing strands (5–9). This strand passing or catalytic activity of topoisomerase II can be assayed *in vitro* by ATP-dependent relaxation of supercoiled DNA, knotting/unknotting of P2 and P4 DNA, or catenation/decatenation of kDNA[3] (5, 6, 10–17). Topoisomerase II activity can also be assayed by measuring enzyme-mediated drug-induced cleavage of appropriate DNA substrates (18, 19). An important feature of topoisomerase II-mediated activity is the formation of a covalent bond between a tyrosyl residue on the enzyme and the phosphate moiety at the 5′ terminus of the DNA at the break site, thereby creating an intermediate DNA-enzyme complex referred to as the "cleavable complex" (18, 20–22).

Several antitumor agents have been postulated to inhibit topoisomerase II activity including DNA intercalators such as Adriamycin and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide and epipodophyllotoxins such as etoposide and teniposide (21, 23–26). Evidence has accumulated to suggest that these drugs interact with topoisomerase II to stabilize the "cleavable complex" resulting in increased DNA scission and inhibition of the rejoining reaction (1, 22, 24, 26); in the presence of protein denaturing agents, the enzyme is trapped in this form which may be observed experimentally as protein-associated DNA strand breaks when assayed by filter elution technology (27–30). It follows that the extent of DNA double-strand breaks induced by topoisomerase-interacting drugs is a function of the levels of topoisomerase II activity as well as the quality of interaction of drug with the enzyme or enzyme-DNA complex. There are several reports in the literature that implicate reduced topoisomerase II activity in the drug resistance phenotype (19, 31–37).

The findings presented here are an extension of two previous reports (38, 39) that have defined the multifactorial nature of drug resistance in cloned cell lines of ADR-sensitive and -resistant P388 leukemia. Among those contributory factors reported, we showed previously (38) that resistance in these cells can be attributed in part to reduced levels of drug-induced double strand breaks. Here, we report that crude nuclear extracts of ADR-resistant P388 cells show markedly reduced levels of topoisomerase II catalytic activity as compared to drug-sensitive cells. In addition, drug-induced topoisomerase II-mediated cleavage of DNA in the crude nuclear extracts was also substantially reduced in the resistant cell lines. The decrease in enzyme activity correlated with reduced immunoreactivity of the topoisomerase II protein as determined by Western blot analysis using a specific antibody. These results are discussed in view of the role of topoisomerase II as a target for the cytotoxic action of ADR.

MATERIALS AND METHODS

Cell Lines and Cultures. Cloned lines of ADR-sensitive (P388/4) and -resistant (P388/ADR/3 or clone 3 and P388/ADR/7 or clone 7) leukemia cells (38) were maintained *in vivo* by weekly transplantation of an inoculum of 10^6 cells i.p. in 6–8-week-old female DBA/2 mice. Clone 3 and clone 7 cells are 5- and 10-fold more resistant to ADR than the drug-sensitive cells and the resistant phenotype is stable for at least 1 year. Suspension cultures *in vitro* were maintained by growing the cells in the absence of drug in RPMI medium supplemented with 15% fetal bovine serum (Grand Island Biological Co., Grand Island, NY). Both sensitive and resistant cells used in these experiments grew exponentially with a doubling time of 11 to 12 h.

Drugs and Chemicals. ADR was obtained from Adria Laboratories, Columbus, OH. [^{3}H]Thymidine (specific activity, 50–80 Ci/mmol) was obtained from New England Nuclear, Boston, MA.

Preparation of DNA Substrates. kDNA was prepared from the trypanosome *Crithidia fasciculata* (purchased from Carolina Biological Supply Co., Burlington, NC). Cultures were grown on a rotary shaker for 72 h at 27°C in trypticase peptone medium supplemented with hemin (10 μg/ml) and liver extract (0.1 mg/ml). For preparation of kDNA labeled with ^{3}H, cultures were grown in the presence of 5 mCi/liter of [*methyl*-^{3}H]thymidine. Stationary phase cells at about 1×10^8 cells/ml were harvested and kDNA was isolated from sarkosyl extracts

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[2] To whom requests for reprints should be addressed, at the Manitoba Institute of Cell Biology, 100 Olivia Street, Winnipeg, R3E 0V9, Canada.

[3] The abbreviations used are: kDNA, kinetoplast DNA; ADR, Adriamycin; NB, nucleus buffer; SDS, sodium dodecyl sulfate; VP-16, etoposide.

of the cells by cesium chloride-ethidium bromide density gradient centrifugation as previously described (11, 16).

Supercoiled plasmid pBR322 dimer was prepared from *Escherichia coli* strain HB101 by standard procedures (10, 40, 41). *Hind*III-linearized pBR322 DNA was end-labeled at the 3'-end by treatment with the Klenow fragment, [^{32}P]dATP and unlabeled dCTP, dGTP, and TTP for 30 min at 25°C as described previously (18, 19). Unincorporated triphosphates were separated from labeled DNA by gel filtration through a Sephadex G-50 column equilibrated in 0.5 M NaCl, 50 mM tris-Cl (pH 7.7), and 0.5 mM EDTA. Labeled DNA samples were concentrated by ethanol precipitation.

Preparation of Crude Nuclear Extracts. Crude nuclear extracts were prepared by a modification of a published report (30). Exponentially growing cells were collected by centrifugation and washed in ice-cold NB (NB consists of 2 mM K_2HPO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, and 0.1 mM dithiothreitol, pH adjusted to 6.5). The washed cells were resuspended in NB and 9 ml of NB supplemented with 0.35% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride were added slowly down the side of the tube. The cell suspension was mixed by rotation for 5 min at 4°C, centrifuged at 1000 × *g* for 10 min, and the nuclear pellet washed in Triton-free NB. Nuclear protein was extracted from the nuclei for 30 min at 4°C with ice-cold NB containing 0.35 M NaCl. DNA and nuclear debris were pelleted by centrifugation at 17,000 × *g* for 10 min and the supernatant collected. Protein concentration in the extract was determined by the method of Bradford (42). Extracts were adjusted to a protein concentration of 20 ng/μl. Glycerol was immediately added to a final concentration of 50%.

Assay of Catalytic Activity of DNA Topoisomerase II. The standard reaction mixture for all types of topoisomerase II assays contained 50 mM tris-Cl (pH 8), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM EDTA, 10 μg/ml of bovine serum albumin, and 1 mM ATP, pH 7.7. The reaction mixture had a final NaCl concentration of 70 mM and a final glycerol concentration of 10% (both components contributed by the nuclear extract).

Topoisomerase II catalytic activity was assayed by decatenation of kDNA into free minicircles. Two types of analysis were performed: a centrifugal analysis in which [^{3}H]kDNA was used as substrate and free minicircles were separated from kDNA by a brief centrifugation; and an electrophoretic analysis in which unlabeled kDNA was used as substrate and minicircles were resolved by gel electrophoresis (7, 17, 43). For both types of analysis, decatenation was carried out by incubating 10 μl of nuclear extract (containing 200 ng total protein) with 2 μg of kDNA in a final volume of 50 μl of reaction mixture at 30°C for 30 min. A time course of enzyme activity using the centrifugal method was linear for at least 40 min for ADR-sensitive and -resistant cell lines (data not shown). At the end of the reaction, samples analyzed by the centrifugal method were treated with 5 μl of 2.25% SDS, centrifuged at 13,000 × *g* for 5 min in a table top microcentrifuge, and the supernatant collected for determining radioactivity by scintillation counting. The results were quantitated by converting the radioactivity liberated in the supernatant to amount of DNA (ng) by using the specific activity of [^{3}H]kDNA. Samples analyzed by gel electrophoresis were treated with 5 μl of a dye solution consisting of 5% SDS, 50% glycerol, and 0.25 mg/ml bromophenol blue; samples were then electrophoresed through 1% agarose in 90 mM tris-borate-2 mM EDTA (pH 8.0) at 25 v for 20 h. After staining with ethidium bromide (5 μg/ml) gels were photographed under UV.

Assay of Cleavage Activity of DNA Topoisomerase II. Topoisomerase II drug-stimulated DNA cleavage activity was assayed qualitatively by the generation of linearized (form III) from supercoiled (form I) pBR322 DNA in a final volume of 50 μl of reaction mixture for 15 min at 37°C as described previously (19). The reaction was terminated by the addition of the dye solution described above and the samples analyzed by electrophoresis through 1% agarose as described for the decatenation assay. The electrophoresis running buffer contained 0.1% SDS in order to dissociate the intercalator (ADR) molecules from DNA (44). Gels were washed three times with water to remove SDS, stained with ethidium bromide (5 μg/ml), destained in water and photographed under UV.

Quantitative analysis of DNA cleavage activity of topoisomerase II was assayed using 3' ^{32}P end-labeled pBR322 restriction fragments and the SDS-KCl precipitation of topoisomerase II-DNA complexes as described previously (18, 19).

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting. Nuclear extracts from sensitive and resistant cells were electrophoresed on a 5–10% gradient polyacrylamide gel according to the procedure of Laemmli (45). Proteins from the gel were electrotransferred onto a nitrocellulose sheet by Towbin's method (46) using an LKB-Nova blot apparatus. The blot was incubated overnight at 37°C with blocking buffer [3% bovine serum albumin in 10.5 mM tris-HCl (pH 7.5)–175 mM NaCl] followed by incubation with polyclonal rabbit antiserum against recombinant human topoisomerase II polypeptide (C terminal half), which was kindly provided by Dr. Leroy Liu, in fresh blocking buffer for 16 h at 0°C. At the end of incubation, the blot was washed with tris-saline buffer and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG at room temperature for 5 h. Bands were visualized after washing the blot with tris-saline buffer and incubating with substrate for alkaline phosphatase as described by the supplier (Bethesda Research Laboratory, Gaithersburg, MD).

RESULTS

Catalytic Activity of DNA Topoisomerase II in Adriamycin-sensitive and -resistant P388 Leukemia

Catalytic activity of DNA topoisomerase II in nuclear extracts of P388 cell lines was assayed by decatenation of kDNA, and separation by gel electrophoresis of kDNA and free minicircles (Fig. 1). In the absence of nuclear extract, kDNA (*k*) is seen as a slow-moving band close to the well (*Lane C*). When nuclear extract is added to kDNA, decatenation occurs and free minicircles (*m*) are observed as a fast-moving band away from the well. The intensity of fluorescence observed indicates that more DNA minicircles are liberated with nuclear extract from drug-sensitive P388/4 cells than that from either of the two resistant clones. The amount of minicircles liberated is a measure of the activity of topoisomerase II in the nuclear extract.

Quantitation of topoisomerase II catalytic activity using the centrifugal method of Sahai and Kaplan (17) was performed (Table 1). Using [^{3}H]kDNA as substrate, topoisomerase II catalytic activity (mean ± SE) was 81 ± 10 units/mg total nuclear protein in sensitive cells, 29 ± 2 units/mg total nuclear

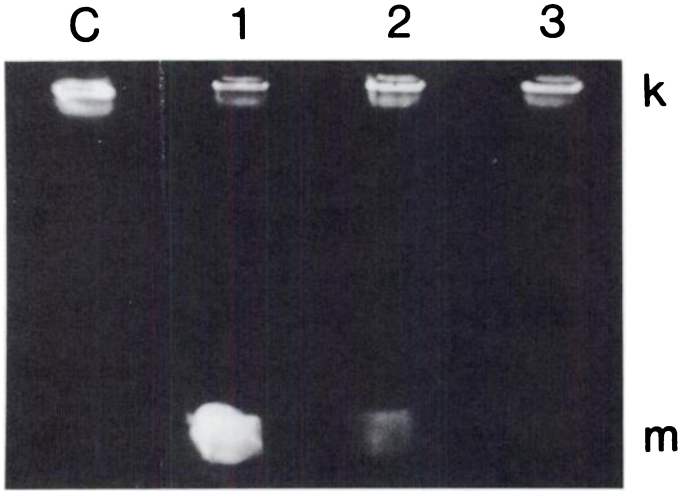

Fig. 1. Decatenation of kDNA by nuclear extracts from sensitive and resistant clone 3 and 7 cells. Decatenation reaction was carried out as described in the text. Reaction mixture was incubated in the absence of nuclear extract (*Lane C*) or in the presence of nuclear extract (containing 200 ng total protein) from ADR-sensitive P388/4 cells (*Lane 1*), or drug-resistant clones 3 and 7 (*Lanes 2* and *3*, respectively). *k*, kDNA band; *m*, free minicircles. Protein concentration in the extracts from all three cell lines was equivalent.

Table 1 *Catalytic activity of topoisomerase II in nuclear extracts of ADR-sensitive and -resistant P388 leukemia*

Experiment[a]	Topoisomerase II catalytic activity[b] (units/mg total nuclear protein)		
	P388/4	P388/ADR/3	P388/ADR/7
1	61[c]	29	16
2	97	33	19
3	84	25	13
Mean ± SE	81 ± 10	29 ± 2	16 ± 2

[a] Each experiment was performed with fresh nuclear extract prepared from exponentially growing cells in mid-log phase. Glycerol was added to a final concentration of 50% to each sample.

[b] Topoisomerase II catalytic activity was assayed quantitatively by the centrifugal method as described in "Materials and Methods." One unit of enzyme is defined as the amount that catalyzes the release of 400 ng of DNA into the supernatant as free minicircles in 30 min at 30°C.

[c] Units of enzyme activity for each experiment is a mean of four determinations.

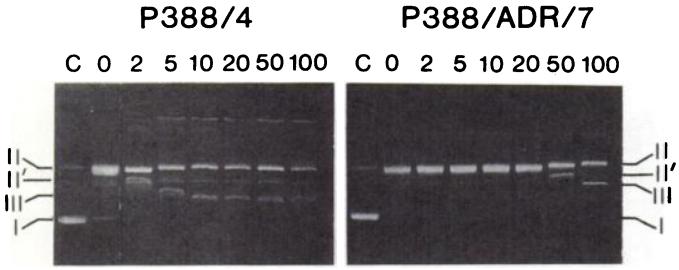

Fig. 2. ADR-induced cleavage activity of DNA topoisomerase II in crude nuclear extracts from drug-sensitive and -resistant P388 leukemia cells. The DNA cleavage assay was performed as described in the text. *Lane C*, control in which the reaction mixture containing supercoiled pBR322 was incubated without nuclear extract or drug. *Numbers*, ADR concentration in micromolar units. Extract dose was 10 μl (200 ng total nuclear protein) in a final volume of 50 μl. The various topological isomers of pBR322 are marked *I* (super-coiled), *II* and *II'* (relaxed and/or nicked), and *III* (linear) forms.

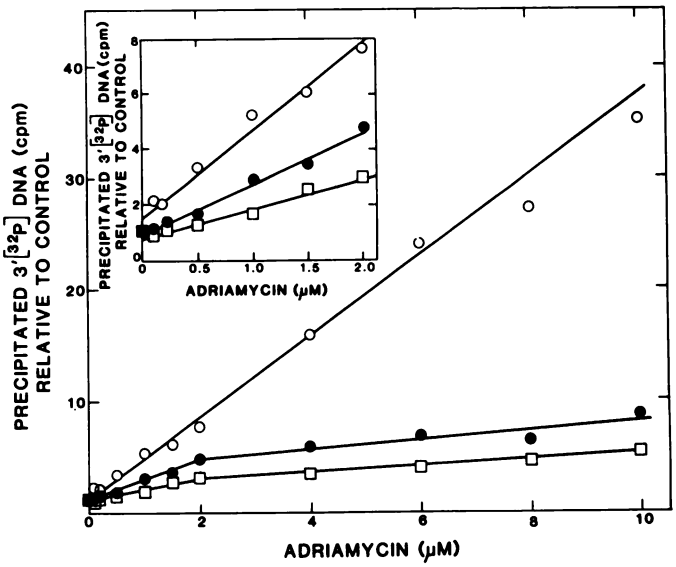

Fig. 3. Quantitative assay of the cleavage activity of DNA topoisomerase II in ADR-sensitive and -resistant P388 leukemia cells. Effect of ADR on the level of ^{32}P-labeled DNA precipitated by SDS and KCl solution in the presence of nuclear extracts of sensitive P388/4 cells (○), resistant clone 3 cells (●), and resistant clone 7 cells (□). 3′ ^{32}P end-labeled pBR322 restriction fragment was incubated in a final volume of 50 μl with 200 ng total protein nuclear extract and varying concentrations of ADR for 15 min at 37°C. At the end of the incubation period, stable DNA-protein complexes were precipitated with SDS-KCl solution as described (18, 19). The amount (cpm) of ^{32}P-labeled DNA precipitated relative to control level observed in the absence of ADR is plotted against ADR concentration. *Inset*, amount (cpm) of ^{32}P-labeled DNA precipitated relative to control over the initial linear phase of the dose-response curve. The linear regression equation of the plot for sensitive P388/4 cells was $y = 3.13x + 1.55$ with a correlation coefficient of 0.988, that for resistant clone 3 cells was $y = 1.82x + 0.95$ with a correlation coefficient of 0.994, and that for resistant clone 7 cells was $y = 1.07x + 0.82$ with a correlation coefficient of 0.987.

protein in resistant clone 3 cells, and 16 ± 2 units/mg total nuclear protein in resistant clone 7 cells; these differences were highly significant ($P < 0.005$). Hence catalytic activity of topoisomerase II varies directly with sensitivity to the intercalator ADR.

Cleavage Activity of DNA Topoisomerase II in Adriamycin-sensitive and -resistant P388 Leukemia

Drug-mediated cleavage activity of DNA topoisomerase II in crude nuclear extracts of ADR-sensitive and -resistant cells was measured (Fig. 2). In the absence of nuclear extract and ADR, pBR322 DNA migrates predominantly in the fast-moving supercoiled state (form I). Addition of nuclear extract without drug results in conversion of supercoiled pBR322 DNA (form I) into the relaxed and/or nicked configurations (forms II and II′). Linearized DNA (form III) is generated when ADR is added in the presence of nuclear extract. Linearized DNA first appears with extracts from sensitive cells at ADR concentrations as low as 5–10 μM, whereas with nuclear extracts from drug-resistant cells, the corresponding drug concentration is 50 to 100 μM drug (Fig. 2).

Topoisomerase II cleavage activity in nuclear extracts of P388 cells was quantitated by precipitation of drug-stabilized DNA-protein complexes with SDS-KCl solution (Fig. 3). In this assay, topoisomerase II in nuclear extracts forms a covalent linkage with the 5′-end of linearized pBR322 DNA that is labeled with [^{32}P]dATP at its 3′ end; in the presence of ADR, this topoisomerase II-DNA complex is stabilized and may be precipitated by addition of SDS-KCl solution. The results in Fig. 3 show that a greater amount of ^{32}P-labeled DNA was precipitated with nuclear extract from sensitive cells than that from either of the two resistant cell lines. A comparison of slopes from the initial linear portion of the curves shows that enzyme activity from sensitive cells was approximately 1.7- and 2.9-fold greater than that from resistant clone 3 and 7 cells, respectively (Fig. 3, *inset*).

Detection of Topoisomerase II by Western Blot Analysis

The amount of DNA topoisomerase II in the three cell types was determined by Western blotting using rabbit antiserum to the C-terminal half of recombinant prepared human topoisomerase II polypeptide (Fig. 4). A marked reduction in the level of topoisomerase II homodimer subunit (M_r 170,000) was detected in resistant clone 3 and 7 cells compared to sensitive P388/4 cells.

DISCUSSION

In this extension of our previous reports (38, 39) we have compared DNA topoisomerase II activity and enzyme content in the three cell lines which differ in their level of sensitivity to the intercalator ADR. The model system that we have used in our studies of mechanism of drug resistance is particularly useful for two reasons: firstly, these are cloned cell lines, so that the various factors contributing to resistance cannot be attributed to different cell subpopulations, and secondly, the use of two resistant cell lines which differ in their degree of resistance, permits quantitative comparisons of various biochemical and molecular features. In addition, the relatively modest levels of resistance in our cells are probably more relevant clinically than that of other systems characterized by much higher levels of drug resistance.

Since DNA topoisomerase II has been identified as an intra-

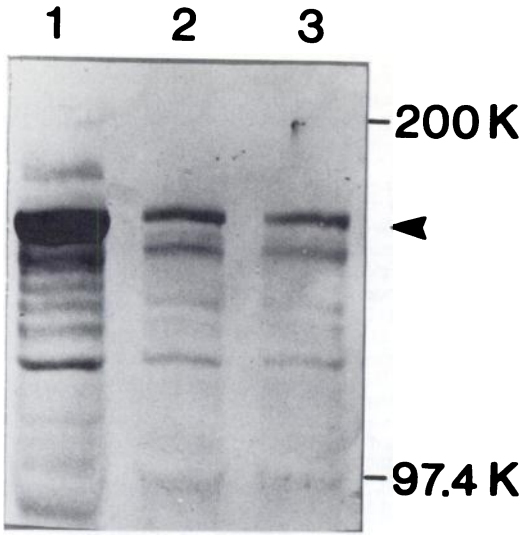

Fig. 4. Western blot analysis of topoisomerase II in nuclear extracts of ADR-sensitive and -resistant P388 leukemia cells. Proteins were separated on a 5–10% gradient gel as described in the text. Each lane was loaded with nuclear extract (50 μg protein) from P388 cells. Nuclear extracts from sensitive P388/4, resistant clone 3 and clone 7 cells were loaded on *Lanes 1–3*, respectively.

cellular target for certain antineoplastic agents (22, 47), a study of catalytic and cleavage activity was undertaken in ADR-sensitive and -resistant P388 leukemia cells. Qualitative assays of both catalytic and cleavage activity of DNA topoisomerase II were markedly reduced in ADR-resistant cells (Fig. 1 and 2). Other cellular factors such as nucleases in the crude extract may influence DNA catalytic and cleavage activity. However, the reaction conditions tended to minimize nuclease activity, as reported previously (17). Furthermore, linear DNA (form III) was not observed in the drug-sensitive and -resistant cells in the presence of crude nuclear extract without ADR (Fig. 2, *Lane 0*). Quantitative assays revealed a direct correlation between drug sensitivity and enzyme activity; catalytic activity was reduced 2.8- and 5-fold in resistant clone 3 and 7 cells respectively (Table 1), whereas cleavage activity was correspondingly reduced 1.7- and 2.9-fold (Fig. 3). These findings are consistent with a report of decreased topoisomerase II activity in amsacrine-resistant P388 leukemia cells (48).

The decrease in topoisomerase II activity reported here correlates closely with the lower levels of DNA strand breaks previously reported in ADR-resistant cells (38). There is growing evidence that drug-induced DNA strand breaks, measured by filter elution technology, are mediated by topoisomerase II (27–30). A number of investigators have suggested involvement of topoisomerase II in the resistance of various cell lines to intercalating drugs and epipodophyllotoxins by correlating drug resistance with reduced levels of protein-associated DNA strand breaks (31, 33–35). Another report has shown that Chinese hamster ovary cell mutants that are hypersensitive to topoisomerase II interacting drugs exhibit enhanced levels of DNA strand breaks (36).

The reduction in catalytic and cleavage activity of topoisomerase II in resistant cells may represent either a quantitative or qualitative change in the enzyme. Western blot analysis of nuclear extracts from the three cell lines using antibody against the C-terminal half of recombinant prepared human topoisomerase II polypeptide showed a lower intensity of staining of a protein band of 170,000 molecular weight in the drug-resistant cells (Fig. 4). This protein band is in the same molecular weight range as that observed by other investigators for topoisomerase II in various mammalian cells (7, 48–50). Our findings are in agreement with those of Drake *et al.* (48), who reported a lower level of the M_r 170,000 protein in amsacrine-resistant P388 leukemia cells, and may be explained by a quantitative reduction of DNA topoisomerase II in ADR-resistant cells. However, this study does not exclude the possibility of posttranslational modifications or other qualitative changes of the protein resulting in both decreased enzyme activity and immunoreactivity.

Other studies have also invoked a role for DNA topoisomerase II in drug resistance (19, 32, 37, 48). However, previously greater emphasis has been placed on cleavage rather than catalytic activity as an index of cytotoxicity (19). In Chinese hamster ovary cells resistant to etoposide (VP-16) decreased cleavage activity of topoisomerase II but with no alteration of catalytic function was reported (19). In this study with ADR-resistant P388 cells, not only cleavage but also catalytic activity was reduced in resistant cells. This apparent discrepancy might represent different mechanisms of resistance for different drugs or might simply reflect differences between cell lines. The data presented here, showing decreased levels of topoisomerase II activity in ADR-resistant P388 leukemia cells suggest that reduction in topoisomerase II may be another property contributing to multifactorial drug resistance in these cells.

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