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Evidence of DNA Topoisomerase II-Dependent Mechanisms of Multidrug Resistance in P388 Leukemia Cells

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SUMMARY

A multidrug-resistant variant of the P388 leukemia cell line exhibits multiple biochemical changes, including reduced drug accumulation and markedly reduced DNA strand breakage induced by anthracyclines. To investigate whether the reduced formation of drug-induced DNA breaks was due to alteration of DNA topoisomerase II activity, nuclear extracts and partially purified enzymes from the sensitive line and the resistant subline were compared. DNA topoisomerase II activity in 0.35 M NaCl nuclear extracts from sensitive cells was approximately 1.7 times higher than that found in extracts from resistant cells, as determined by ability to unknot P4 phage DNA. In addition, it was found that teniposide-stimulated topoisomerase II DNA cleavage activity of nuclear extract from resistant cells was at least 10-fold lower than that from sensitive cells. This differential sensitivity paralleled a similar drug response of nuclei, as determined by the alkaline elution method. However, partially purified DNA topoisomerase II showed similar drug sensitivity in both cell lines. This finding suggests the presence of a modulating factor, which may be lost during purification. These results, indicating a reduction of both catalytic activity and DNA cleavage activity of DNA topoisomerase II in P388 multidrug-resistant cells, emphasize the importance of DNA topoisomerase function in the resistance mechanism. Thus, the concomitant involvement of multiple mechanisms could explain the high degree of resistance of these cells.

A common feature of MDR is a decreased intracellular drug accumulation in resistant cells. This is often the result of increased drug efflux, ascribed to an overexpression of a membrane glycoprotein (GP 170) (1). However, there is evidence that a decrease in drug accumulation does not completely explain the MDR phenomenon. For example, a moderate change in intracellular drug accumulation does not correlate with the high degree of resistance observed in some selected sublines, including P388/DX leukemia cells (2). Some resistant cell lines show a marginal (if any) reduction in drug accumulation (3–5). An atypical MDR phenotype has been described, with a different pattern of cross-resistance and unimpaired ability to accumulate drug within cells (6). These observations and recent findings indicate that multiple factors may be involved in MDR (7, 8). Studies from our laboratory in MDR murine leukemia P388 cells have shown an almost complete insensitivity to anthracycline-induced DNA cleavage (2). Because markedly reduced levels of protein-associated DNA strand breaks were found also in isolated nuclei, we proposed that at least two mechanisms of drug resistance could be operative in MDR P388 cells (9). These include overexpression of P-glycoprotein (10) and possible alterations of nuclear DNA topoisomerase II. Topoisomerase II has been suggested to be the cellular target of several classes of antitumor compounds, including anthracycline antibiotics and epipodophyllotoxins (11). The enzyme is involved in DNA replication, transcription, and recombination. During the DNA relaxation process, the enzyme introduces DSB into DNA, forming at the cleavage site a transient covalent bound with the 5′ terminus of the broken strand. Antitumor drugs such as doxorubicin and VM-26 interfere with topoisomerase II activity during the breakage-reunion reaction by stabilizing the enzyme-DNA complex and causing the accumulation of these covalent complexes (11).

The present study is an extension of a previous investigation on the mechanisms of anthracycline resistance in the leukemia P388 cell line. To test the hypothesis that drug resistance in the variant P388 cell line is also due to alterations in topoisomerase II activity, this study provides a detailed analysis of VM-26-induced cytotoxic effects and DNA strand breaks in isolated nuclei from P388 and P388/DX cells, topoisomerase II activity in nuclear extracts of both cell lines, and VM-26-induced cleavage of pBR322 plasmid DNA in the presence of nuclear extracts. Moreover, topoisomerase II has been partially

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ABBREVIATIONS: MDR, multidrug resistance; TBE, Tris/borate/EDTA buffer; P388/DX, doxorubicin-resistant subline of P388 leukemia; DNA DSB, DNA double-strand break; VM-26, teniposide; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

purified from P388 and P388/DX cells and compared in relation to VM-26 ability to induce DNA cleavage.

Materials and Methods

Chemicals. VM-26 was obtained from Bristol Italiana (Latina, Italy). Plasmid pBR322 DNA was purchased from Boehringer Mannheim (West Germany) and restriction endonuclease *Eco*RI from Bethesda Research Laboratories (Eggenstein, West Germany). Deoxycytidine-5′-[α-^{32}P]triphosphate (specific activity, 3000 Ci mmol^{-1}) and [2-^{14}C]thymidine (50 mCi/mmol) were purchased from Amersham International (Amersham, UK). All other chemicals of analytical reagent grade were from commercial sources.

Cell lines and cytotoxicity assay. Murine leukemia P388 cells sensitive (P388) and resistant to doxorubicin (P388/DX) were maintained *in vivo* and cultured as previously described (2). Sensitive and resistant cells grew *in vitro* with a doubling time of around 18 and 25 hr, respectively. The sensitivity of P388 and P388/DX cells to VM-26 was determined by the growth inhibition test. Exponentially growing cells (10^6 cells/ml) were exposed to the drug for 1 hr at 37°. After drug treatment, cells were centrifuged, washed twice, resuspended, cultured in drug-free medium for 72 hr, and then counted with a Coulter Counter (Coulter Eletronics Ltd., Luton, UK).

Topoisomerase II activity assay. Topoisomerase II strand-passing activity in nuclear extracts was assayed by using the P4 unknotting assay. Naturally knotted DNA, used as the substrate for the strand-passing activity, was isolated from tailless capsids of P4 phage according to the method of Liu and Davis (12). Serial 1:2 dilutions of nuclear extracts were made in lysis buffer (see below). The same volume (1 μl) of nuclear extracts or of dilutions was added to reaction mixtures containing 20 mM Tris·HCl, pH 7.5, 80 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/ml bovine serum albumin, 1 mM ATP, and 0.2 μg of knotted P4 DNA (total volume, 20 μl). Reaction mixtures were incubated at 37° for 30 min and the reaction was stopped by the addition of 4 μl of 5% (w/v) SDS, 10% (w/v) Ficoll, 0.2% bromophenol blue (w/v), and 0.25% (w/v) xylene cyanol. DNA was loaded into a 0.7% agarose gel in TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA). Electrophoresis was at 2 V/cm for ≃16 hr DNA was visualized by UV illumination after ethidium bromide staining. Enzyme catalytic activity was detected by electrophoresis as a conversion of a smear into a band that reflects the topological change of complex knots of P4 DNA into unknotted circles. One unit of topoisomerase II activity was defined as the amount of protein that completely unknotted 0.2 μg of knotted P4 DNA under the above conditions at 37° in 30 min.

Nuclear extracts from P388 and P388/DX cells. About 10^8 cells were collected from tumor-bearing mice and, after lysis of erythrocytes, were pelleted at 500 × *g* for 10 min. All steps during preparation were carried out at 0–4°. The cell pellet was resuspended in 5 ml of nuclear buffer [100 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol, and 0.5 mM phenylmethylsulfonyl fluoride, pH 6.4]; 45 ml of nuclear buffer containing 0.35% Triton X-100 was then added and the suspension was incubated for 30 min at 0°. Nuclear suspensions were centrifuged (900 × *g* for 10 min) and nuclei were washed once with nuclear buffer. Nuclei were resuspended in about 1 ml of lysis buffer [100 mM NaCl, 5 mM KH_2PO_4, 0.5 mM EGTA, 10 mM 2-mercaptoethanol, 10% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 mM $NaHSO_3$, pH 7], and NaCl was slowly added to a final concentration of 0.35 M. Nuclei were salt-extracted for 1 hr by gentle rotation and then discarded by centrifugation at 12,000 × *g* for 15 min. Nuclear extracts were always assayed immediately for protein concentration, P4 unknotting activity, and DNA cleavage activity. Protein concentration was measured according to the method of Bradford (13), using a Bio-Rad kit.

Partial purification of topoisomerase II from P388 and P388/DX cells. All steps during the purification procedures were carried out at 4°. DNA topoisomerase II was partially purified from nuclear extracts. Polymin P (10%) was slowly added to the nuclear extract (20 μl of polymin P/ml of nuclear extract), and after 30 min of continuous stirring samples were centrifuged for 10 min at 14,000 × *g* and the pellets were discarded. Proteins were then precipitated by the slow addition of ammonium sulfate (0.4 g/ml) under constant stirring. After 30 min the precipitate was collected by centrifugation at 16,000 × *g* for 20 min, resuspended in 20 ml of buffer A [20 mM KH_2PO_4, pH 7, 200 mM NaCl, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol (v/v), 0.5 mM EGTA, and 10 mM $NaHSO_3$], and dialyzed overnight against buffer A. The precipitate formed during dialysis was removed by centrifugation at 12,000 × *g* for 15 min. The clarified dialysate was loaded onto a P11 phosphocellulose column (1.5 × 10.5 cm) equilibrated with buffer A. The column was washed with buffer A and then eluted with 200 ml of a linear gradient from 0.2 to 1 M NaCl in buffer A. Enzyme activities in elution fractions were detected by means of the P4 unknotting assay for topoisomerase II and the pBR322 DNA relaxation assay for topoisomerase I. DNA relaxation activity was assayed in a manner identical to that for unknotting activity, except that supercoiled plasmid pBR322 was used as the DNA substrate and ATP was omitted from the reaction. Fractions containing only topoisomerase II activity were pooled and diluted about 2.5-fold with buffer A without NaCl. Topoisomerase II was then concentrated by loading the pooled fractions onto a 1 × 2.5 cm P11 phosphocellulose column equilibrated with buffer A, followed by elution with 1 M NaCl in buffer A. Fractions with topoisomerase II activity were pooled, dialyzed against storage buffer [20 mM potassium phosphate, pH 7, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, and 50% (v/v) glycerol] and stored at −20°. The partially purified topoisomerase II obtained after the steps described above was completely topoisomerase I-free.

Filter elution method. DNA DSB in isolated nuclei were measured by the DNA nondenaturating elution method, as described by Bradley and Kohn (14). Briefly, cellular DNA was labeled with 0.04 μCi/ml [2-^{14}C]thymidine for 24 hr, followed by an overnight chase period before nuclei isolation (9, 15). Nuclei, resuspended at 10^6 nuclei/ml, were exposed to VM-26 for 30 min at 37°, and then filter elution assays were carried out immediately. Nuclei were layered on 0.8-μm pore-size polycarbonate membranes (Nucleopore, Pleasanton, CA) and lysed with a solution of 2% SDS, 0.1 M glycine, 25 mM disodium EDTA (pH 9.6), and 0.5 mg/ml proteinase K (Merck, Darmstadt, FRG). The DNAs were then eluted from the filter with 0.1% SDS, 20 mM EDTA (acid form), tetrapropylammonium hydroxide (Eastman Kodak, Rochester, NY), at pH 9.6. DNA DSB were expressed as rad-equivalents calculated as already reported (9).

Topoisomerase II-mediated DNA cleavage assay. Drug-stimulated DNA cleavage activity was assayed by the fragmentation of *Eco*RI-linearized pBR322 DNA. Six nanograms of linear pBR322 DNA were incubated with the indicated amount of nuclear extract proteins or with partially purified topoisomerase II in the reaction mixture (20 μl) containing 40 mM Tris·HCl, pH 7.5, 80 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, and 15 μg/ml bovine serum albumin, in the presence of 1 mM ATP and VM-26 when indicated. Reactions were incubated at 37° for 30 min and then terminated by the addition of 1 μl of 10% SDS and 1μl of 0.1 mg/ml proteinase K; the incubation was allowed to proceed for 30 min at 50°. Samples were then fractionated by electrophoresis through a 0.7% agarose gel in TBE. The DNA fragments were denatured (30 min in 0.5 M NaOH, 1.5 M NaCl), neutralized (1 hr in 0.5 M Tris·HCl, pH 7, 1.5 M NaCl, 1 mM EDTA), and finally blotted overnight to Hybond-N (Amersham International). The filters were baked at 80° and then prehybridized for at least 4 hr at 42° in 50% formamid, 6× standard sodium citrate, 5× Denhardt solution, 0.5% SDS, 500 μg/ml salmon sperm DNA (Sigma, St. Louis, MO). Hybridizations were conducted for 18–20 hr at 42° with denatured nick-translated pBR322 DNA (specific activity, 2–4 × 10^8 cpm/μg of DNA) in the presence of 10% dextran sulfate. The final wash of filters was at 65° in 0.1× standard sodium citrate and 0.1% SDS; autoradiography was at −70° on Amersham MP films.

Results

Cytotoxicity studies. Cytotoxic effects of 1-hr exposure of P388 and P388/DX cells to VM-26, determined by the growth inhibition test, are shown in Fig. 1. The leukemia P388/DX cell line is highly resistant to the selecting agent doxorubicin and is characterized by a MDR pattern (10). VM-26 concentrations required for 50% cell growth inhibition, deduced from the dose-response curves, were 0.17 and 9.5 μM for sensitive and resistant cells, respectively. Thus, P388/DX cells were about 56-fold more resistant than P388 cells to VM-26.

VM-26-induced DNA cleavage in isolated nuclei from P388 and P388/DX cells. DNA DSB induced by VM-26 were measured in isolated nuclei from P388 and P388/DX cells with the filter elution technique (Fig. 2). Treatment of isolated nuclei with drug concentrations above 0.5 μM effectively induced DNA cleavage in P388 cells, whereas under the same conditions VM-26-induced DNA cleavage was markedly reduced in P388/DX cells. These observations are in good agreement with previous findings on anthracycline-induced DNA strand breakage in these cell lines. Because DNA breaks are mediated by DNA topoisomerase II, DNA cleavage was examined in the presence of ATP (0.1 mM). ATP clearly affected the DNA cleavage activity induced by VM-26 in isolated nuclei, because at least a 2-fold increase in DNA DSB was observed in the P388 cell line (Fig. 2). Appreciable VM-26-induced DNA cleavage was observed in the P388/DX cell line only in the presence of ATP. In both experimental conditions, DNA break levels were always higher in P388 cell nuclei than in P388/DX cell nuclei (Fig. 2).

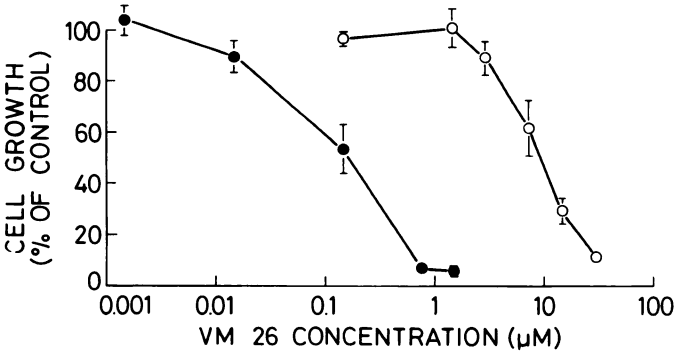

Fig. 1. Growth inhibitory effects of VM-26 on P388 (●) and P388/DX (○) cell lines. Cells were exposed to drug for 1 hr at 37°. After treatment, cells were cultured in drug-free medium for 72 hr and then counted. *Points*, mean of two or three independent determinations; *bars*, ± standard deviation.

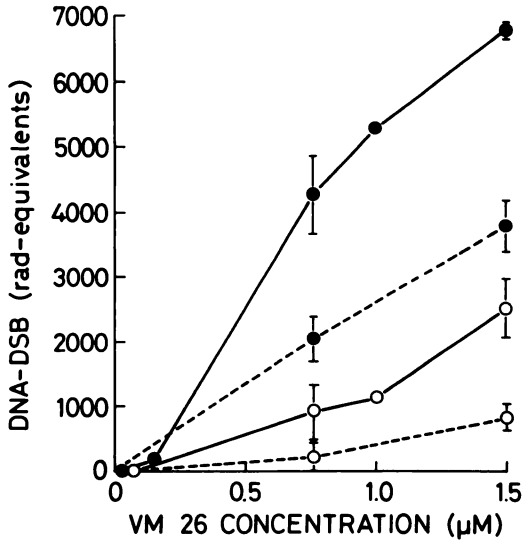

Fig. 2. DNA DSB induced by VM-26 in isolated nuclei from P388 (●) and P388/DX (○) cells. Isolated nuclei were incubated with the drug for 30 min at 37° with (——) or without (– – –) 0.1 mM ATP, lysed on the filter in the presence of proteinase K, and eluted at pH 9.6. *Points*, mean of two or three independent determinations; *bars*, ± standard deviations.

DNA topoisomerase II activity in P388 and P388/DX cells. To investigate topoisomerase II catalytic activity in P388 and P388/DX cell lines, 0.35 M NaCl extracts were prepared from isolated nuclei. This method, conventionally used for selective extraction of nonhistone proteins, has been shown to extract topoisomerase activity from nuclear chromatin (16). A quantitative analysis of topoisomerase II strand-passing activity in nuclear extracts was assayed by measuring the ability of the extracts to unknot P4 phage DNA. In this assay (12), knotted P4 DNA migrates as a smear reflecting a heterogeneous population of DNA molecules (Fig. 3, *control lane*). The strand-passing activity of topoisomerase II removes the knots, therefore converting P4 DNA into a circular form that migrates as a single band. Nuclear extracts from P388 and P388/DX cells were serially (1:2) diluted to determine the maximum dilution at which P4 unknotting activity could be measured (Fig. 3). The results obtained in three independent experiments consistently showed that topoisomerase II activity in nuclear extracts of P388 cells, when normalized to equivalent protein content, was about 1.7-fold higher than that found in P388/DX nuclear extracts (Table 1).

VM-26-induced cleavage of pBR322 DNA in the presence of nuclear extracts from P388 and P388/DX cells. Because a marked reduction in DNA cleavage induced by VM-26 (Fig. 2) or by anthracycline analogs (9) has been observed in isolated nuclei from P388/DX cells as compared with P388 cells, 0.35 M NaCl nuclear extracts from both cell lines were also prepared, to directly examine DNA cleavage activity in an *in vitro* system.

Fig. 4 shows that cleavage of pBR322 DNA is stimulated by VM-26, in the presence of nuclear extracts from P388 cells, because DNA fragments smaller than the full-length plasmid DNA could be visualized by autoradiography. DNA cleavage

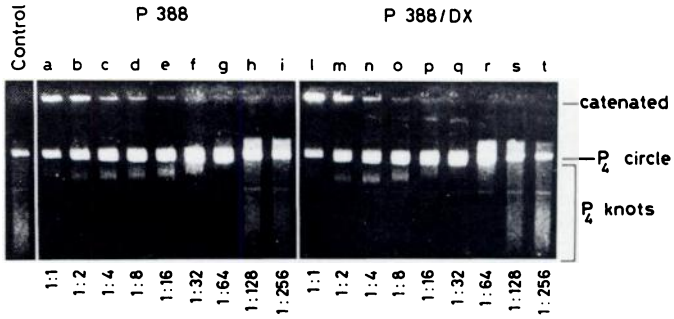

Fig. 3. Strand-passing activity of 0.35 M NaCl nuclear extracts from P388 and P388/DX cells. A serial 1:2 dilution of a 0.35 M NaCl nuclear extract (containing, in this representative experiment, 3.0 and 2.6 mg/ml protein from P388 and P388/DX cells, respectively) was incubated with 0.2 μg of knotted P4 DNA in the presence of 1 mM ATP for 30 min. The samples, which were stopped with an SDS-Ficoll solution, were electrophoresed on 0.7% agarose gels. *Control*, P4 DNA incubated in the absence of nuclear extracts.

TABLE 1

Comparison of sensitive and resistant P388 cells: VM-26 cytotoxic effects, DNA topoisomerase II activity, and sensitivity to VM-26-induced DNA cleavage

	P388	P388/DX
VM-26 ID_{50} (μM)[a]	0.17	9.5
VM-26-induced DSB (rad equivalents)[b]	4313 ± 625	914 ± 465
DNA topoisomerase II activity of nuclear extracts (0.35 M NaCl) (units/mg of protein)[c]	5950 ± 1520	3486 ± 1098
VM-26 concentration required to induce pBR322 DNA cleavage (μM)[d]	1	>10

[a] Determined by the growth inhibition test. Drug treatments were for 1 hr at 37°.
[b] Determined in isolated nuclei at 0.75 μM VM-26 in the presence of ATP (0.1 mM).
[c] Determined by the P4 unknotting assay.
[d] In the presence of 1 μg of protein of nuclear extracts from P388 or P388/DX.

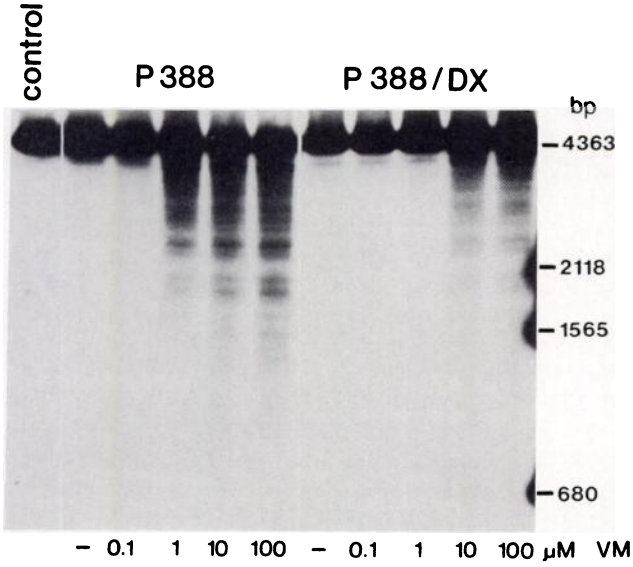

Fig. 4. DNA cleavage activity of 0.35 M NaCl nuclear extracts from P388 or P388/DX cells in the presence of VM-26. pBR322 DNA (6 ng) was incubated with 1 μg of protein of nuclear extracts for 30 min in a reaction mixture containing the indicated VM26 concentrations. The reactions were stopped with SDS (0.5% final concentration) and samples were treated with proteinase K and, after further incubation (30 min at 50°), were electrophoresed on a 0.7% agarose gel. The DNA was then transferred onto a nylon filter and probed with nicked-translated pBR322 DNA. *Control*, DNA incubated without nuclear extracts and drug.

was evident at 1 μM VM-26 with 1 μg of proteins of nuclear extracts from sensitive cells, whereas a 10-fold higher VM-26 concentration (10 μM) was required to induce appreciable DNA breaks with the same amount of protein of nuclear extracts from P388/DX cells. Because topoisomerase II activity measured in nuclear extracts was about 2-fold reduced in P388/DX cells as compared with P388 cells, VM-26-induced DNA cleavage was also examined in additional experiments using different amounts of nuclear extracts (0.5 and 1 μg of proteins) (Fig. 5). A comparison of drug-induced effects in the presence of different amounts of nuclear protein indicated that cleavage of pBR322 DNA was always reduced in nuclear extracts from the resistant line. Although several protein concentrations have been used, only results with optimal protein concentrations are presented, because amounts of extracted protein above 1 μg partially inhibited the DNA cleavage activity (data not shown). Because ATP increased VM-26-induced DNA DSB in isolated

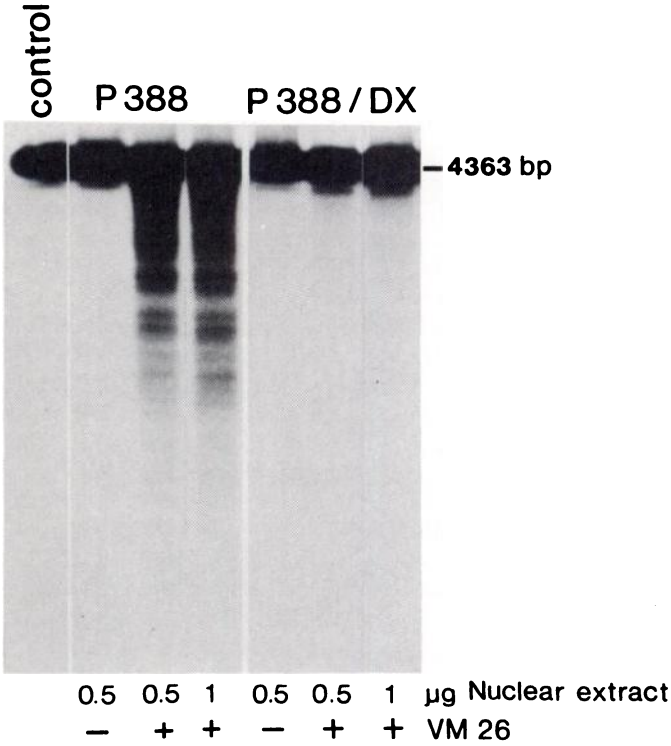

Fig. 5. DNA cleavage induced by VM-26 (10 μM) in the presence of different amounts of nuclear extracts. Experimental details are indicated in Fig. 4.

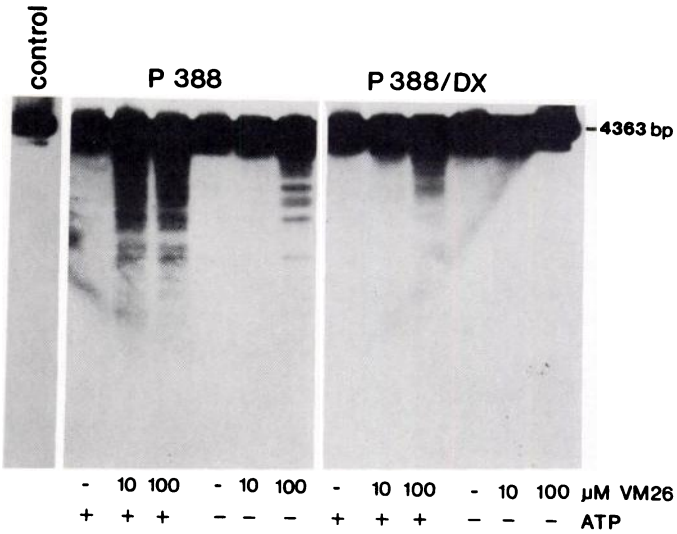

Fig. 6. Effects of ATP (1 mM) on DNA cleavage induced by VM-26 in the presence of 1 μg of protein of nuclear extracts from P388 and P388/DX cells. Experimental details are indicated in Fig. 4.

nuclei (Fig. 2), ATP effects on DNA cleavage activity of nuclear extracts in the presence of VM-26 were also examined (Fig. 6). The results were in agreement with the filter elution experiments, inasmuch as increased DNA break levels were observed in the presence of 1 mM ATP in nuclear extracts from both P388 and P388/DX cells. This ATP enhancement is expected if the DNA cleavage is mediated by topoisomerase II (17). Moreover, cleavage of pBR322 DNA was never observed with 1 μg of protein of nuclear extracts from P388/DX cells in the absence of ATP. Thus, in these experiments, both the stimulation by VM-26 and the ATP effect on DNA cleavage docu-

ment that DNA fragmentation produced by the nuclear extracts is mediated by DNA topoisomerase II.

VM-26-induced cleavage of pBR322 DNA in the presence of partially purified topoisomerase II. To clarify the basis of DNA topoisomerase II alterations in P388/DX cells, topoisomerase II was partially purified from fresh 0.35 M NaCl nuclear extracts of both cell lines. DNA cleavage induced by VM-26 in the presence of equivalent enzyme activity (4 units of topoisomerase II activity) is shown in Fig. 7. In contrast to the results obtained with nuclear extracts (Figs. 4 and 5), no difference between "sensitive" and "resistant" enzyme was detected in the ability of VM-26 to induce DNA breaks in pBR322 DNA. Again, ATP increased DNA fragmentation (not shown). However, also in the presence of ATP, we were unable to detect appreciable differences in cleavage activity of the two enzymes.

Discussion

Cellular drug resistance may arise from a variety of mechanisms that may be concomitantly operative in the same resistant cells (18–20). MDR in P388 leukemia cells occurs as a result of at least two distinct mechanisms, (a) a reduced drug accumulation, related to overexpression of membrane P-glycoprotein (10), and (b) a reduced sensitivity to drug-stimulated DNA cleavage (9). The reduced ability of anthracyclines to induce DNA damage led us to propose a modification of DNA topoisomerase II activity in resistant cells. Several lines of evidence have implicated this enzyme in the resistance of different cell lines to intercalating agents and epipodophyllotoxins (5, 21, 22). Alterations of DNA topoisomerase II activity in resistant cells may be the result of (a) quantitative changes (i.e., reduced level of enzyme activity) or (b) reduced sensitivity of the enzyme to drug action (i.e., altered drug-DNA-enzyme interaction or alteration in enzyme regulation).

The results presented in this work indicate that both quantitative and qualitative changes occur in the P388 resistant line (Table 1). Indeed, nuclear extracts from resistant cells contained approximately 1.7-fold less topoisomerase II catalytic activity than did extracts from sensitive cells. In addition, as documented by pBR322 plasmid DNA cleavage induced by VM-26 in the presence of nuclear extracts (Fig. 4), the different drug concentrations required to affect enzyme activity are consistent with a differential drug sensitivity. These observations are in agreement with decreased DNA DSB induced by VM-26 (Fig. 2) and anthracyclines in isolated nuclei from resistant cells (9). The molecular basis for reduced ability to induce enzyme-mediated DNA cleavage in the presence of drug still remains unclear. In an attempt to determine whether the difference observed in P388 cells is due to a change in the target enzyme, drug-stimulated cleavage of pBR322 plasmid DNA was examined in the presence of partially purified DNA topoisomerase II from sensitive and resistant cells. No appreciable differences were observed in enzyme sensitivity to VM-26 stimulation of DNA cleavage. The patterns of the cleaved DNA fragments were very similar for the two enzymes. The observation that purified topoisomerases from sensitive and resistant cells are equally sensitive to drug (VM-26), in contrast to nuclear extracts, is consistent with the hypothesis of the presence of a nuclear factor that modulates the sensitivity of topoisomerase II to inhibitory drugs (22). This hypothesis is also supported by the comparison of purified enzymes from intercalator-resistant Chinese hamster cells and parental sensitive cells reported by Pommier *et al.* (23), who also failed to find differences in drug sensitivity of the two enzymes. This possibility seems to be substantiated by the observation that 4′-(9-acridinylamino)methane-sulfon-*m*-aniside cytotoxicity is critically dependent on RNA and protein synthesis (24). A labile protein may enhance the drug effect by increasing the persistence of the cleavable complex. If this is the case, modulating factors may be lost during the purification process. An alternative explanation for the apparent discrepancy between results obtained with nuclear extracts and with purified enzymes may be a partial proteolytic degradation during the purification process. It is possible that only the undegraded enzyme is characterized by a differential sensitivity to drug action. Due to the limited amount of enzyme, a molecular characterization of the protein was not attempted. It should be pointed that an amsacrine-resistant P388 cell line contains two distinct forms of the enzyme and the relative amount of these forms appears to be different as compared with the sensitive line (25). It is also possible that the differences observed between the two cell lines in DNA cleavage activity is the result of a differential protein extraction from chromatin. If this is the case, the differential extraction could be related to a specific alteration in the variant cell line, because a different cleavage activity was consistently observed in all experiments. Under our extraction salt condition, a contamination with substantial amounts of histone proteins is not expected (26). However, it is evident that the presence, if any, of DNA-condensing agents (H1 histone) did not affect the DNA strand-passing activity of topoisomerase II in the nuclear extracts, because DNA catenation was observed with high concentrations of nuclear proteins from both cell lines (Fig. 3, *lanes a* and *l*).

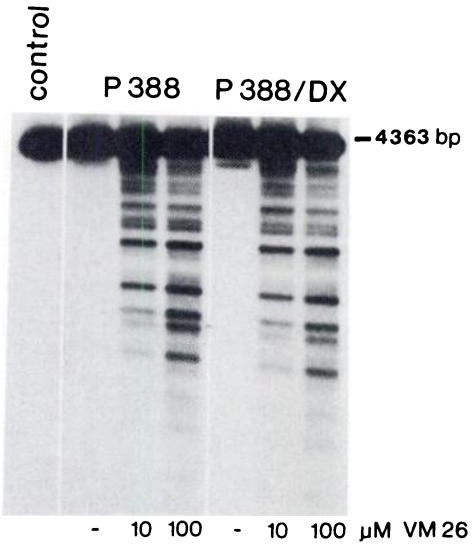

Fig. 7. VM-26-induced DNA cleavage in the presence of topoisomerase II partially purified from P388 and P388/DX cells. pBR322 DNA (6 ng) was incubated with the same enzyme activity (4 units) from P388 or P388/DX cells for 30 min at 37° in the presence or absence of VM-26 and without ATP. Experimental details are indicated in Fig. 4. *Control*, DNA incubated without enzyme.

In summary, our results show that not only catalytic activity but also DNA cleavage activity is reduced in resistant P388 cells. Taken together with results obtained in different cell lines (27–30), these observations are further support of the concept that the topoisomerase II-dependent mechanism of

resistance is a frequent mechanism of drug resistance. A better understanding of the underlying mechanisms is expected to promote the development of rational strategies to prevent or overcome resistance to topoisomerase II inhibitors.

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